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Optimization of culture media for *Desmodium incanum* micropropagation

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Desmodium incanum DC., popularly known as pega-pega, is a wide spread leguminous plant in fields in the state of Rio Grande do Sul in Southern Brazil and is well accepted by the cattle as forage. However, its forage potential is currently threatened, due to the replacement of natural grasslands by agricultural crop and grazing lands and because of the poor pasture management associated with high stocking rate, making it necessary to search for alternatives for the preservation of this genetic resource. The aim of this study was to test variations in the composition of culture media in order to optimize the development of *in vitro* plantlets of *D. incanum.* The study was divided into three stages: the first test, evaluated different compositions of MS medium, varying concentrations of nutrients, especially macronutrients (MS 50%; MS 50% and macronutrients at 25%; MS 100% and macronutrients at 50%; MS 100% and macronutrients 25%); the 2nd test evaluated different concentrations of MS medium nutrients, especially micronutrients (MS 50%; MS 100% and micronutrient at 50%; MS 50% and micronutrients at 25%); and the 3rd test, in which varying concentrations of IBA (0, 0.5, 1.0 and 1.5 mgL^{-1}) were evaluated. In Test 1, the culture media with components diluted to 50% provided better development for *D. incanum* in relation to the media with 100% of the components, and MS 50% was the best treatment. In Test 2, the MS medium with 25% of micronutrients and 50% of other components provided the best growth. In Test 3, the species *D. incanum* responded positively to the addition of IBA with an increase in root development.

Key words: *In vitro,* forage, pampa biome, leguminous plant, mineral nutrition.

INTRODUCTION

Desmodium incanum, popularly known as pega-pega, is a perennial, summer crop and native leguminous plant which presents a prostrated and growing growth habit

(Oliveira, 1983). According to Burkart (1939), the species is widely spread throughout fields in the state of Rio Grande do Sul (RS) fields in Southern Brazil and Damé

(1999) found that *D. incanum* was predominant in the Fabaceae family, with a frequency above 50%, except during the winter period, when its population was considerably diminished. It is an important forage due to its favorable qualitative characteristics and its good acceptance by the animals (Boldrini, 1993). The effective dispersion of *D. incanum* is associated to the trichomes present on the fruit epicarp, which facilitate t h e spread of seeds through the epizoocoria (Souza et al., 2006).

However the information available about the biodiversity of native grasslands is still lacking (Overbeck et al., 2007), and their forage potential is still neglected by most technicians and producers, due to improper management of native pastures, combined with intense pressure from grazing due to high stock densities, leading to the extinction of many field species.

Between 1970 and 2005, Pillar et al. (2009) estimated that 4.7 million hectares of native grasslands in the state had been converted to other agricultural uses, such as farming and afforestation areas. In this context, there is a need for preservation of forage and *D. incanum* appears as a promising alternative because of its potential for recovery of native grassland areas affected by crops (Favreto, 2004).

One efficient strategy for the preservation of germplasm is *in vitro* growth, where whole plants can be obtained from the growth of cells, tissues or plant organs (Pence, 2011). *In vitro* growth techniques allow for the control of climate variables such as light intensity, temperature and humidity, and also the availability of nutrients and vitamins that the plant can acquire from the culture medium. Nutritional requirements vary among different species, making it necessary to perform initial tests and adjustments for the *in vitro* establishment of any species.

In order to induce the processes responsible for generating tissues and organs, growth regulators are often used to stimulate the formation of shoots and roots (Kielse et al., 2009). Auxins act on cell expansion, elongation and division, their main function being root induction (Blakesley et al., 1991; Werner and Pla, 2012).

Successful *in vitro* development necessitates knowledge of a species' nutritional requirements, thus it is necessary to verify the best growth medium composition for each species. As *D. incanum* is native to acid soils with low fertility (Miotto, 2011), it can be assumed that it does not have a high nutritional requirement, but this information needs to be tested. In addition, little is known about the tolerance of this species to salinity (Marques, 1991). Thus, the aim of this study was to test variations in the nutritional composition of growth media in order to optimize the *in vitro* development of seedlings of *D. incanum*.

MATERIALS AND METHODS

In all tests, the growth media were based on variations of MS growth medium (Murashige and Skoog, 1962) (Table 1). Besides the macro and micronutrients and vitamins, 30 gL^{-1} of sucrose and 100 mgL^{-1} of myo-inositol were added to the MS media. After dilution, the media were completed to a volume of 1 L. The pH of the culture media was adjusted to 5.8 and 7 gL^{-1} of agar were added before autoclaving. The seeds of *D. incanum* were collected at the Iwar Beckman Research Center - Fepagro Campanha, lat. 31.48'S and long. 53.88'W, mechanically scarified on sandpaper number 120 (sand grains cm^{-2}), and then disinfected according to the methodology proposed by Maldaner et al. (2014). After inoculation, the material was kept in a growth room at a temperature of 25 ± 2°C, photoperiod of 16 h of light and photon fluency rate of 35 µmol m^{-2}s^{-1} provided by cool-white fluorescent lamps.

All tests were conducted in a completely randomized design with 20 replications, and the experimental unit consists of a glass flask (100 ml) with approximately 20 ml of culture media containing three seeds of *D. incanum*. Subsequently, the tallest seedling of each flask was used for evaluations.

Test 1: Nutrient concentration - Effect of macronutrient in *D. incanum* development

Four different growth medium compositions were evaluated as shown in Table 2. At the thirtieth day of growth, the following aspects were evaluated: height, number of nodes and number of leaves of *D. incanum* seedlings. The resulting data were subjected to analysis of variance and means of each treatment were compared by Tukey test at 5% probability (p ≤ 0.05). Statistical analyses were performed using the software ASSISTAT 7.7 (Silva and Azevedo, 2002).

Test 2: Nutrient concentration - Effect of micronutrients in *D. incanum* development

The different growth medium compositions are presented in the Table 3. After thirty days of growth, the following morphological data were recorded: height, number of roots, number and color of leaves, number of shoots, root dry weight and shoot dry weight. For the color of leaves notes were given from 1 to 4, with 1 for lightest and 4 for darkest green. The resulting data were subjected to analysis of variance and means of each treatment were compared by Tukey test at 5% probability (p ≤ 0.05). Statistical analyses were performed using the software ASSISTAT 7.7 (Silva and Azevedo, 2002).

Test 3: Effect of different concentrations of indolebutyric acid – IBA in *D. incanum* development

After being sterilized and scarified, the seeds were germinated in a growth medium with half of the concentration of all mineral nutrients, vitamins and FeEDTA of the MS culture medium (MS to 50%), and subjected to different concentrations of indolebutyric acid (IBA) concentrations (0, 0.5, 1.0 and 1.5 mgL^{-1}). At the end of thirty days, height, number of shoots, number of leaves, number of roots, root dry weight and shoot dry weight were evaluated. The resulting data were subjected to analysis of variance and when there was a significant difference, with α = 0.05, the means of each treatment were subjected to regression analysis. Statistical analyses were performed using the software ASSISTAT 7.7 (Silva and Azevedo, 2002).

RESULTS

The treatments in Tests 1 and 2 were chosen based on

Table 1. Composition of MS culture midium (Murashige and Skoog, 1962).

Component	Formula	Concentration (mg L^{-1})
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride	$CaCL_2.2H_2O$	441
Magnesium sulphate	$MgSO_4.7H_2O$	370
Potassium phosphate	KH_2PO_4	170
EDTA disodium	Na_2EDTA	37,25
Potassium iodide	KI	0,83
Micronutrients		
Iron sulfate	$FeSO_4.7H_2O$	27.85
Manganese Sulfate	$MnSO_4.H_2O$	16.9
Zinc sulfate	$ZnSO_4.7H_2O$	8.6
Boric acid	H_3BO_3	6.2
Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.25
Cobalt Chloride	$CoCL_2.6H_2O$	0.025
Copper sulphate	$CuSO_4.5H_2O$	0.025
Vitamins		
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine hydrochloride	$C_6H_{12}ClNO_2$	0.5
Thiamine hydrochloride	$C_{12}H_{18}CL_2N_4OS$	0.5
Glycine	$C_2H_5NO_2$	2.0
Myo-inositol	$C_6H_{12}O_6$	100.0
Agar		7.000
Sucrose	$C_{12}H_{22}O_{11}$	30.000

Source: Adapted from Oliveira et al. (2005).

Table 2. Treatments (T1, T2, T3 and T4) that have been derived from the standard composition of MS medium (table 1) and their respective percentage of each constituent.

Test 1	Vitamins (%)	Macronutrients (%)	Micronutrients (%)	FeEDTA (%)
T1	50	25	50	50
T2 – standard*	50	50	50	50
T3	100	50	100	100
T4	100	25	100	100

T2 was choice as standard treatment due results from previous experiments.

Table 3. Treatments (T1, T2 and T3) that have been derived from the standard composition of MS medium (table 1) and their respective percentage of each constituent.

Test 2	Vitamins (%)	Micronutrients (%)	Macronutrients (%)	FeEDTA (%)
T1 –standard *	50	50	50	50
T2	100	50	100	100
T3	50	25	50	50

*T1 was choice as standard treatment due results from previous experiments.

results of previous experiences (Schwalbert et al., 2014), in which reducing the medium components to half (MS medium to 50%) showed better results than the full culture medium for *D. incanum*. Therefore the medium
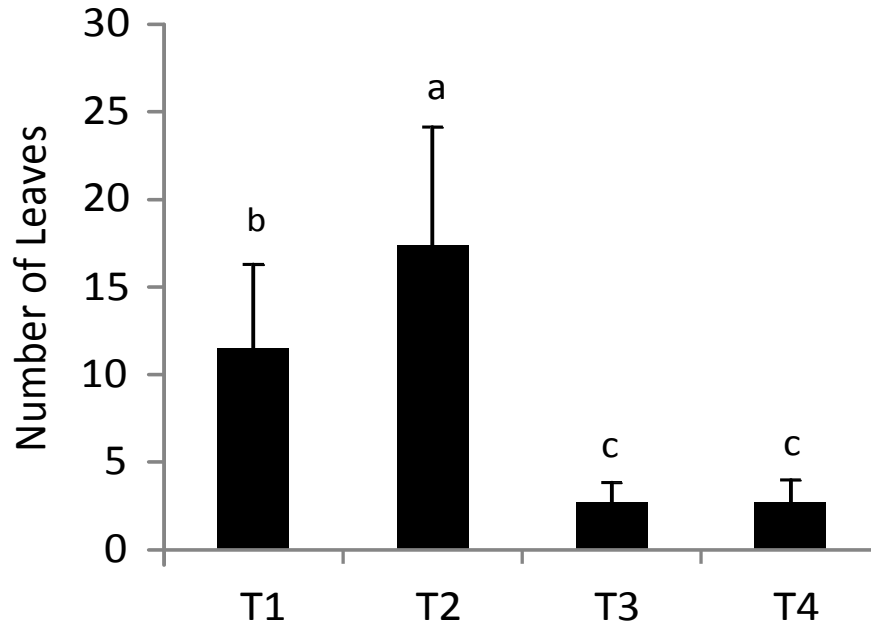
Figure 1. Effect of different compositions of MS culture media in seedling height (A), number of nodes (B) and the number of leaves (C) of *D. incanum* cultivated *in vitro*. Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

with half of all the components was adopted as the standard in both tests. The other treatments were an attempt to find out which medium component, when reduced in relation to the complete medium, favored the growth of the species. The best treatment found in Tests 1 and 2 was used for Test 3.

Test 1: Nutrient concentration - Effect of macronutrient in *D. incanum* development

Variation of macronutrient concentrations significantly affected the height of shoots, as well as the number of nodes and the number of leaves of *D. incanum* grown *in vitro*. Half of the concentration of the basic nutrient composition of MS culture medium (T2) promoted growth in terms of height and production of nodes and leaves (Figure 1) when compared to the other treatments.

Test 2: Nutrient concentration - Effect of micronutrients in *D. incanum* development

The darker color of leaves, which visually indicates a healthy pattern was obtained with 50% of the concentration of micronutrients, keeping the other components of MS medium at the standard concentration (T2). However, when all nutrients were reduced by 50% (T1), the color of green was less intense, while the reduction to one fourth part of micronutrients and 50% of macronutrients, vitamins and FeEDTA (T3) produced clearer leaves, which were visibly affected and chlorotic (Figure 2).

On the other hand, reducing the concentration of micronutrients to one fourth part and macronutrients, vitamins and FeEDTA to one half (T3), promoted the development of shoots, leading to a higher number of roots and increasing dry weight of roots and shoots (Figure 3). The number of *D. incanum* roots was 1.2 times higher in the more diluted micronutrient treatment (T3) when compared to T2, which was the second best treatment (Figure 3C), while the root dry weight in T3 exceeded T2 by 4.2 times (Figure 3E), demonstrating that the plants not only produced new roots, but showed increased weight of the root system. According to Silveira and Monteiro (2011), under a low supply of nitrogen, the plant produces longer roots, while an increased supply of this nutrient reduces the length of the roots.

The number of leaves and the number of shoots per plant was not significantly affected by the treatments (Figure 3D, B). However, the plant morphology changed, despite these variables not being statistically different, as plant height and shoot dry mass were increased in treatment 3 Figure 3A, F), with lengthened internodes and larger leaves.

Test 3: Effect of different concentrations of indolebutyric acid – IBA in *D. incanum* development

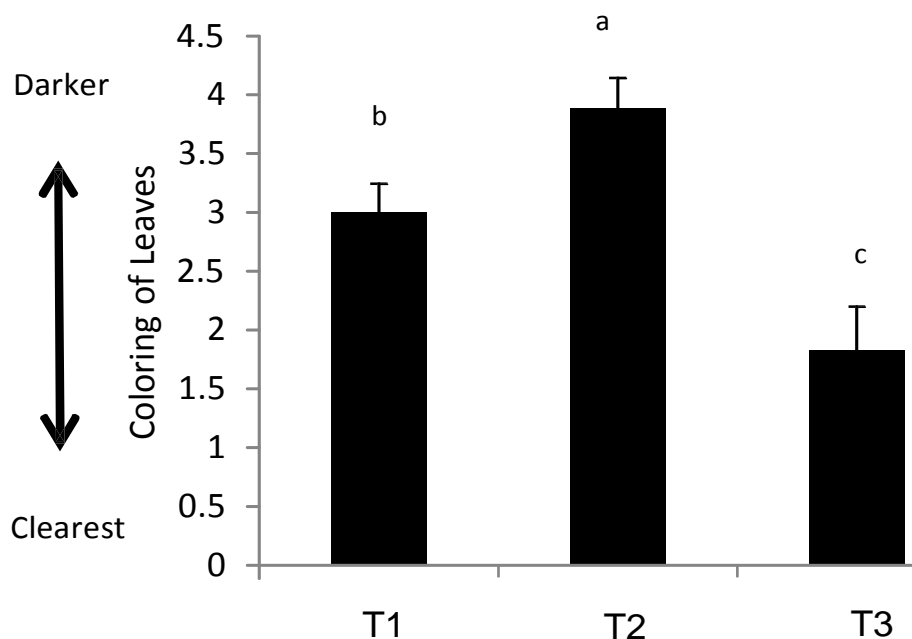Regression analysis resulted in a linear fit for root dry

Figure 2. Effect of different compositions of MS culture media in the coloration of *Desmodium incanum* leaves cultivated *in vitro.* Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

weight and IBA concentrations (Figure 4). Root dry weight was increased linearly by IBA concentrations (Figure 4). On the other hand, IBA concentrations were not sufficient to promote significant differentiation in the components of shoot morphology. However, a tendency to increase was observed for height of shoots and shoot dry weight (Figure 5).

DISCUSSION

Test 1 results indicate a likely intolerance of the species to high concentrations of nutrients, what may be attributed to the fact that the species *D. incanum* is native to acid soils with low fertility and adapted to these conditions (Miotto, 2011). In addition, the results suggest some toxicity especially from micronutrients, because the reduction only in the concentration of macronutrients (treatments 3 and 4) led to a decrease in all parameters (Figure 1).

The toxicity of micronutrients has long been studied. Increasing the availability of manganese in the soil, for example, leads to an increase in its content in the aerial part of the plant, reducing the production of chlorophyll, and therefore the photosynthetic capacity, hindering the growth of roots and total dry weight (Smith et al., 1983). Copper toxicity appears primarily in the roots, damaging the permeability of membranes (Seliga, 1993). Corn plants fertilized with high doses of boron showed toxicity symptoms as chlorosis in older leaves evolving to necrosis in a "V" (Leite et al., 2003).

Antagonistic effects on the absorption of some elements may also occur due to an increase in the concentration of others. According to Malavolta (1994), the presence of zinc can prevent the absorption of other ions by competition and thus can cause chlorosis of the leaves. Moreover, Disarz and Corder (2009) observed that a 25% reduction of the nutrient concentration in the MS medium was beneficial for the formation of buds and leaves of *Acacia mearnsii.* Other authors also found satisfactory results by reducing the macronutrient content in MS culture medium for several species (Mercier and Kerbauy, 1992).

Tamaki and Mercier (2007) noted that *Ananas comosus* plants grown in MS culture medium with a fifth of the nutrient concentration had absorbed and assimilated sufficient nitrogen quantity for normal development of plants, as that obtained in basic MS medium.

In Test 2, the nitrogen content probably interfered directly in the staining of leaves, since according to Booij et al. (2000), the amount of chlorophyll is directly correlated with the N concentration in the plant. Similarly, other micronutrients were also correlated to chlorophyll content in tissues (Taiz and Zeiger, 2004). For this variable, the reduction of the concentration of micronutrients to 25% (T3) appears to be too high, resulting in chlorotic leaves, which may indicate some micronutrient deficiencies. Many authors have reported the symptoms of chlorosis with a deficiency of micronutrients such as boron, zinc, iron and manganese (Malavolta et al., 1997). The other results from Test 2 (Figure 3), agree with those presented in Test 1,
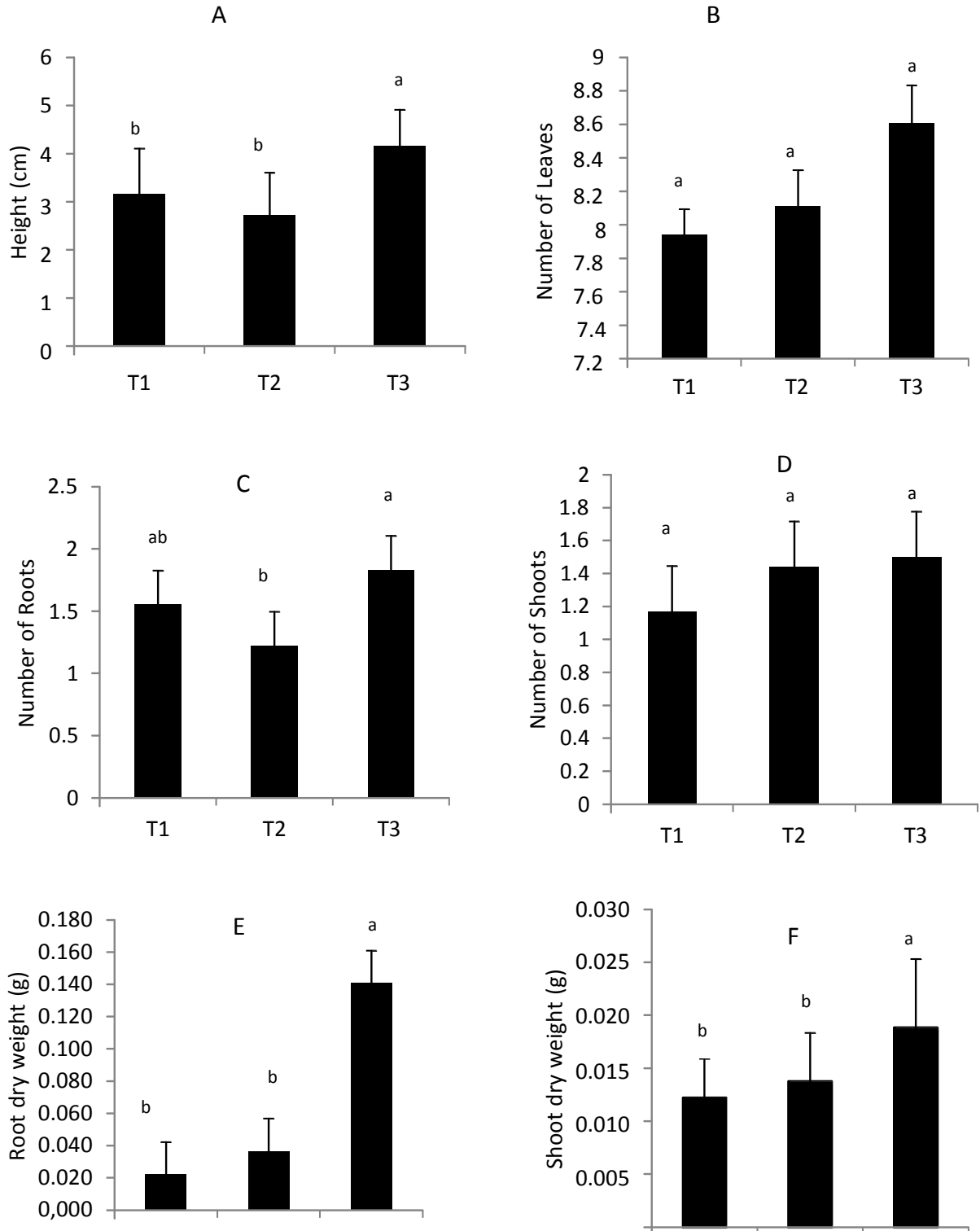
Figure 3. Effect different compositions of MS culture media in height (A), number of leaves (B), number of roots (C), number of shoots (D), roots dry weight (E) shoot dry weight (F) of *Desmodium incanum* cultivated *in vitro.* Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

indicating that the reduction in the concentration of nutrients, particularly micronutrients is favorable for the

growth in height of *D. incanum*. Comparing the results for height of shoots (Figure 3A) and number of roots
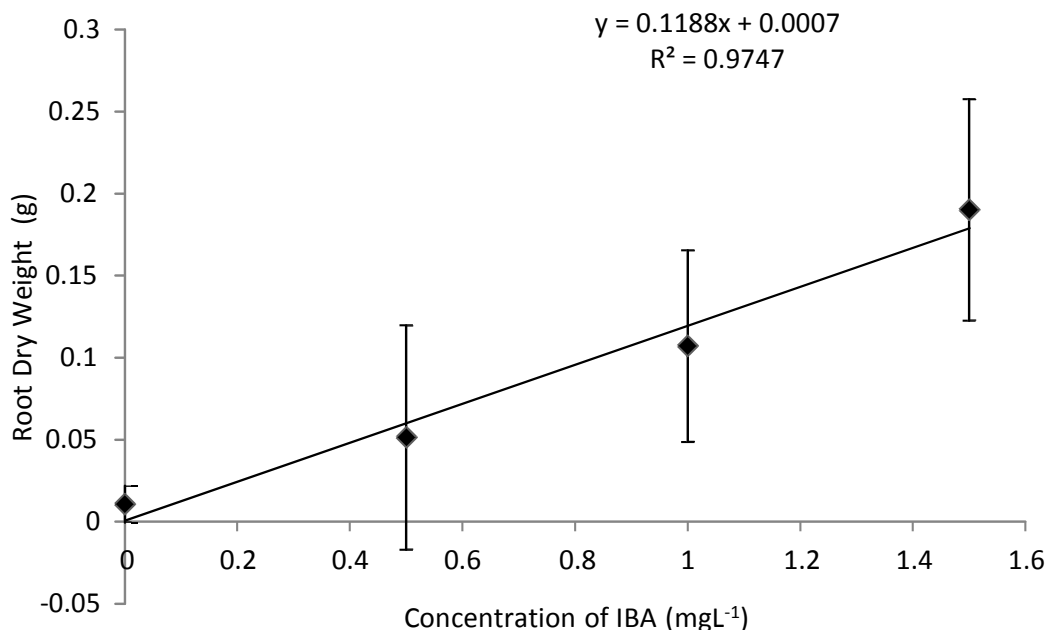
$$y = 0.1188x + 0.0007$$
$$R^2 = 0.9747$$

Figure 4. Effect of different concentrations of IBA (mgL^{-1}) in root dry weight of *Desmodium incanum* cultivated *in vitro* p<0.01*.

per plant (Figure 3C) it is also possible to link the increased height in treatment 3 with the highest number of roots also verified in this treatment. Plants grown in saline media often have restricted vegetal growth, when the concentration of mineral ions reaches levels that limit water availability or exceed the appropriate amount of a particular nutrient. The mechanisms by which plants tolerate salinity are complex and involve molecular synthesis, enzyme induction and membrane transport (Taiz and Zeiger, 2009).

In some cases, a restriction of nutrients leads to increased mass allocation to the roots when compared to the shoot, in unrestricted light conditions (Chapin, 1980). In most cases, the explants do not start the rooting process in culture media with high concentrations of salts, even in the presence of auxin. Reducing the salt concentrations in the culture media to 1/2, 1/3 or 1/4, enables enhanced rooting (Hu and Wang, 1983).

The increase in plant height and number of roots and the healthy coloration of the plants when the concentration of micronutrients was reduced to 50% may also indicate the toxicity of some micronutrient(s) as already discussed with regard to the results obtained in Test 1. For shoot dry weight (Figure 3F), similar results were found by Kanashiro et al. (2007) in *Aechmea blanchetiana* seedlings, in which this variable decreased linearly with increasing nitrogen concentration in modified MS medium. Moreover, the number of leaves was increased, according to the quadratic regression. According to Illenseer and Paulilo (2002), with a change in nutritional regimen, species can show morphological

and physiological changes to maximize the dry weight gain in the new conditions, or vary the distribution of biomass between root and shoot (Osunkoya et al., 1994). In addition, plants subjected to low levels of nitrogen showed higher use efficiency of this nutrient (Illenseer and Paulilo, 2002).

For Test 3, the results presented in Figure 4 can be explained by the change in the standard balance of auxin/cytokinin. For differentiation and tissue formation, as well as growth *in vitro*, a suitable balance between auxins and cytokinins is necessary, maintaining the auxin/cytokinin ratio less than one (Pierik, 1990). When the auxin level is high in relation to cytokinin, root formation occurs, while the opposite leads to shoot formation and even when the proportions are approximately the same, a mass of callus is produced (Krikorian, 1995). Similarly, Nascimento et al. (2008) found the highest percentage of root formation (60%) of *Eugenia pyriformis* in a treatment with 1.0 mgL^{-1} of IBA. Machado et al. (2011) observed an increase in lavender rooting percentage with increasing concentrations of IBA, up to 5.0 mM.

Although it is possible to observe an upward trend in height and shoot dry weight in response to IBA concentration (Figure 5D), available data did not show statistical differences. A similar t r e n d was observed by Silva et al. (2011). Moreover, Machado et al. (2011) observed that increasing concentrations of IBA reduced plant height and root length of lavender plants. The number of leaves was constant for all IBA concentrations tested, with no significant difference between means
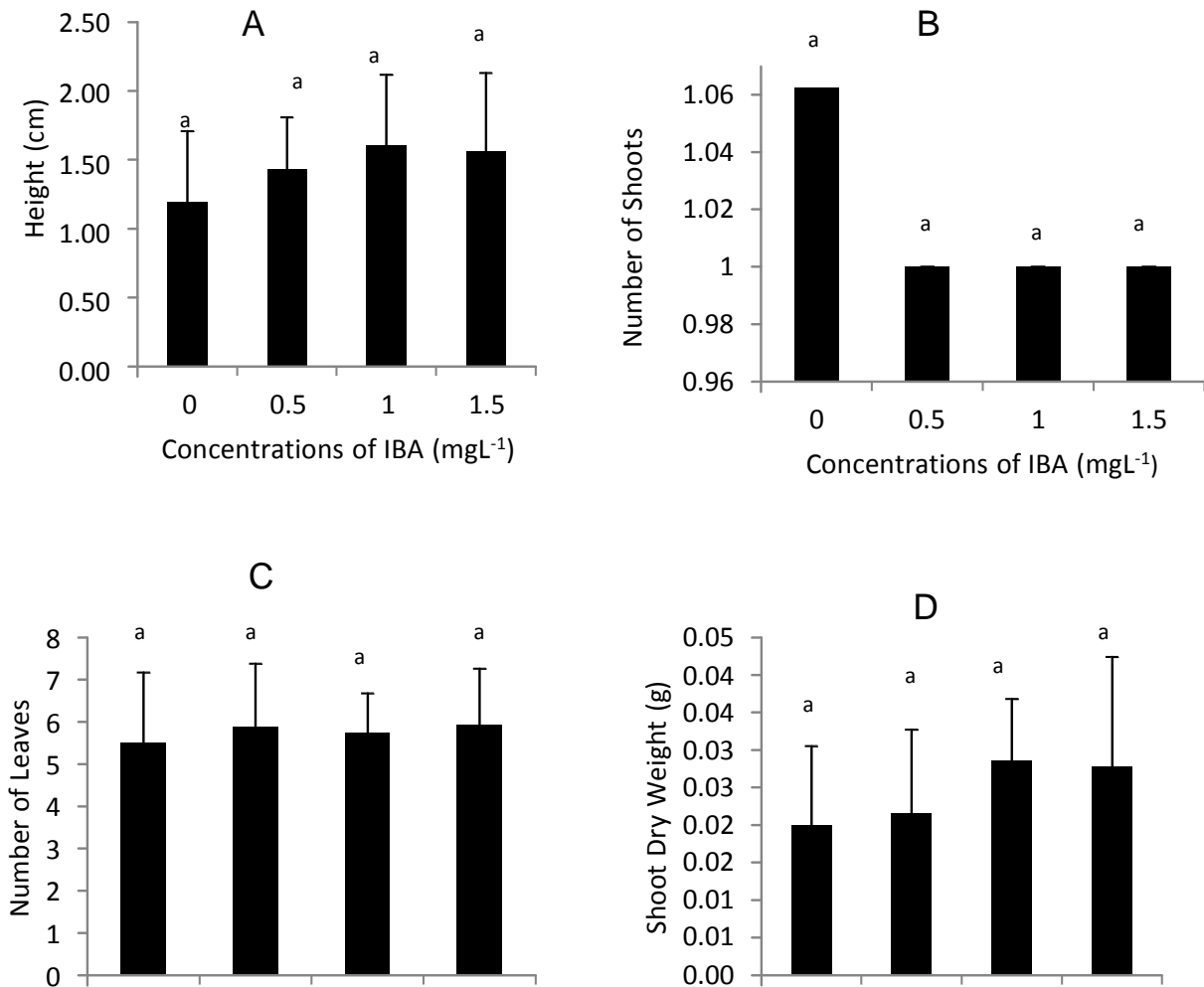
Figure 5. Effect of different IBA concentrations (mg L^{-1}) on height (A), number of shoots (B), number of leaves (C) and shoot dry weight (D) of *Desmodium incanum* cultivated *in vitro*. Statistical test: Tukey at 5% probability (p ≤ 0.05). Replication number: 20.

(Figure 5C). Similar results were found by Horbach et al. (2011). The number of shoots tended to increase in the absence of IBA, although without statistical difference (Figure 5B). Similar results were observed by Maia and Botelho (2008), with a constant number of one shoot, regardless of IBA dose.

Conclusion

Reducing the concentration of macronutrients, micronutrients, vitamins and FeEDTA to 50% provided the greatest growth and development of *D. incanum* grown *in vitro*. Moreover, a greater reduction (to 25%) of micronutrients appears to have further promoted the growth of this species. In addition, *D. incanum* responded positively to the addition of IBA in terms of root growth.

Conflict of Interests

The authors have not declared any conflict of interests.

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