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Scientific Research

Optimization of the Protocol for the *in Vitro* Cultivation of *Piper aduncum* L.

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Abstract

***Piper aduncum* L. (Piperaceae) has great potential for economic exploration because of the proven use of its essential oil in the agriculture and in the human health. A technique that improves its propagation, as the tissue culture, becomes necessary. Some parameters must be determined for the successful cultivation *in vitro.* Thus, this study aimed to determine the salts concentration of MS medium, temperature, luminosity and light quality for *in vitro* culture of this species. The following treatments were conducted: 1/4MS, 1/2MS, MS and 2MS; 20˚C, 25˚C, 30˚C and 35˚C; monochromatic blue, red and white lights and the combination of red and blue, using light emitting diodes (LEDs); luminosities of 17, 37, 48 and 73 µmol·m^{-2}·s^{-1} obtained with cool white fluorescent lamp. After 35 days, the treatments were analyzed. To determine the salts concentration of MS, the variables were evaluated: number of shoots, leaves and roots, length and width of leaves, length and dry mass of root and shoots and percentage of death by necrosis. To the plantlets under different temperatures, length and dry mass of shoot and root, number of shoots, number, length and width of leaves and survival and rooting percentages were measured. The plantlets that were maintained under different luminosities were evaluated for length of shoot and root, dry mass of leaf and root. To evaluate the growth under the LEDs, the length and dry mass of shoot and root, number of shoots and roots, percentage of rooting and sprouting were assessed. The medium 1/4MS and the medium 1/2MS showed better responses for number and length of root, leaf width and shoot length. The temperature 25˚C provided the highest number of leaves, length of shoot and root, root dry mass and rooting percentage. The luminosity 73 µmol·m^{-2}·s^{-1} provided higher values in length of root and dry mass of leaf and root. The red LED provided plantlets with greater growth. Thus, for the *in vitro* cultivation of *Piper aduncum*, 1/4MS, environment temperature of 25˚C, light intensity of 73 µmol·m^{-2}·s^{-1} and environments with red light to obtain high quality plantlets for propagation of this species are recommended.**

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Keywords

Tissue Culture, LEDs, Monkey Pepper

1. Introduction

Piper aduncum (Piperaceae) is a shrub that is native to tropical regions of the Americas and was introduced in Asia during the 19th century [1]. It produces an essential oil that has been used and reported as medicinal for the treatment of many diseases [2]-[4] and has been highlighted, especially, by showing an insecticidal, larvicidal, leishmanicidal, molluscicide, antibacterial and antifungal activities [4]-[9].

Most of the medicinal plant species that are used by the population are obtained from natural habitats. The ability to sustain production of raw vegetable materials that are of high quality under controlled conditions is important because of the need to produce standardized herbal medicines [10]. Additionally, there is disordered extraction of species in natural environments which promotes the extinction of these species. The tissue culture is a technique that allows the production of secondary metabolites *in vitro*, ensuring alternative way for the sustainable exploitation of species, especially from threatened ecosystems. Among the perspectives there is the obtainment of a germoplasm that is competitive and adapted to various growing methods, and the possibility of choosing new species that will serve as a source of biologically active compounds and improve the production of phytochemicals [11].

However, tissue culture requires the analysis of some parameters, such as explants, aseptic nutrient medium, light conditions and temperature [12]. Regarding the luminosity, the light quality has also been a concern for the *in vitro* culture. Recently, light emitting diodes (LEDs) have drawn considerable interest as an alternative light source for *in vitro* propagation. The advantages of LEDs are their large photo fluxes of red photons, their light weight [13] and their high electrical energy conversion efficiency [14]. Pure R-LEDs are a potential light source for growing plants in space-flight systems because of their safety, small mass and volume, wavelength specificity and longer life [15]. Despite these attractive features of the LEDs system, studies on growth and morphogenesis of different species under different LEDs systems are very limited.

Given the importance of these factors on *in vitro* culture, this work aimed to study the effects of salts concentrations in the culture medium MS, the effects of temperature, luminosity and light quality (with the use of LEDs), in the *in vitro* growth of *Piper aduncum* L. in order to optimize the protocol for *in vitro* cultivation of this species.

2. Materials and Methods

2.1. Plant Material

For the experiments, the *Piper aduncum* L. seeds were established at Laboratory of Tissue Culture and Medicinal Plants of the Federal University of Lavras (UFLA). For asepsis, the seeds were immersed in a solution of sodium hypochlorite, 60% (v/v) for 15 minutes under constant stirring. After the seeds were washed 5 times with distilled and autoclaved water within laminar flow. After the germination, the seedlings were subcultured and inoculated into test tubes containing MS medium [16] with 30 $g \cdot L^{-1}$ of sucrose, 0.6% agar and pH adjusted to 5.7 ± 0.1.

Nodal segments containing two buds were inoculated and conducted to four conditions of *in vitro* culture: different salts concentrations of MS medium, temperatures, luminosities and wavelengths of LEDs. In all the experiments, were used tests tubes containing culture medium MS, supplemented with 30 $g \cdot L^{-1}$ of sucrose, solidified with 0.6% agar and pH adjusted to 5.7 ± 0.1. The plantlets were maintained under photoperiod of 16 hours of light in all the experiments. The luminosity was 16 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for the temperature experiment and 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for the salts concentration one. A temperature of 26˚C ± 1˚C was used for the salts concentrations, luminosity and LEDs experiments. The segments were inoculated in a horizontal position, and were carried out on a completely randomized design and evaluated after 35 days.

2.2. Salts Concentrations of Medium MS

To determine the effect of salts concentrations of medium MS, the segments were inoculated in the following

treatments: 1/4MS, 1/2MS, MS and 2MS, with 5 replications and 4 tubes per replicate. The number of shoots, leaves and roots, length and width of leaves, length and dry mass of shoot and root and percentage of death by necrosis, were evaluated.

2.3. Temperatures

In the experiment aiming to analyzing the effect of temperature, the explants were taken to four growth chambers, with white fluorescent light and temperatures of 20˚C, 25˚C, 30˚C and 35˚C ± 1˚C with 5 replicates and 5 tubes for each replication. The length and dry mass of shoot and root, number of shoots, number, length and width of leaves and survival and rooting percentages were evaluated.

2.4. Growth under Different Luminosities

The explants were subjected to the following treatments: 17, 37, 48 and 73 μmol·m^{-2}·s^{-1}, obtained with cool white fluorescent lamp. The experiment was performed with 4 replicates and 4 tubes for each replicate. The plantlets were evaluated for growth: length of shoot and root, dry mass of leaf and root.

2.5. Growth under Different Light Emitting Diodes

The explants were cultured under different spectra of LEDs, totaling 6 treatments: blue, red, white, 28% blue + 72% red, 72% blue + 28% red and 50% blue + 50% red (**Figure 1**) with 4 replications and 4 tubes per replication. The length and dry mass of shoot and root, the number of sprouting and root and percentages of rooting and shoots were assessed.

2.6. Statistical Analysis

The data were subjected to ANOVA by F ($p < 0.05$), using the Sisvar® software [17]. The growth variables were analyzed by regression (luminosity) and by the Scott-Knott test (MS salt concentrations, temperature and growth under LEDs).

3. Results and Discussion

Lower concentrations of salts (1/4MS and 1/2MS) favored the *in vitro* growth of the species (**Figure 2**). It was observed that the high salts concentration in the culture medium is detrimental to *P. aduncum*, because it decreased rooting and shoot growth (**Table 1**). No influence of the concentration of salts was observed in the number of shoots, and the death for necrotic occurred only in cultured explants in 2MS, which showed 25% of necrosis.

Plants grown *in vitro* under high salt concentrations may reduce the synthesis of carbohydrates altering growth. Furthermore, the high salt concentration may promote osmotic effect in the culture medium *in vitro* which hinders the absorption of water by plantlets. Thus, the negative effects caused by higher concentrations of MS medium are probably due to a higher amount of salt than what naturally the species receive, becoming inadequate to the morphogenic process [18].

Sousa [19] found that during the multiplication of explants from *P. aduncum* plants cultivated *in vitro*, concentrations of salts of the MS (1/2, 3/4 and 100%) can directly influence the process of micropropagation of the species by microcuttings relative to height and number of buds. *P. aduncum* showed better results, both in the number of buds and in height when grown in environments with concentrations of 1/2 and 3/4 of MS salts. As in the present work, the species showed better development in salt concentrations lower than the full MS. Ghimire [20] studying *Solanum aculeatissimum*, medicinal plant, obtained the results for the number of shoots, in descending order: 1/3MS, 1/2MS, MS, 1/4MS, respectively. And root length, also in descending order, with the cultivation of the species in: MS, 1/2MS, 1/3MS, and 1/4MS, respectively showing that the ideal salt concentration of MS depends on the species under study.

Regarding the temperature, the explants cultured under 35˚C did not survive. The temperature of 25˚C favored the growth of the species, with higher means for number of leaves, length of shoot and root, root dry mass and rooting percentage (**Table 2** and **Figure 3**). Silva [21] evaluated *P. aduncum* under temperatures of 10˚C, 20˚C and 25˚C, and it was observed that after six months of storage *in vitro* occurred an influence of temperature on the survival, shoot length and number of buds per shoot. The highest temperatures tested (20˚C and 25˚C) promoted an increase in variables.

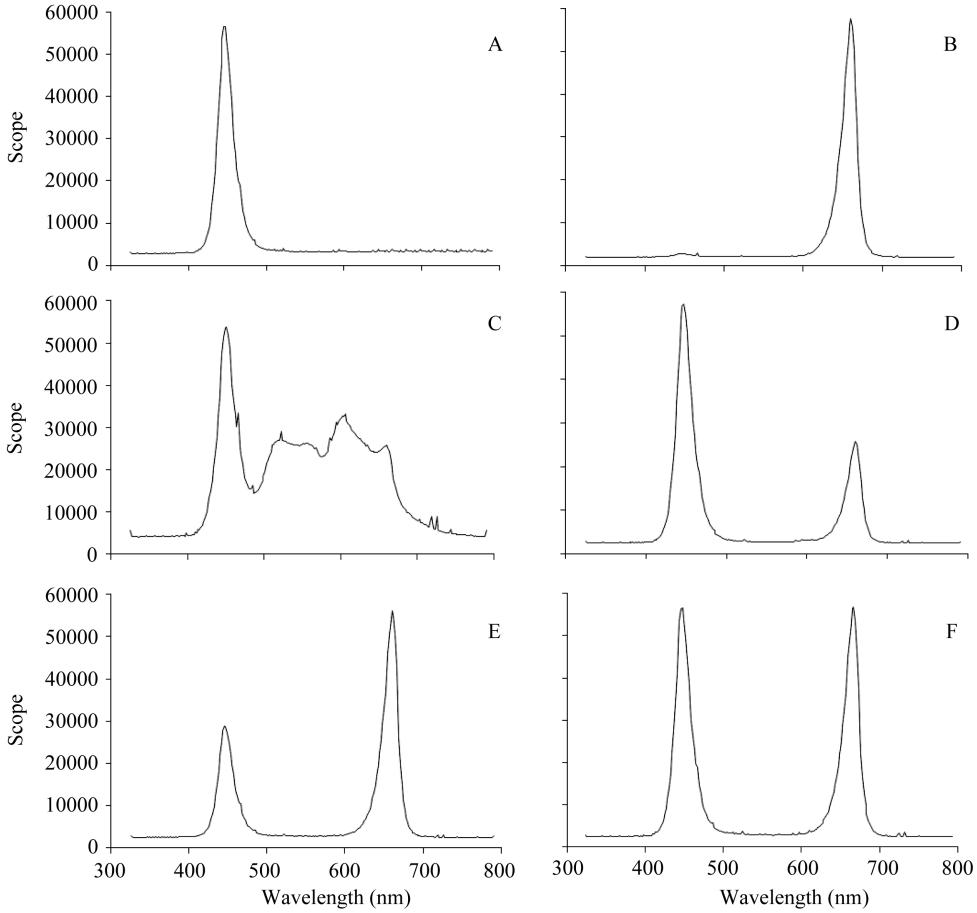

Figure 1. Relative spectral emission of light sources used in the experiments (scope in function of wavelength (nm)). A = blue; B = red; C = white; D = 72% blue + 28% red; E = 28% blue + 72% red; F = 50% blue + 50% red.

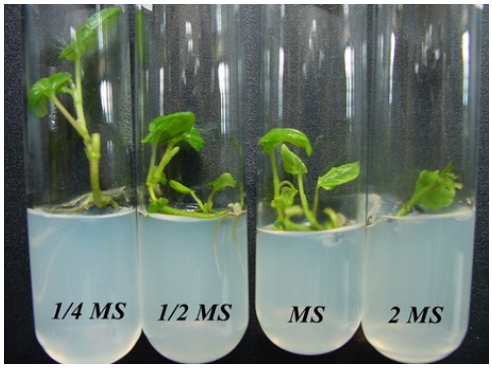

Figure 2. Overall aspect of *P. aduncum* subjected to different salts concentrations of MS (1/4MS, 1/2MS, MS and 2MS). Lavras, 2014.

Plants exposed to high temperatures suffered injury, exhibiting reduction or stop of growth and the degree of injury can vary with plant species [22]. This effect is due to the suppression of elongation growth rate of cells, because of an irreversible inhibition exerted by high temperature due to protein damage [23]. In addition, somatic embryos of *Eleutherococcus senticosus* exposed at 12˚C, 16˚C, 24˚C and 30˚C showed reduction in growth at high temperatures, because it resulted in a reduction of the activity of antioxidant enzymes that protect

Table 1. Growth of *P. aduncum* in different concentrations of MS medium. Lavras, 2014.

Salts	Variables (means ± DP)									
	NS	NR	LN	RL	LL	LW	SL	N	DMS	DMR
				(cm)				(%)	(mg)	
1/4MS	1.20 ± 0.33 a	5.60 ± 0.72 a	2.50 ± 0.42 a	1.81 ± 0.20 a	1.60 ± 0.31 a	1.22 ± 0.24 a	1.80 ± 0.28 a	0.00 b	7.91 ± 2.13 a	1.18 ± 0.33 b
1/2MS	1.30 ± 0.21 a	5.05 ± 1.04 a	3.15 ± 0.42 a	2.47 ± 0.28 a	1.76 ± 0.13 a	1.33 ± 0.16 a	1.73 ± 0.19 a	0.00 b	8.17 ± 1.07 a	2.40 ± 0.46 a
MS	1.32 ± 0.21 a	2.23 ± 0.95 b	2.55 ± 0.45 a	1.51 ± 0.89 a	1.14 ± 0.25 b	0.84 ± 0.12 b	1.32 ± 0.38 b	0.00 b	7.31 ± 1.95 a	0.19 ± 0.19 c
2MS	1.05 ± 0.45 a	0.15 ± 0.02 c	1.62 ± 0.72 b	0.05 ± 0.05 b	0.91 ± 0.54 b	0.59 ± 0.13 b	0.67 ± 0.38 c	25.00 ± 15.00 a	4.83 ± 1.65 b	0.00 c

NS: number of shoots; RL: root length, NR: number of roots; LL: leaf length; LW: leaf width; LN: leaf number; SL: shoot length; N: necrosis; DMS: dry mass of shoot; DMR: dry mass of root. Means followed by the same letter do not differ statistically from each other, by the Scott-Knott ($p < 0.05$).

Table 2. Growth of *P. aduncum* under different temperatures. Lavras, 2014.

T (˚C)	Variables (means ± DP)									
	NS	NL	SL	LL	WL	RL	DMS	DMR	S	R
			(cm)				(mg)		(%)	
20	1.24 ± 0.36 a	1.76 ± 0.38 b	0.27 ± 0.16 c	1.32 ± 0.25 a	0.86 ± 0.19 a	0.09 ± 0.03 b	14.32 ± 4.22 b	0.00 b	96.00 ± 8.94 a	8.00 ± 3.95 b
25	1.20 ± 0.20 a	3.20 ± 1.24 a	1.20 ± 0.32 a	0.68 ± 0.41 b	0.58 ± 0.41 a	2.22 ± 0.87 a	49.00 ± 12.6 a	9.72 ± 5.10 a	96.00 ± 8.94 a	68.00 ± 22.80 a
30	1.12 ± 0.27 a	1.80 ± 0.58 b	0.72 ± 0.40 b	0.00 c	0.00 b	0.12 ± 0.08 b	28.60 ± 9.70 a	0.06 ± 0.01 b	72.00 ± 12.80 b	8.00 ± 3.95 b
35	0.00 b	0.00 c	0.00 c	0.00 c	0.00 b	0.00 b	0.00 b	0.00 b	0.00 c	0.00 b

NS: number of shoots; NL: number of leaves; SL: shoot length; LL: leaf length; WL: width of leaves; RL: root length; DMS: dry mass of shoot; DMR: dry mass of roots; S: survival; R: rooting. Means followed by the same letter do not differ statistically from each other, by the Scott-Knott ($p < 0.05$).

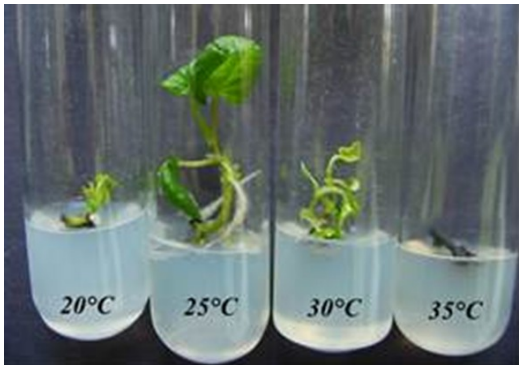

Figure 3. Overall aspect of *P. aduncum* cultivated *in vitro* under different temperatures. Lavras, 2014.

cells against damage from the stress of high temperatures [24].

Changes in luminosity affected the growth variables analyzed (**Figure 4** and **Figure 5**). The increase of the luminosity resulted in an increase of the length and dry mass of root, and dry mass of leaf (**Figure 4**). In contrast, the shoot length reduced with the increasing of the luminosity. The highest production of dry mass obtained under higher luminosity indicates that low luminosity may be limiting for the *in vitro* growth of *Piper aduncum* plants. In moderate luminosity, plants generally bear longer internodes, and are less resistant than those grown under intense light [25]. This may reflect the reduction in shoots length in high luminosity.

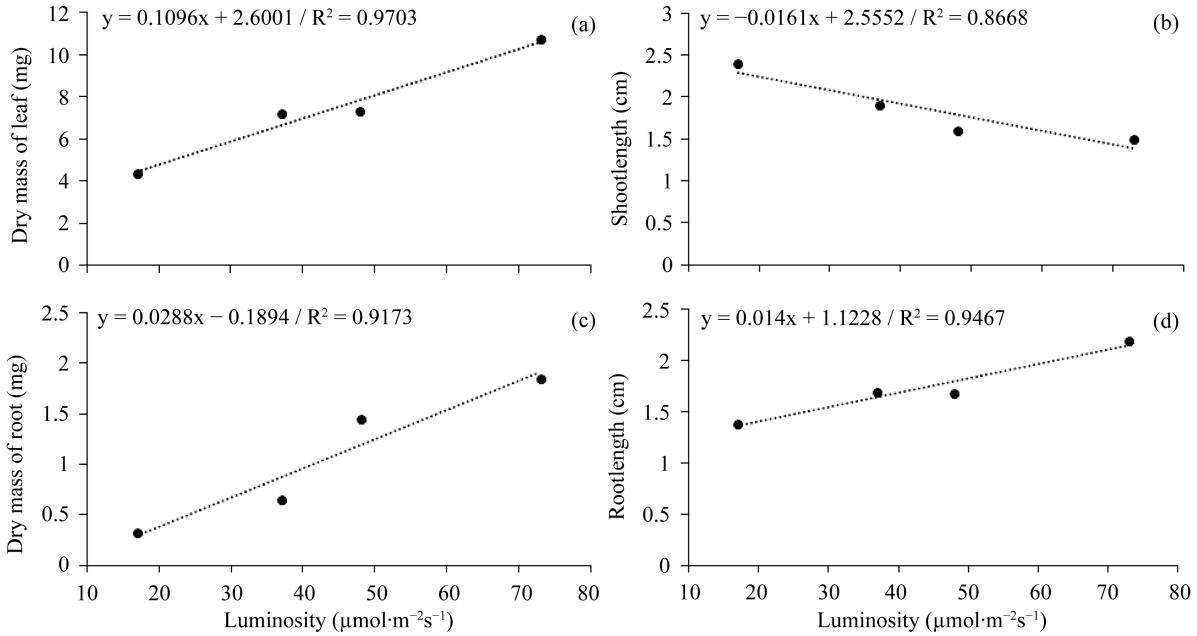

Figure 4. Dry mass of leaf (a), shoot length (b), dry mass of root (c), and root length (d) of plantlets of *P. aduncum* cultivated under different luminosities (µmol·m^{-2}·s^{-1}). Lavras, 2014.

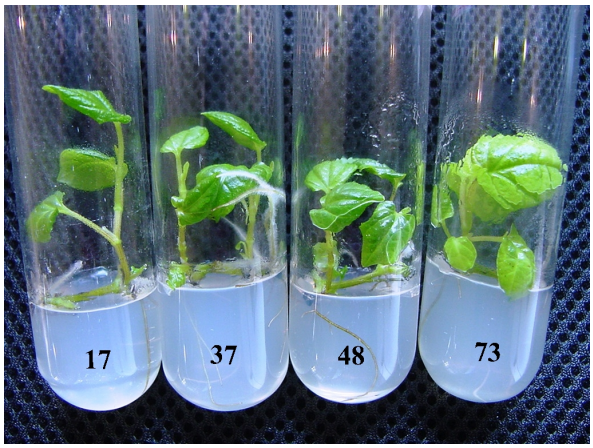

Figure 5. Overall aspect of *P. aduncum* L. cultivated under different luminosities: 17, 37, 48 and 73 µmol·m^{-2}·s^{-1}, respectively. Lavras, 2014.

Light is the ultimate substrate for photosynthetic energy conversion [26]. Thus, plant dry mass decreases with decreasing luminosity [27]. In addition, the acclimatization to high irradiances usually results in an increased photosynthetic activity [28]. This improvement is not only associated with more carbon available for growth but also with the fact of that light promotes photomorphogenic responses [29].

The growth parameters varied in response to the different LEDs (**Table 3** and **Figure 6**). The root length, number of roots, number of shoots, dry mass of shoots and root and percentage of survival were higher under the red light, white and 28% blue + 72% red (**Table 3**). It can be stated that the red light applied alone or in combination favored the development of *Piper aduncum* cultivated *in vitro*. Growth induction by red light has been reported in several species such as strawberry (*Fragaria x ananassa*) and grapes (*Vitis ficifolia*) [30] [31].

Hence, the present study revealed that a longer wavelength is required for the root elongation of monkey pepper. Light may influence root elongation through photomorphogenic action, *i.e.*, root elongations may be controlled by phytochrome [32].

Table 3. Growth of *P. aduncum* under different LEDs. Lavras, 2014.

Light espectrum	Variables (means ± DP)							
	NS	NR	SL	RL	DMS	DMR	S	R
			(cm)		(mg)		(%)	
Red	1.48 ± 0.21 a	1.96 ± 0.56 a	1.41 ± 0.22 a	1.33 ± 0.23 a	7.92 ± 1.90 a	1.15 ± 0.72 a	93.75 ± 12.50 a	87.50 ± 14.43 a
Blue	1.09 ± 0.94 b	0.98 ± 0.25 b	0.90 ± 0.71 c	0.94 ± 0.43 b	4.08 ± 1.47 b	0.82 ± 0.28 b	56.25 ± 22.70 b	37.50 ± 12.27 b
White	1.31 ± 0.64 a	1.63 ± 1.00 a	1.05 ± 0.50 b	1.18 ± 0.62 a	5.95 ± 2.93 a	0.97 ± 0.32 a	68.75 ± 13.94 a	56.25 ± 13.24 b
28% blue + 72% red	1.34 ± 0.32 a	1.60 ± 0.68 a	1.08 ± 0.49 b	1.35 ± 0.57 a	8.04 ± 3.04 a	1.04 ± 0.52 a	81.25 ± 13.94 a	75.00 ± 20.41 a
72% blue + 28% red	0.96 ± 0.69 b	1.04 ± 0.87 b	0.87 ± 0.19 c	1.03 ± 0.45 b	2.88 ± 1.47 b	0.79 ± 0.64 b	37.50 ± 15.00 b	37.50 ± 15.00 b
50% blue + 50% red	0.93 ± 0.36 b	1.14 ± 0.98 b	0.89 ± 0.84 c	1.03 ± 0.42 b	3.58 ± 1.50 b	0.85 ± 0.49 b	37.50 ± 14.43 b	31.25 ± 12.50 b

NS: number of shoots; NR: number of roots; SL: shoot length; RL: root length; DMS: dry mass of shoot; DMR: dry mass of roots; S: survival; R: rooting. Means followed by the same letter do not differ statistically from each other, by the Scott-Knott ($p < 0.05$).

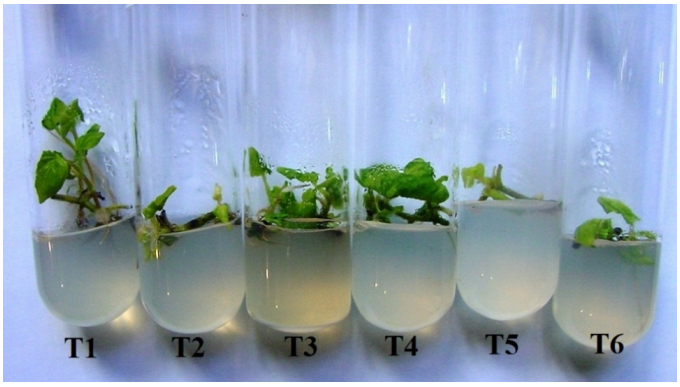

Figure 6. Overall aspect of the species *Piper aduncum* L. maintained on different treatments with light emitting diodes (LEDs). T1 = red; T2 = blue; T3 = white; T4 = 28% blue + 72% red; T5 = 72% blue + 28% red; T6 = 50% blue: 50%. Lavras, 2014.

4. Conclusions

It is concluded that the 1/4MS medium is the most suitable for the *in vitro* cultivation of *Piper aduncum* L. The ideal temperature for growing *in vitro* is 25˚C.

Luminosity also affected the *in vitro* growth of *P. aduncum*. The species has a maximum length of shoots when grown under 17 µmol·m^{-2}·s^{-1}, and greater root length, dry mass of leaves and roots when grown at higher luminosities 73 µmol·m^{-2}·s^{-1}.

It appears that red LEDs might be effective for increasing length, number and dry mass of shoots and root and percentages of shooting and rooting. Hence, the red light is recommended for cultivation *in vitro* of *Piper aduncum*.

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