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Growth regulators, DNA content and anatomy *in vitro*-cultivated *Curcuma longa* seedlings

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Curcuma longa L., from the Zingiberaceae family, generally reproduces through its rhizomes, which are also utilized for therapeutic purposes because they are rich in terpenoids. Its conventional propagation has low efficiency due to the small number of seedlings and their contamination by pathogens. Therefore, this study aimed to evaluate the influence of growth regulators on the development of *in vitro*-cultivated *C. longa* as well as to determine their influence on DNA content and foliar anatomy. Shoots were inoculated in MS culture medium with the addition of 30 g/L of sucrose and 6.5 g/L of agar, and a pH adjusted to 5.8. Two assays were built to study the multiplication and rooting phases of growth. The first assay evaluated the influence of eight concentrations of cytokinins and auxins on the multiplication phase. Leaf samples were analyzed for DNA content through flow cytometry, utilizing two reference standards, green pea, and tomato. Characteristics of leaf anatomy were also measured in four time periods. The second assay analyzed the influence of six auxin concentrations on the rooting phase. The first assay showed that the root systems grew more in treatment 3 (4.44 μ M benzylaminopurine [BAP], 0.46 μ M kinetin [KIN]) and reached greater dry mass in T8 (8.88 μ M BAP, 0.92 μ M KIN, 2.16 μ M naphthalene acetic acid [NAA]). The largest fresh matter of the main shoot was found in T2 (4.44 μ M BAP). The estimated DNA content varied depending on the presence of supplemental growth regulators, from 2.38 to 2.77 pg, and was greater in T4 (4.44 μ M BAP, 1.08 μ M NAA) and T5 (4.44 μ M BAP, 0.46 μ M KIN, 1.08 μ M NAA). Results from the latter two treatments were not significantly different. Estimates of DNA content were precise, as indicated by coefficients of variation that were much lower than 5%. The results also showed quantitative variation of evaluated anatomical traits. In general, there was a thin epidermis layer with rectangular cells, followed by parenchyma with octahedral cells and differentiated xylem and phloem. In leaf parenchyma, the presence of idioblasts containing phenolic compounds was observed in all growth stages. In the rooting phase, the supplementary auxins affected the dry matter of the aerial part and roots. The highest averages were observed in treatments with 2.0 μ M NAA.

Key words: Turmeric, micropropagation, flow cytometry, vegetal anatomy.

INTRODUCTION

Curcuma longa L. from the Zingiberaceae family, popularly known as turmeric, is a perennial plant native to Southern and Southeastern Asia (Pinto and Graziano,

2003). It is a medicinal plant whose rhizomes, generally rich in terpenoids, are utilized for therapeutic purposes (Nicoletti et al., 2003). Curcumin is its main compound of

interest (Chainani, 2003). Among the pharmaceutical applications, *C. longa* has anti-inflammatory, antioxidant, and antitumor properties (Kainsa et al., 2012; Green and Mitchell, 2014). It is indicated for the treatment of gastritis, ulcers, and food poisoning, acting mainly on the digestive system. *C. longa* has also been included in the treatment of cancer, hepatitis, inflammations, and painful diseases like arthritis, to name but a few (Mata, 2004). Moreover, this species stands out as a spice and food coloring for pasta and it is used for decoration due to its durability, beauty, and the exuberance of its inflorescences (Costa et al., 2011).

One of the problems *C. longa* producers face is the conventional propagation system. This system is limited by the phytosanitary quality of rhizomes, leading to dissemination of soil pathogens like fungi and nematodes (Bharalee et al., 2005; Faridah et al., 2011). The propagation is long and costly a rhizome can only produce four plants and has a period of dormancy, which is common in Zingiberaceae (Zhang et al., 2011). In addition, this rhizome, necessary for propagation, is also the commercialized part of the plant (Bharalee et al., 2005). Micropropagation could be a possible solution for this problem as *in vitro* methods are frequently utilized to complement traditional methods (Ahmadian et al., 2013). This technique can provide a greater number of pathogen-free seedlings in a shorter amount of time (Yildiz, 2012).

Several protocols have been used, altering the basal media and using different concentrations of growth regulators to meet the needs of each species. Santos (2003) stated that supplementary growth regulators replace the hormones missing from explants isolated from the mother plant. The different types of regulators work as stimuli for the expression of genes that control plant development, resulting in the induction of shoot and root growth and tissue differentiation (Depuydt and Hardtke, 2011). The most utilized vegetal regulators in tissue culture are auxins and cytokines, and among them, 3-indoleacetic acid (IAA), naphthalene acetic acid (NAA), Kinetin (KIN) and benzylaminopurine (BAP) have been the most utilized in assays (Neelakandan and Wang, 2012). Results for other species of Zingiberaceae showed that these regulators were paramount to promote growth and higher numbers of shoots of *Zingiber zerumet* (Faridah et al., 2011) and *Etilingera elatior* (Abdelmageed et al., 2011), and increased rooting rate and root length of *Zingiber officinale* (Abbas et al., 2011), and *Curcuma soloensis* (Zhang et al., 2011).

Tools like flow cytometry (Doležel and Greilhuber, 2010a) and structural and morphological analyses of tissues also help explain the effect of these regulators on

seedling development because the regulators influence tissue differentiation (Aloni et al., 2004; Aloni et al., 2006). In addition, studies that anatomically describe Zingiberaceae, including *C. longa*, are scarce. According to Aloni et al. (2006), auxins and cytokinins control the differentiation of xylem and phloem, and other hormones, like gibberellins and ethylene, may also be involved in this process. Assays demonstrated that the addition of BAP along with KIN in the culture medium increased the thickness of parenchyma cells, both spongy and palisade, and consequently the thickness of foliar limbs in *Annona glabra* (Oliveira et al., 2008). The quantitative analysis of leaf tissues of two species in Bromeliaceae showed that 0.5 mg/L of BAP resulted in a greater distance between the xylem and phloem (Galek and Kukulczanka, 1996). Thus, it is expected that, such as in other species of Zingiberaceae, auxins and cytokinins may have an effect on the development and anatomy of *C. longa*.

The ideal propagation protocol promotes better development and health vigorous seedling without the occurrence of abnormalities. However, the excess of growth regulators might be toxic to plant tissue and trigger an abnormal seedling development (Anjanasree et al., 2012). It was observed in *Elaeis guineensis* that the addition of 0.05 NAA + 0.05 BAP + 0.05 GA₃ + 2000 activated carbon (mg/L) (Suranthran et al., 2011).

In grapes, the addition of 10 μmol de GA₃ + 10 μmol IAA in MS medium promoted 56% of abnormal seedlings (Ji et al., 2013). In this way, monitoring through anatomical observations ensure a better understanding about the process, what is usually done visually. In addition, this would help in the comprehension of regulators action on the *C. longa* morphogenesis, once there are few studies about it.

Flow cytometry is used to characterize vegetal material for several purposes, such as DNA content analysis, ploidy verification, and cell cycle acquisition (Ochatt, 2008). Specifically, in tissue culture, this technique has been important to verify genetic stability, identify hybrids, check ploidy, and quantify genome size (Doležel and Greilhuber, 2010b; Pasqual et al., 2012). Growth regulators through *in vitro* culture, may cause somaclonal variations which might be from genetic or epigenetic (Miguem and Marum, 2011; Georgiev et al., 2014); this mechanism regulation influences the genetic expression affecting the phenotype. Furthermore, errors in DNA reading frame, might affect tissue analysis. Recently, researchers have discussed about the occurrence of self-tanning (Bennett et al., 2008) on the tissues *in vitro* analysis. Thus, flow cytometry, may contribute to verify the occurrence of somaclonal variation and is also

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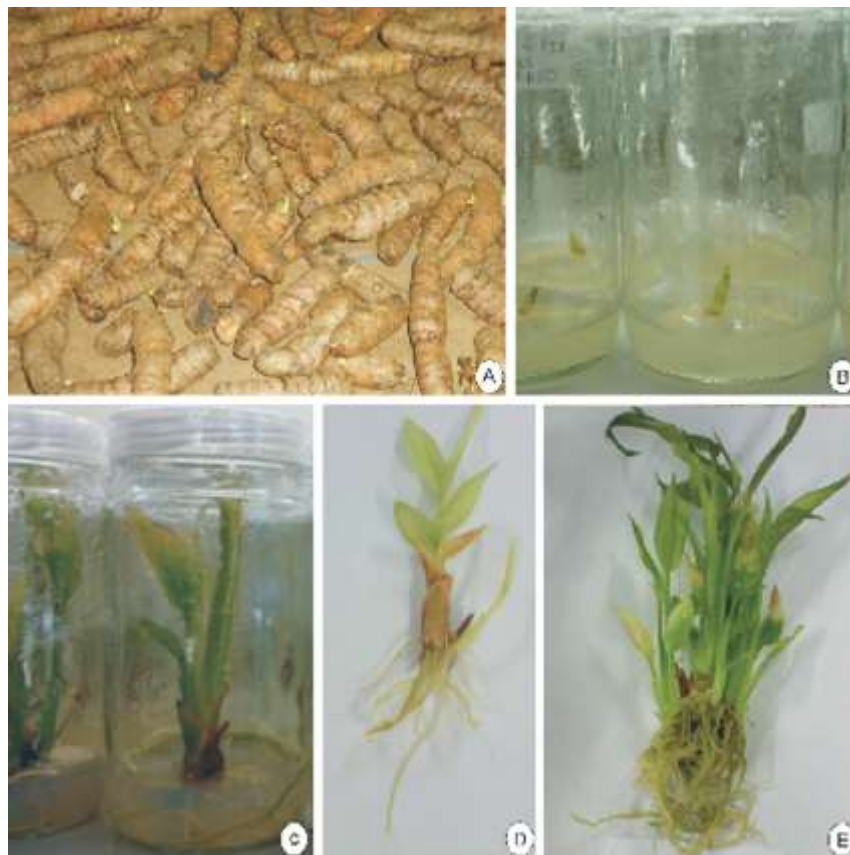


Figure 1. Rhizomes, shootings and developing seedlings of *C. longa* in function of the concentration of cytokinins and auxins. (A= Rhizomes, B= Inoculated shootings, C= developing seedlings, D=seedling submitted to Treatment 2 and E= seedling submitted to Treatment 8).

important to the monitoring of self-tanning in *C. longa* in studies with growth regulators.

Thus, the aim of this study was to evaluate the influence of growth regulators on the development of *in vitro* *C. longa*, as well as to verify the DNA contents and foliar anatomy of this species.

MATERIALS AND METHODS

C. longa rhizomes were obtained in the city of Mara Rosa (Figure 1A), GO, Brazil, and transported in bags to the Laboratory of Molecular Biology and Vegetal Tissue Culture of Paranaense University (UNIPAR), campus of Umuarama, PR, Brazil.

Shoot asepsis

The rhizomes were kept in the laboratory for a month at room temperature prior to selection for culture. Rhizomes that were cracked or had symptoms of infection by pathogens were discarded. The remaining rhizomes were washed in running water to remove soil fragments. After emergence, the shoots were removed from the rhizomes and standardized to 2.0 ± 0.2 cm in length and 0.5 ± 0.2 cm in diameter. In an aseptic chamber, the shoots were immersed in a solution of 2% (v/v^{-1}) sodium

hypochlorite for 20 min under manual agitation and then submitted to three successive washings in distilled water.

Phase 1: Multiplication phase

Axillary shoots from the asepsis phase were inoculated in 350 ml clear glass flasks (Figure 1B) containing MS culture medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g/L of sucrose and 6.0 g/L of agar, and adjusted to a pH of 5.8. Three growth regulators, BAP, NAA, and KIN, were added to the culture medium at different concentrations (Table 1). Inoculation was done in an aseptic chamber after autoclaving of the flasks at 121°C for 20 min. The shoots were individually placed in flasks with 50 ml of culture medium. The flasks were then closed with transparent plastic lids and sealed with PVC plastic. The material was kept in a growth chamber for 101 days at a temperature of $25 \pm 2^\circ\text{C}$, in the presence of light for 24 h per day.

After 101 days, the following characteristics were evaluated: leaf number (LN), shoot number (SN), aerial part length (APL), base diameter (BAD), root length (RL), fresh matter of main shoot (FMMS), root fresh matter (RFM), total dry matter of aerial part (DMAP), and root dry matter (RDM). Data for length were measured by a digital pachymeter and dry matter measurements were obtained after drying in an air circulation oven at 65°C until measurements were constant.

The experiment had a complete randomized design with eight

Table 1. Concentration of different growth regulators, BAP, KIN and NAA added to the culture medium (MS).

Treatment	BAP (μM)	KIN (μM)	NAA (μM)
T1	0.0	0.0	0.0
T2	4.44	0.0	0.0
T3	4.44	0.46	0.0
T4	4.44	0.0	1.08
T5	4.44	0.46	1.08
T6	8.88	0.0	0.0
T7	8.88	0.92	0.0
T8	8.88	0.92	2.16

treatments, one shoot in each flask, three shoots per plot, and four replicates. Leaf number, base diameter, and aerial part length were submitted to an analysis of variance by a Kruskal Wallis test ($p \leq 0.05$), whereas the other traits were submitted to an analysis of variance (ANOVA, $p \leq 0.05$). The averages were compared using Tukey's test ($p \leq 0.05$).

DNA content estimate by flow cytometry

At 101 days after assay implementation, a leaf portion of approximately 1 cm from each treatment was removed and ground in a Petri dish with 1 ml cold Marie buffer in order to release nuclei (Marie and Brown, 1993). The buffer solution consisted of 50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM Na₂ EDTA, 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES (pH 7.2), and 1% (m/v) polyvinylpyrrolidone-10 (PVP-10). The nuclei suspension was aspirated through two layers of cotton gauze using a plastic pipette and filtered through a 50- μm mesh. The nuclei were then stained by adding 25 μl of 1 mg/ml propidium iodide to each sample.

To compare DNA content in picograms, two other species, *Pisum sativum* with 9.09 pg (Pasqual et al., 2012) and *Solanum lycopersicum* with 1.86 pg were used as external reference standards, using the same procedure for nucleus suspension. For each sample, 10,000 nuclei were evaluated through a logarithmic scale. The analysis was carried out in a FACS Calibur cytometer (BD, Biosciences, San Jose, CA, USA) and the histograms were obtained by Cell Quest software and statistically analyzed by WinMDI 2.8 software (Scripps 43 Research Institute, 2011). Nuclear DNA content (pg) was estimated as the ratio between the fluorescence intensities of G1 nuclei from the reference standard and G1 nuclei from the sample, multiplying this ratio by the DNA amount of the reference standard. Estimated DNA contents and coefficients of variation (CV%) were submitted to an analysis of variance and the averages were compared using Tukey's test ($p \leq 0.05$) in Sisvar statistical program (Ferreira, 2011). All analyses were done in quadruplicates.

Anatomical evaluation

Samples of the vegetal material from each of the eight treatments in the multiplication phase were collected at different periods. Samples for period 1 were collected immediately after *in vitro* inoculation; period 2 at 35 days after inoculation; period 3 at 56 days; and period 4 at 101 days.

Each sample was fixed in FAA 50 solution (formaldehyde, 50% ethanol, acetic acid, 1:1:18, v/v) for 24 h and stored in 70% ethanol (Johansen, 1940). For permanent slide preparation, the material was dehydrated in a butylic series (Johansen, 1940) and embedded

Table 2. Concentration of different growth regulators, NAA and IAA, added to the culture medium (MS).

Treatment	NAA (μM)	IAA (μM)
1	0.0	0.0
2	1.0	0.0
3	2.0	0.0
4	0.0	34.0
5	0.0	44.0
6	1.0	34.0

in Paraplast (Kraus and Arduin, 1997) in an oven at 60°C. Transversal cuts (7 μm) were done in a rotary microtome at the Pathology Histotechnical Laboratory of Unipar – Campus 2. The histological cuts were placed in a hot water bath at 45°C and immediately removed with glass slides. The slides with fixed vegetal material were placed in a water bath in butyl acetate to remove the excess Paraplast. For complete removal of Paraplast the samples were dehydrated in an ethylic series. Next, they were stained with safrablau, a mixture of astra blue and safranin (9:1, v/v) modified to 0.5% (Bukatsch, 1972). Acrilex colorless glass varnish was used for adhesion of coverslips (Paiva, 2006).

The prepared slides were utilized to measure epidermis, parenchyma, xylem, phloem of the aerial part of the plant. These measurements were made from images of the longitudinal sections captured by a digital camera coupled to an optical light microscope, Olympus BX-60, using Motic Images Plus 2.0 software. To calculate averages, 10 cells from each slide were used in three replicates divided into the aerial part for each of the following variables: epidermis, parenchyma, xylem, and phloem. The averages were compared by Scott-Knot's test ($p \leq 0.05$).

Phase 2: Rooting phase

Aseptic shoots were inoculated in MS culture medium (Murashige and Skoog, 1962). The medium was supplemented with 30 g/L of sucrose and 6.0 g/L of agar, and adjusted to a pH of 5.8. Two growth regulators, NAA and indoleacetic acid (IAA), were added to the culture medium at different concentrations (Table 2). The culture media were autoclaved at 121°C for 20 min.

In an aseptic chamber, the shoots were individually placed in flasks containing 50 ml of culture medium, closed with clear plastic lids, and sealed with PVC plastic. The material was kept in a growth chamber for 60 days at a temperature of 25 \pm 2°C and submitted to 24 h of light per day.

After 60 days, the following characteristics were evaluated: leaf number (LN), base diameter in mm (BAD), root length in mm (RL), fresh matter of main shoot (FMMS), root fresh matter (RFM), shoot number (SN), dry matter of aerial part (DMAP), and root dry matter (RDM). Data for length were measured by a digital pachymeter. Dry matter measurements were obtained after drying in an air circulation oven at 65°C until measurements were constant. The experiment had completely randomized design (CRD) with six treatments, three shoots per plot, and four replicates. Data were submitted to an ANOVA ($p \leq 0.05$), and averages were compared by Tukey's test ($p \leq 0.05$).

RESULTS

Phase 1: Multiplication phase

There were significant differences for several of the

Table 3. Growth measurements obtained from *in vitro* *Curcuma longa* seedlings cultivated with differing concentrations of auxins and cytokinins in the multiplication phase.

Treatment	LN	SN	BAD (mm)	APL (mm)	RL (mm)	FMMS (g)	RFM (g)	DMAP (g)	RDM (g)
T1	7.75 ^a	1.5 ^a	8.12 ^a	67.28 ^{ab}	54.49 ^{ab}	1.07 ^b	1.23 ^b	0.10 ^a	0.08 ^b
T2	7.25 ^a	0.75 ^a	5.13 ^a	36.11 ^b	38.94 ^{ab}	13.31 ^a	2.32 ^{ab}	0.14 ^a	0.08 ^b
T3	5.75 ^a	2.5 ^a	6.39 ^a	69.72 ^{ab}	65.19 ^a	1.08 ^b	2.28 ^{ab}	0.11 ^a	0.12 ^b
T4	6.25 ^a	1 ^a	7.89 ^a	88.92 ^a	54.49 ^{ab}	1.13 ^b	3.07 ^{ab}	0.10 ^a	0.17 ^{ab}
T5	8.25 ^a	2.25 ^a	9.62 ^a	55.99 ^{ab}	27.58 ^b	1.29 ^b	5.42 ^{ab}	0.12 ^a	0.31 ^{ab}
T6	3.25 ^a	0.75 ^a	5.82 ^a	38.36 ^{ab}	30.09 ^b	0.44 ^b	2.26 ^{ab}	0.08 ^a	0.16 ^{ab}
T7	5.5 ^a	0.75 ^a	6.92 ^a	68.36 ^{ab}	65.79 ^a	1.22 ^b	4.09 ^{ab}	0.12 ^a	0.14 ^{ab}
T8	10.75 ^a	2.5 ^a	4.64 ^a	69.72 ^{ab}	33.17 ^{ab}	2.29 ^b	7.77 ^a	0.26 ^a	0.36 ^a

*Analysis by Kruskal Wallis' test, (LN) test value=8.472, p (0.05)=14.070; (APL) test value=14.205, p (0.05)=14.070; (BAD)=test value=7.983, p (0.05)=14.070. *Other characteristics, averages followed by the same letter do not differ statistically by Tukey's test at p<0.05. LN: Leaf number; SN: shoot number; APL: aerial part length; BAD: base diameter; RL: root length; FMMS: fresh matter of main shoot; RFM: root fresh matter; DMAP: total dry matter of aerial part; RDM: root dry matter. T1: Control, T2: 4.44 μ M BAP, T3: 4.44 μ M BAP +0.46 μ M KIN, T4: 4.44 μ M BAP +1.08 μ M NAA, T5: 4.44 μ M BAP + 0.46 μ M KIN + 1.08 μ M NAA, T6: 8.88 μ M BAP, T7: 8.88 μ M BAP + 0.92 μ M KIN, and T8: 8.88 μ M BAP + 0.92 μ M KIN +2.16 μ M NAA.

evaluated growth characteristics ($p \leq 0.05$), depending on the types and concentrations of growth regulators. Leaf and shoot number, base diameter, and dry matter of aerial part did not show significant differences (Table 3). However, root and aerial part lengths, fresh matter of main shoot, and root fresh and dry matter were influenced by growth regulators (Table 3).

The treatments that caused greater root growth were treatment 3 (4.44 μ M of BAP and 0.46 μ M of KIN) and T7 (8.8 μ M of BAP and 0.92 μ M of KIN), whereas T5 and T6 were the least efficient for root growth. The highest average aerial part length was seen in T4 (4.44 μ M/L of BAP added with 1.08 μ M/L of NAA). Treatment 2 (4.44 μ M of BAP) was the most efficient for increasing the fresh matter of the main shoot. The final averages were similar for the other treatments. Treatment 8 was the most effective for improving root mass; T4, T5, and T6 did not differ among themselves for root fresh matter (RFM) and root dry matter (RDM), whereas T1 was less efficient for RFM. Treatment 1, T2, and T3 were inefficient for improving root mass (Table 3).

Regarding RFM and RDM, high concentrations of cytokinins combined with auxins, such as in T8, caused a decrease of root growth, but favored mass gain with the emergence of several lateral and secondary roots (Figure 1E). Skala and Wysokinska (2004) and Garlet et al. (2011) also observed root length reduction in *Salvia nemorosa* L. and *Mentha gracilis* S. plants when higher concentrations of BAP were used during *in vitro* proliferation of these species. The combination of cytokinins and auxin (NAA) was important for the root system, making synergism between these regulators evident. Treatments with low concentrations of NAA had fewer lateral roots and the control treatment showed the lowest root mass, probably due to the absence of growth regulators.

Figure 2 shows the tendency of the length development of aerial part and root of *C. Longa* seedlings submitted to combinations and concentrations of cytokinins and auxins. For root length, an increment of approximately 40 mm was observed in the first 35 days; this behavior was similar to the other treatments. After that, there was a growth distinction for the aerial part which was influenced by the regulator and its concentration. After 56 days, the treatments with 4.44 μ M of BAP added with 0.46 μ M of KIN, and 8.88 μ M/L of BAP added with 0.92 μ M/L of KIN provided length of approximately 52 mm, and at the end of the assay, there was a more evident growth, but a smaller increase for the aerial part in the treatment with 8.88 μ M/L of BAP (Figures 1D and 2). Regarding root length, no similar root growth standard was observed for the aerial part in the first 35 days; the control stood out from the others, reaching 65 mm, and the roots in T3 and T7 developed more than in other treatments after 56 days (Figure 2). At 101 days, there was an increase of 32.01 mm in root length when comparing T3 to T8, but root length was 35.1 mm in T3 (Figure 2).

DNA content varied according to treatment for *in vitro*-cultivated *C. longa*. All treatments presented CVs lower than 5% and, consequently, highly reliable DNA indexes (Table 4). Sample quality can influence CV; a lower value indicates greater reliability in the estimates. The highest DNA content was observed in T4 and T5, and the smallest in T3. The results were the same regardless of whether *P. sativum* or *S. lycopersicum* were used as the reference (Table 4).

Figures 3 and 4 provide histograms of flow cytometry using each reference standard. In the histogram of Figure 3, the first peak indicates peak G1 of the interphase of leaves from the experimental treatments whereas the second one indicates peak G1 of the reference standard (*P. sativum*). In Figure 4, the first peak indicates peak G1

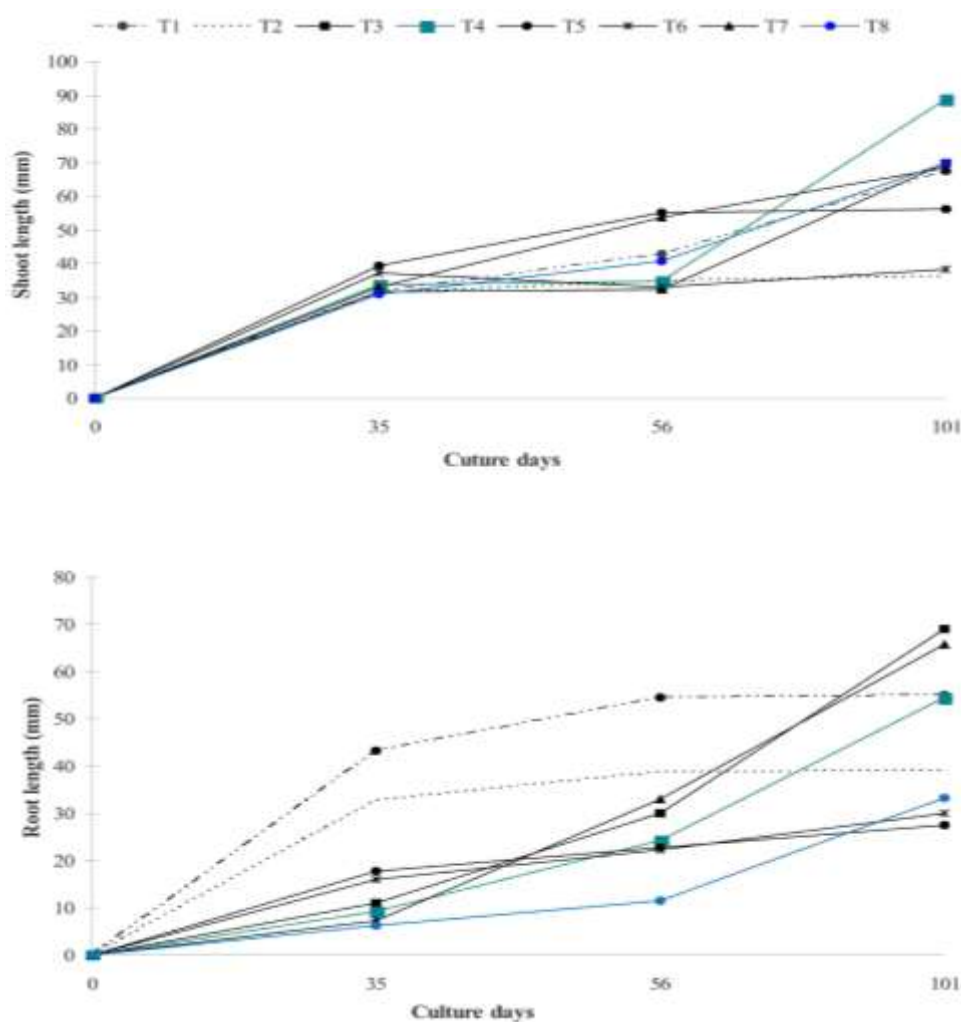


Figure 2. Aerial part length (mm) and root length (mm) evaluated in four distinct periods and at different concentrations of auxins and cytokinins during multiplication phase. **T1:** Control, **T2:** 4.44 ($\mu\text{M/L}$) of BAP, **T3:** 4.44 ($\mu\text{M/L}$) of BAP+0.46 ($\mu\text{M/L}$) of KIN, **T4:** 4.44 ($\mu\text{M/L}$) of BAP+1.08 ($\mu\text{M/L}$) of NAA, **T5:** 4.44 ($\mu\text{M/L}$) of BAP+0.46 ($\mu\text{M/L}$) of KIN+1.08 ($\mu\text{M/L}$) of NAA, **T6:** 8.88 ($\mu\text{M/L}$) of BAP, **T7:** 8.88 ($\mu\text{M/L}$) of BAP+0.92 ($\mu\text{M/L}$) of KIN, and **T8:** 8.88 ($\mu\text{M/L}$) of BAP+0.92 ($\mu\text{M/L}$) of KIN+2.16 ($\mu\text{M/L}$) of NAA.

Table 4. DNA content and Coefficient of Variation (CV) of *in vitro*-cultivated *Curcuma longa*.

Treatment	Green pea (<i>Pisum sativum</i> . L)		Tomato (<i>Solanum lycopersicum</i>)	
	DNA (pg)	CV (%)	DNA (pg)	CV (%)
1	2.4450 ^{ab}	0.49 ^a	2.7025 ^{ab}	0.49 ^a
2	2.4650 ^{ab}	0.49 ^a	2.7300 ^{ab}	0.49 ^a
3	2.3850 ^b	0.49 ^a	2.6275 ^b	0.49 ^a
4	2.5275 ^a	0.49 ^a	2.7775 ^a	0.50 ^a
5	2.5300 ^a	0.48 ^a	2.7575 ^a	0.50 ^a
6	2.4500 ^{ab}	0.48 ^a	2.6825 ^{ab}	0.50 ^a
7	2.4950 ^{ab}	0.48 ^a	2.7500 ^{ab}	0.49 ^a
8	2.4500 ^{ab}	0.46 ^a	2.7125 ^{ab}	0.48 ^a

*Averages followed by the same letter do not statistically differ among themselves by Tukey's test at $p(<0.05)$. T1: Control, T2: 4.44 μM BAP, T3: 4.44 μM BAP+0.46 μM KIN, T4: 4.44 μM BAP+1.08 μM NAA, T5: 4.44 μM BAP+0.46 μM KIN+1.08 μM NAA, T6: 8.88 μM BAP, T7: 8.88 μM BAP+0.92 μM KIN, and T8: 8.88 μM BAP+0.92 μM KIN+2.16 μM NAA.

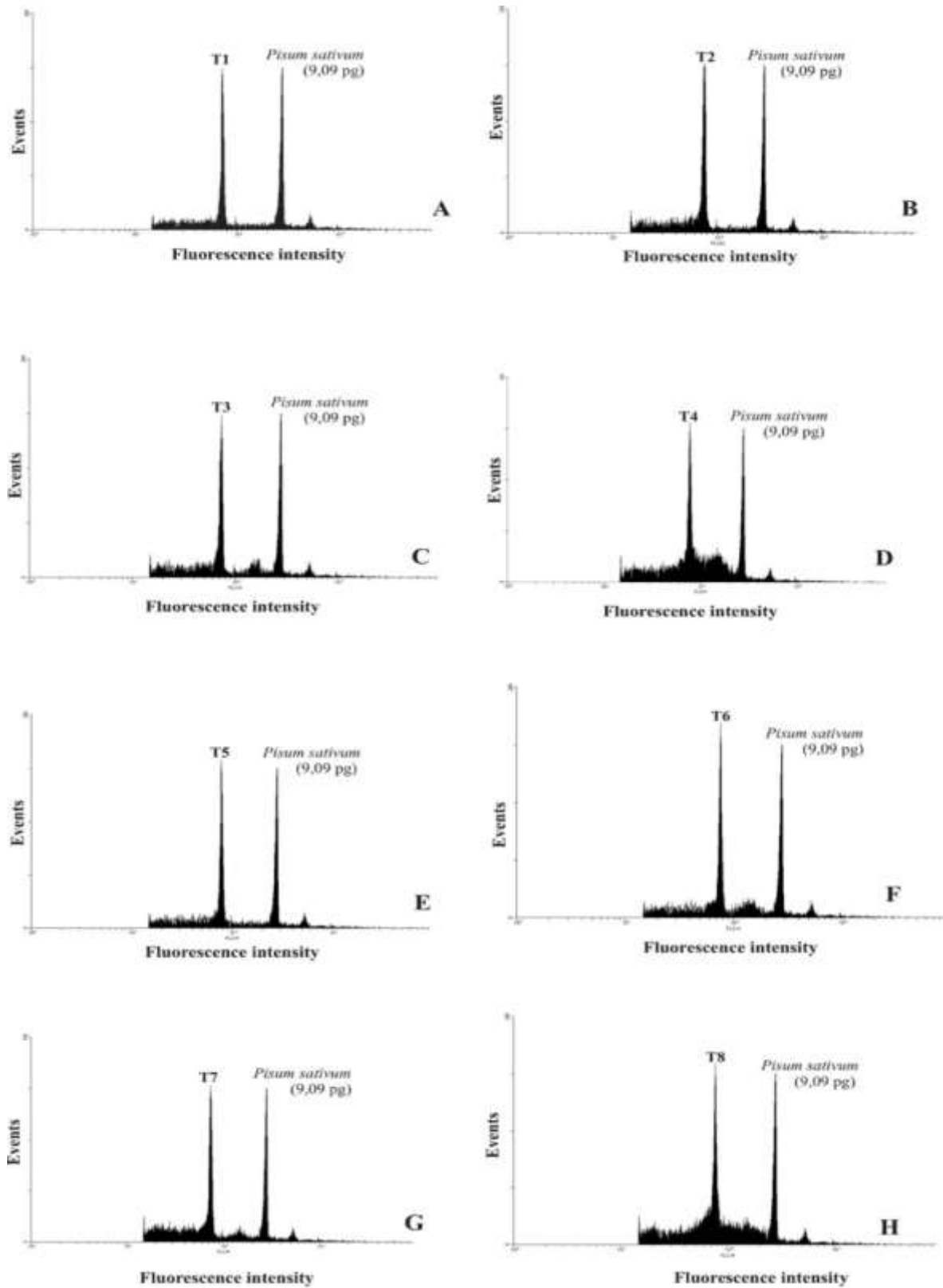


Figure 3. Histograms of flow cytometry of *Curcuma longa* leaves grown with different combinations and concentrations of auxins and cytokinins in the multiplication phase (green pea reference standard). A=T1: Control, B=T2: 4.44 μ M BAP, C=T3: 4.44 μ M BAP+0.46 μ M KIN, D=T4: 4.44 μ M BAP+1.08 μ M NAA, E=T5: 4.44 μ M BAP+0.46 μ M KIN+1.08 μ M NAA, F=T6: 8.88 μ M BAP, G=T7: 8.88 μ M BAP+0.92 μ M KIN, and H=T8: 8.88 μ M BAP+0.92 μ M KIN+2.16 μ M NAA.

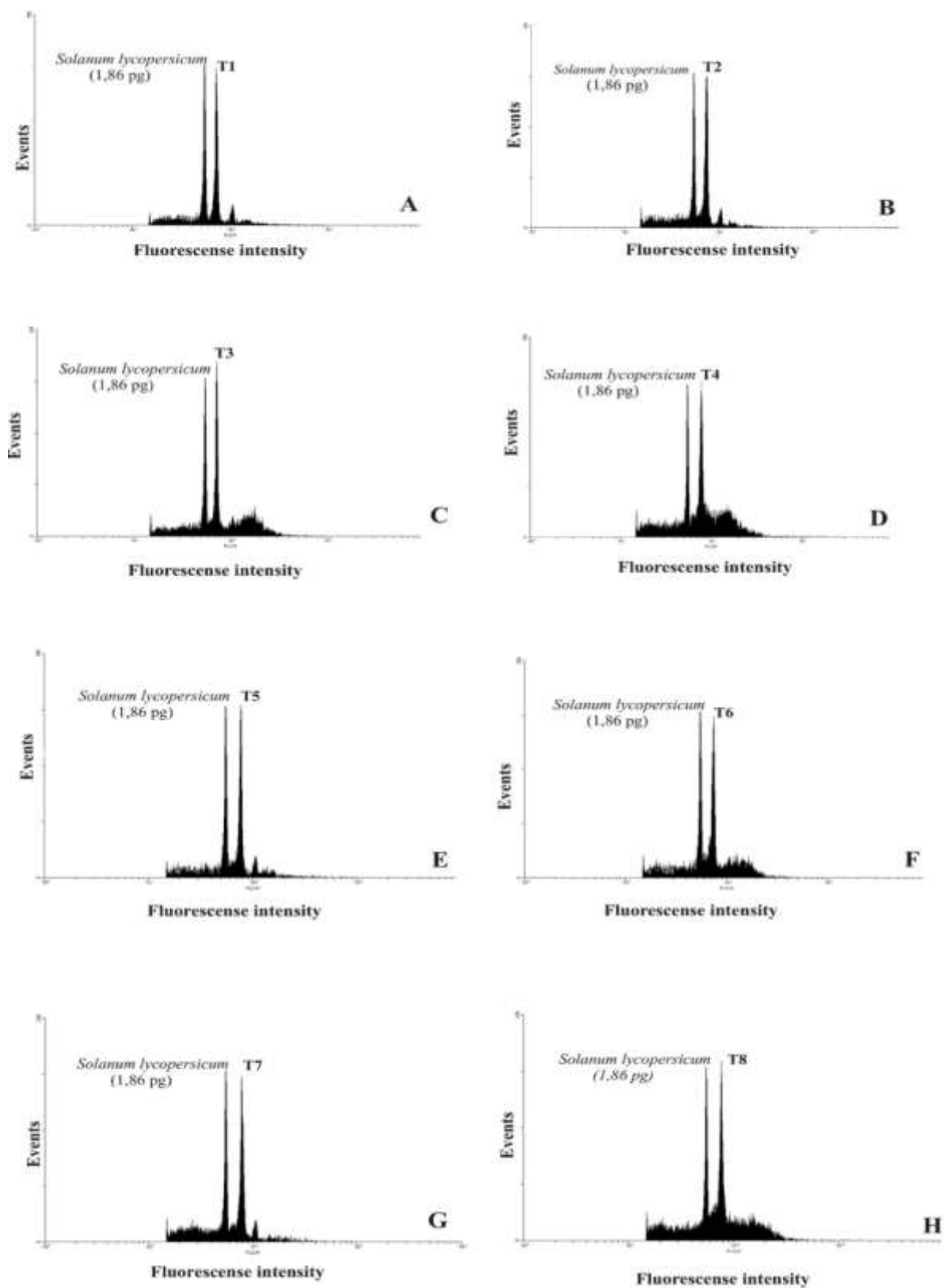


Figure 4. Histograms of flow cytometry of *Curcuma longa* leaves grown with different combinations and concentrations of auxins and cytokinins in the multiplication phase (tomato reference standard). A=T1: Control, B=T2: 4.44 μ M BAP, C=T3: 4.44 μ M BAP +0.46 μ M KIN, D=T4: 4.44 μ M BAP+1.08 μ M NAA, E=T5: 4.44 μ M BAP+0.46 μ M KIN+1.08 μ M NAA, F=T6: 8.88 μ M BAP, G=T7: 8.88 μ M BAP+0.92 μ M KIN, and H=T8: 8.88 μ M BAP+0.92 μ M KIN+2.16 μ M NAA.

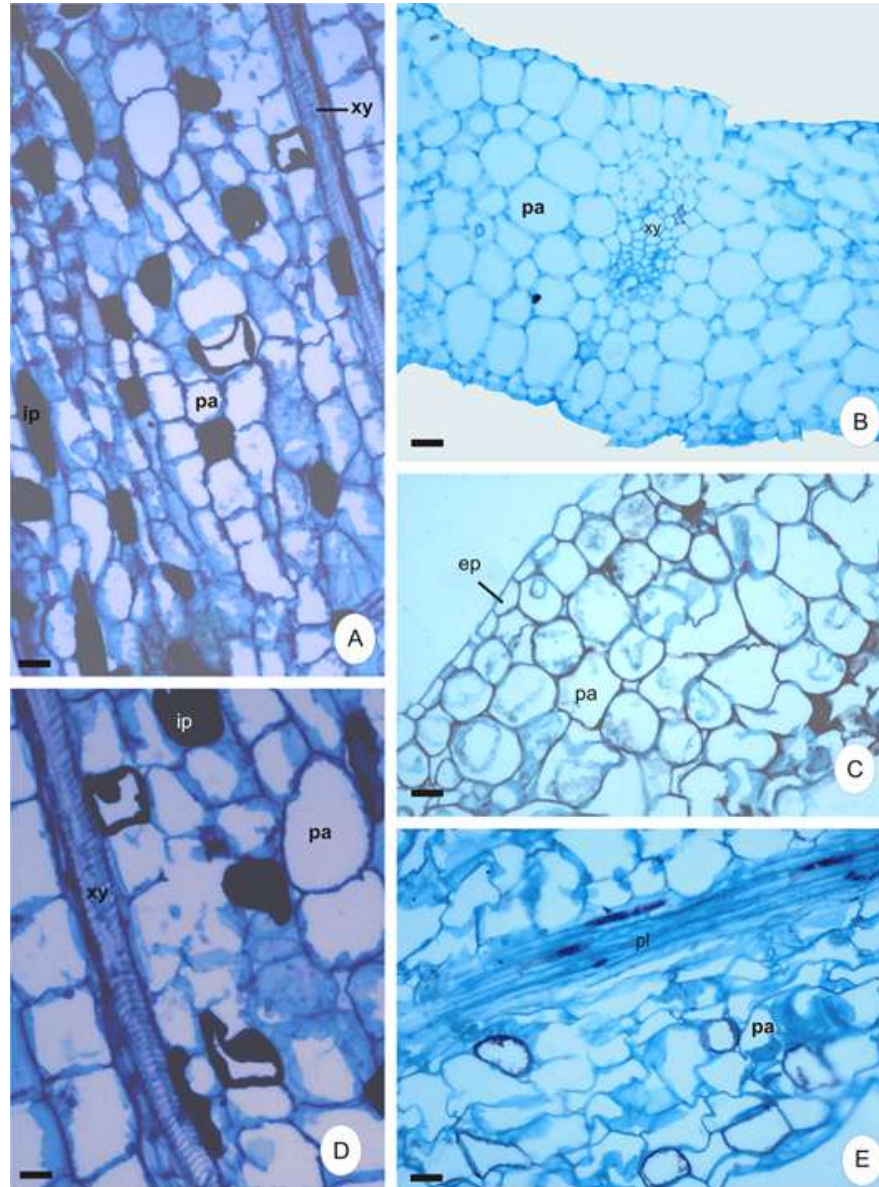


Figure 5. Longitudinal sections of seedling tissues from initiation of the multiplication phase of *in vitro*-cultivated *Curcuma longa*. (A - D) Aerial parts; (E,F) Root. Ep: Epidermis, Pl: phloem, Ip: idioblast containing phenolic compounds, Pa: parenchyma, Xy: xylem.

of the reference standard (*S. lycopersicum*), while the second peak indicates the G1 phase of the interphase of leaves from the experimental treatments. In general, the graphs were of excellent quality with thin peaks and little dragging, consistent with the low CV for all treatments and standards.

Regarding to seedling development and anatomical cuts, tissue abnormalities was not verified in treatments with development regulators analyzed. However, in quantitative terms, some differences was observed mainly in parenchyma and in the epidermis. In our study, the epidermis of *C. longa* presented juxtaposed rectangular

cells, with thin cell walls with little or no wax (Figure 5A). These results corroborate the ones by Thong et al. (2009) for *C. longa* seedlings kept *in vitro* in 2-4D medium. Epidermis thickness varied according to the treatment and time period (Table 5). At 35 days after cultivation, T4, T5, T7, and T8 had the thickest epidermis cells and were not statistically different from each other. At 56 and 101 days, T2, T4, T6, and T8 had the highest average thickness (Table 5).

The parenchyma of *C. longa* consisted of voluminous cells along both sides of the leaves; the tissue had thin cell walls and the cells had an isodiametric shape with

Table 5. Measurements in μm of longitudinal sections of epidermis, parenchyma, xylem, and phloem of *Curcuma longa* leaves submitted to eight combinations of auxins and cytokinins in the multiplication phase.

Treatments	Epidermis	Parenchyma	Xylem	Phloem
	Aerial part period/2			
1	635,5 ^b	915,1 ^c	508,9 ^a	1.485,0 ^a
2	553,3 ^b	1.079,7 ^c	450,6 ^a	1.084,8 ^b
3	465,5 ^b	1.202,2 ^c	348,1 ^b	1.345,1 ^a
4	784,7 ^a	1.523,4 ^b	397,5 ^b	1.496,0 ^a
5	705,6 ^a	1.483,9 ^b	431,4 ^a	1.346,4 ^a
6	598,2 ^b	1.855,8 ^a	466,0 ^a	1.626,3 ^a
7	711,1 ^a	1.962,1 ^a	495,6 ^a	1.503,8 ^a
8	747,6 ^a	1.164,5 ^c	368,8 ^b	1.504,0 ^a
Period 1	757,3	1.734,7	519,1	1.783,1
Aerial part period/3				
1	471,6 ^b	1.155,1 ^c	418,7 ^a	1.377,1 ^b
2	733,2 ^a	1.961,7 ^a	471,8 ^a	1.383,5 ^b
3	534,3 ^b	920,0 ^c	409,9 ^a	1.161,6 ^b
4	656,9 ^a	1.441,3 ^b	464,3 ^a	1.600,5 ^a
5	501,6 ^b	1.462,2 ^b	478,1 ^a	1.863,8 ^a
6	697,2 ^a	1.644,3 ^b	390,8 ^a	1.289,3 ^b
7	443,7 ^b	1.319,1 ^c	410,5 ^a	1.651,8 ^a
8	700,9 ^a	1.475,5 ^b	492,7 ^a	1.499,5 ^b
Period 1	757,3	1.734,7	519,1	1.783,1
Aerial part period/4				
1	471,6 ^b	1.155,1 ^c	385,5 ^a	1.377,1 ^b
2	733,2 ^a	1.961,7 ^a	526,7 ^a	1.383,5 ^b
3	534,3 ^b	920,0 ^c	466,8 ^a	1.161,6 ^b
4	656,9 ^a	1.441,3 ^b	497,4 ^a	1.600,5 ^a
5	501,6 ^b	1.462,2 ^b	443,6 ^a	1.863,8 ^a
6	697,2 ^a	1.644,3 ^b	537,1 ^a	1.289,3 ^b
7	443,7 ^b	1.319,1 ^c	375,7 ^a	1.651,8 ^a
8	700,9 ^a	1.475,5 ^b	545,8 ^a	1.499,5 ^b
Period 1	757,3	1.734,7	519,1	1.783,1

*Averages followed by the same letter are not significantly different according to Skott-Knott's test ($p \leq 0.05$). Period 1: assay implementation, period 2: 35 days after cultivation, period 3: 56 days, and period 4: 101 days.

small intercellular spaces observed in the longitudinal sections (Figure 5B to C). After 35 days in the culture medium, the cells became bigger with more intercellular spaces, and the cell walls got thicker. Idioblasts with phenolic compounds were observed in the leaf parenchyma of the seedlings utilized at the beginning of the assay (Figure 5A and D). The parenchyma, as well as the epidermis, presented significant differences quantitatively among the studied treatments and time periods. The greatest averages at 35 days obtained in this tissue were observed in T6 and T8, whereas T2 had

the greatest average at 56 and 101 days (Table 5).

The only significant differences in xylem thickness in the present study were found at 35 days, with differences in the averages for T1, T2, T5, T6, and T7 (Table 5). The phloem had already been differentiated at the assay implementation; at that time, T2 had a significantly lower average phloem thickness compared to the rest of the treatments. At 56 and 101 days, T2 remained the lowest, whereas T3, T4, and T7 had significantly higher phloem thickness compared to the rest of the treatments (Table 5).

Table 6. Growth measurements obtained from *in vitro* *Curcuma longa* seedlings cultivated with differing concentrations and combinations of auxins and cytokinins in the rooting phase.

Treatments	SN	LN	APL (mm)	RL (mm)	BAD (mm)	FMMS (g)	FMAP (g)	RFM (g)	DMAP (g)	RDM (g)
1	1. ^{20b}	8.33 ^a	68.24 ^a	119.24 ^a	8.99 ^{ab}	2.18 ^a	2.67 ^a	0.1596 ^a	0.1160 ^c	2.3060 ^{bc}
2	1.20 ^b	8.56 ^a	63.24 ^{ab}	55.97 ^b	8.17 ^b	2.24 ^a	2.80 ^a	0.1648 ^a	0.1340 ^{bc}	2.1580 ^c
3	1.80 ^{ab}	8.95 ^a	58.55 ^{ab}	72.39 ^b	9.82 ^{ab}	2.33 ^a	2.94 ^a	0.1715 ^a	0.2300 ^a	4.4120 ^a
4	2.20 ^a	8.93 ^a	52.86 ^b	74.08 ^b	9.16 ^{ab}	2.33 ^a	3.04 ^a	0.1786 ^a	0.2080 ^a	2.5780 ^{bc}
5	1.80 ^{ab}	8.90 ^a	58.03 ^{ab}	77.80 ^b	10.95 ^{ab}	2.54 ^a	3.13 ^a	0.1784 ^a	0.2080 ^a	3.8160 ^{ab}
6	1.60 ^{ab}	8.80 ^a	71.74 ^a	81.56 ^b	11.72 ^a	2.63 ^a	3.25 ^a	0.1780 ^a	0.1960 ^{ab}	3.6520 ^{abc}

*Averages followed by the same letter do not differ statistically by Tukey's test at $p < 0.05$. LN: Leaf number, SN: shoot number, APL: aerial part length, RL: root length, BAD: base diameter, FMMS: fresh matter of main shoot, FMAP: fresh matter of aerial part, RFM: root fresh matter, DMAP: dry matter of aerial part, RDM: root dry matter. T1: Control (absence of regulator), T2: 1.0 (μM L) NAA, T3: 2.0 (μM L) NAA, T4: 34.0 (μM L) IAA, T5: 44.0 (μM L) IAA and T6: 1.0 (μM L) NAA added with 34.0 (μM L) IAA.

Phase 2: Rooting phase

The results showed significant differences among the six treatments of this phase ($p \leq 0.05$) for the following characteristics: number of shoots, length of aerial part and root, base diameter, and dry matter of aerial part and root. The rest of the characteristics did not vary in response to changes in supplemental regulators (Table 6). The largest final shoot diameter was seen in T6 (1.0 μM NAA + 34.0 μM IAA). However, the highest shoot number (SN) was found in T4 (34.0 μM /L IAA), followed by T5 (44.0 μM IAA), and T6 (1.0 μM NAA + 34.0 μM IAA). The control and T2 resulted in the lowest averages (Table 6). The longest aerial part length (APL) values were seen in the control (T1) and T6 (1.0 μM NAA + 34.0 μM IAA; Table 6) and were similar between those two treatments.

It is important to point out that the root system of *C. longa* formed several lateral and secondary roots and large amounts of root hair; therefore, the influence of regulators on growth was also measured using dry mass. The largest values for the dry matter of aerial part (DMAP) were found in three treatments, T3 (2.0 μM NAA), T4 (34.0 μM IAA), and T5 (44.0 μM IAA; Table 6). The DMAP was twice as small in the control treatment compared to T3, suggesting the importance of supplementary growth regulators (Table 6). Only isolated auxin, 2.0 μM of IAA, was efficient enough to increase RDM, since the lowest average was observed in T2 (Table 6).

DISCUSSION

Phase 1: Multiplication phase

C. longa shoots presented a well-developed root system with many lateral roots and an aerial part with a great number of leaves (Figure 1E). Depuydt and Hardtke (2011) reported that different plant organs do not necessarily respond similarly to the action of growth

regulators, and these responses can occur distinctly depending on the regulator. The cytokinin BA influences mitosis, whereas auxins affect DNA replication; combined, the two types of growth regulators regulate cell division (Nishiyama et al., 2011; Simon and Petrášek, 2011). This is a complex mechanism since these regulators act as signals for gene expression during development (Depuydt and Hardtke, 2011).

The most utilized growth regulators in assays within Zingiberaceae have been BAP, NAA, and KIN; however, other combinations could be tested in future assays. With concentrations close to the ones used in our study, Prakash et al. (2004) obtained high shoot growth rates in *Curcuma amada*, because a medium supplemented with (4.44 μM BAP + 1.08 μM NAA) and another to (8.88 μM BAP + 2.70 μM NAA) resulted in 80 and 72% regeneration, respectively when compared with a medium without growth regulators. In *Kaempferia galangal*, 8.87 μM of BAP added with 2.46 μM of IBA resulted in an average of 8.3 shoots (Chithra et al., 2005). However, Yunus et al. (2012), utilizing 13.32 μM BAP in *E. elatior*, observed two 3.15-cm shoots and approximately 3 leaves after 12 weeks of cultivation. A significant difference in leaf number among the treatments was not found in our study, and the average across all treatments was 6.5 leaves per shoot.

The results for these species indicated better outcomes with the utilization of only BAP, whereas the combination with an auxin is necessary in other species (Nayak, 2000; Bharalee et al., 2005; Kambaska and Santilata, 2009). In our assay, the addition of BAP led to greater gain in mass for the largest shoot (Figure 1D), improved root length when combined with KIN, and increased root mass when combined with NAA. The superiority of BAP for inducing responses, especially in shoots, can either be attributed to the capacity of the plant tissues to metabolize it more easily than other growth regulators, or to the capacity of this substance to induce the production of endogenous hormones like zeatin (Varshney et al., 2013). However, when comparing the control treatment to the others, it was observed that the average values for leaf and shoot

numbers were not significantly different. In the case of these two traits, the exogenous dose may not have been enough to induce endogenous hormones and, consequently, there was no response. Yunus et al. (2012) similarly verified that shoots had high survival rates and equal average shoot numbers in treatments with and without supplemental growth regulators in *E. elatior*. The same was observed for *C. longa* (Jala, 2012) and *Matthiola incana* (Kaviani, 2014).

Recently, flow cytometry has been used in the assays which evolves tissue culture, mainly when evolves growth development, where there is the possibility of occurrence of somaclonal variation in tissues.

Recently, flow cytometry has been used in assays involving tissue culture (Miguem and Marum, 2011) and self-tanning (Bennett et al., 2008). Moreover, studies utilizing flow cytometry for members of the Zingiberaceae family are scarce. For *C. longa*, DNA content ranged from 2.38 to 2.77 pg, regardless of the utilized standard. Across *Curcuma*, some variation in DNA content has been found depending on the studied species. Islam (2004) reported that *C. amada* varied from 3.2 to 5.3 pg, whereas *Curcuma angustifolia* and *Curcuma attenuata* reached 3.28 and 3.18 pg, respectively. The values obtained for these diploid species are higher than for *C. longa* (2n) and genome size is greater yet for polyploid species like *Curcuma malabarica*, varying from 4.30 to 8.84 pg (Nayak et al., 2006).

Estimates of DNA content in the present study varied slightly depending on the treatment. Possible explanations for this include intrinsic factors of the vegetal matter, such as hormonal interference and a possible induction of somaclonal variation (Miguel and Marum, 2011) and external factors that involve sample preparation prior to the cytometer readings (Camolesi et al., 2007); somaclonal variation may induce morphological, physiological and biochemical variation (Miguel and Marum, 2011; Georgiev et al., 2014) which may cause positive or negative characteristics. In morphology, the main alteration are the occurrence of variations which is common in ornamental plants and in pineapple (Rodrigues et al., 2007) or with over growth as in *Musa acuminata* cv *prata-anã* (Albany et al., 2005). In this study, DNA variations might be associated to these phenomena. However, future assays will be necessary to prove it and when detected, to identify which type of alterations was induced.

Some authors suggest that chemicals in the tissue can interfere with the analysis (Bennett et al., 2008). A phenomenon known as self-tanning weaves phenolic compounds into the DNA, resulting in reading errors in the equipment (Doležel and Greilhuber, 2007). In our study, the chemical composition of *C. longa* was not analyzed; however, numerous phenolic idioblasts were observed in foliar tissues (Figure 5A to D) and they may have caused the small variation in measurements.

DNA content in the absence of supplemental growth

regulators was larger or smaller from that seen for the other treatments, regardless of the utilized reference standard. The presence of idioblasts was observed even in the control treatment (Figure 5A); they occur naturally in this species. Although T2, T3, T7, and T8 resulted in the greatest shoot and root length and root mass, T4 and T5 showed the highest DNA content. The same result was reported for *Butia capitata*, in which idioblasts containing phenolic compounds were observed in all development phases of *in vitro* plants. Analysis by flow cytometry showed changes in DNA content at various stages of seedling development except at the embryo stage; the authors attributed the presence of idioblasts containing phenolic compounds as responsible for altering the DNA content (Magalhães et al., 2015).

Tannins have been noted as the main chemical compounds that interfere with cytometer readings (Loureiro et al., 2006; Doležel and Greilhuber, 2007; Bennett et al., 2008). However, these same authors reported that DNA content can be estimated by cytometry without problems if one takes precautions. To mitigate the effect of these compounds, extraction buffers can be used to promote a greater removal of phenolic compounds (Loureiro et al., 2006), including Galbraith's buffer and LB01 (Galbraith et al., 1983; Doležel et al., 1989), Otto buffer, and other adaptations (Otto, 1990; Doležel and Göhde, 1995). Other vegetal materials like dry roots and seeds (Jedrzejczyk and Sliwiska, 2010) can also be used for analyses, but colored tissues such as flowers and fruits should be avoided (Bennett et al., 2008).

Due the importance of the influence of growth regulators in plant development in our assay, it was visually observed that the treatments did not cause abnormal seedling formation, what was confirmed in anatomical analysis of the main *C. longa* tissues (xylema, phloem, parenchyma and epidermis). All showed an appropriated pattern of development. At the end of the assay, the seedling was healthy and vigorous (Figure 1E). External epidermis tissue followed the pattern according to *Alpinia zerumbet* (Albuquerque and Neves, 2004). In parenchyma, the presence of idioblasts was observed with phenols. Thong et al. (2009) described the presence of tannin idioblasts in the basal regions of *Alpinia purpurata* seedlings. Albuquerque and Neves (2004) reported a great number of tannin idioblasts in the fundamental parenchymatic tissue of all studied vegetal parts in *A. zerumbet*. The same authors mentioned that occasionally tannins can be found in trichomes of the leaf edge, but trichomas were not observed in *C. longa* in the present study.

In monocots like *C. longa*, veins and bundles stretch along the leaf, forming parallel or striated nervation. Albuquerque and Neves (2004) observed that all secondary veins branch out from the main vein and grow parallel toward the edges in *A. zerumbet*. The conducting tissue, inserted in the parenchyma, has phloem facing

the abaxial face and xylem oriented towards the adaxial face of the leaf.

Xylem of *C. longa* was differentiated at shoot emergence, with the presence of numerous vein elements. Compared to other anatomical measurements, the xylem and phloem had less variation among the treatments (Figure 5D to E). This might have been because of the time that the plants were kept *in vitro* was not enough to realize a more pronounced differentiation of tissues. Obviously, the xylem differentiation process would be resumed, the slower the differentiation of xylem and phloem appears to occur at the time tested here. Another factor is due to the action of gibberellins which has a direct role in the differentiation of xylem; according to McKenzie and Deyholos (2011), the exogenous addition of the gibberellin GA₃ was essential to initiate thickening of xylem cells in *Linum usitatissimum* L along the cultivation of this species. The authors reported that the presence of GA₃ was essential to promote the differentiation process of this tissue as well as promoting thickness gains. In this work, no exogenous gibberellin was not added to confront the analyzed treatments.

Phase 2: Rooting phase

Although there was no cytokinin in the media for the rooting phase, SN responded to the supplemental auxin. The presence of a growth regulator seems to be necessary to increase SN in Zingiberaceae. Bharalee et al. (2005) did not observe any shoot growth from *Curcuma caesia* in base medium. The same was verified for *Z. officinale* by Kambaska and Santilata (2009) and for *C. amada* by Prakash et al. (2004). Recent studies have shown the role of auxins not only in cell elongation but also in allowing cells to progress through the G1/S transition of the cellular cycle (Perrot-Rechenmann, 2010).

In contrast, the longest root length (RL) values were seen in the control treatment, with no benefits seen with the addition of growth regulators. In this case, the amount of endogenous auxin may have been sufficient to stimulate cell elongation in the roots but not in the aerial part. This difference between the aerial part and roots may be due to the existence of at least five TIR1/AFB families of IAA receptors in the nucleus (Simon and Petrášek, 2011). It is likely that different receptors are active in the different parts of the plant (Vierstra, 2009). Villa et al. (2008) obtained similar results for *Rubus* species in which exogenous auxin did not improve root length total but did increase fresh matter of aerial part.

The results demonstrate that the auxin is important to provide mass increments *C. longa* root. The average total root dry mass was twice as high in the treatment 3 compared to treatment 2 and control. This is consistent with other studies demonstrating the effects of auxin in increasing root mass and number (Aslam et al., 2013; Aroonpong and Chang, 2015).

Conclusion

In the multiplication phase, the addition of BAP to the culture medium increased the fresh mass of the mother plant. The combination of auxin and cytokinins was vital for increasing the mass of the root system, and resulted in seedlings with a greater number of lateral roots.

DNA content varied in samples according to the treatments. When using *P. sativum* as an external reference standard, the content varied from 2.53 pg (in T5) to 2.38 pg (in T3), and when using *S. lycopersicum*, it ranged from 2.77 pg (in T4) to 2.62 pg (in T3).

There was quantitative variation in the measured anatomical characteristics. Epidermis and parenchyma were the tissues most affected by the action of regulators. In general, there was a thin layer of epidermis with rectangular cells, followed by parenchyma with octahedral cells. Xylem and phloem had already been differentiated with extensive leaf branching.

In the rooting phase, auxins were not necessary to increase length, but they were essential to increase root system mass and the presence of lateral roots.

Conflict of interest

The authors declare that they have no conflict of interest.

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