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Nutrient concentrations of *in vitro* culture is crucial for *Desmodium incanum* DC. Acclimatization

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Abstract - *Desmodium incanum* is a perennial species, native in grassland of Rio Grande do Sul, Brazil and popularly known as "pega-pega". Due its good characteristics as forage, the degradation of the natural fields became an alarming reality. The conservation of these species requires the knowledge of the nutritional requirements and the abiotic conditions that the species is adapted. So, besides *in vitro* culture as germplasm conservation, it is suggested practices such as acclimatization of plants to increase the survival percentage in *ex vitro* environment. The plant nutritional status in the period prior to the acclimatization may influences the results of survival and development during this process. To this end, it was tested the influence of nutrition of the seedling during *in vitro* cultivation on the *D. incanum* acclimatization through variations in the salt composition of the culture medium. Our results showed that the culture medium which provided the greatest survival rate and better plant growth was MS medium with half the standard concentration of salts. On the other hand, we observed that the iron halving, in culture medium, during *in vitro* culture was highly detrimental to the acclimatization process of *D. incanum*. So we could confirm the importance of nutritional status on *in vitro* culture for acclimatization stage.

Key words: Grassland. Pampa biome. Acclimatization. Nutritional requirements.

Concentração de nutrientes durante o cultivo *in vitro* é crucial para a aclimatização de *Desmodium incanum* DC.

Resumo - *Desmodium incanum* é uma espécie perene, nativa em pastagens do Rio Grande do Sul, Brasil, e popularmente conhecida como "pega-pega". Devido suas boas características como a forragem, a degradação dos campos naturais tornou-se uma realidade alarmante. A conservação das espécies nativas requer o conhecimento sobre as exigências nutricionais e demais condições abióticas a que a espécie está adaptada. Assim, além do cultivo *in vitro* como forma de conservação do germoplasma, práticas como a aclimatização

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de plantas são interessantes para aumentar as taxas de sobrevivência no ambiente *ex vitro*. Com o objetivo de observar o quanto o estado nutricional, ao qual as plantas estão expostas no período anterior a aclimatação, afeta os resultados de sobrevivência e desenvolvimento das mesmas durante este período, testou-se a influência do suprimento nutricional de mudas de *Desmodium incanum* produzidas *in vitro* durante a aclimatação, através de variações na composição de sais do meio de cultura. Os resultados mostraram que o meio de cultura que proporcionou a maior taxa de crescimento e a sobrevivência das plantas foi meio MS com metade da concentração padrão de sais. Por outro lado, observou-se que a redução para metade do ferro, em meio de cultura, durante o cultivo *in vitro* foi altamente prejudicial para o processo de aclimatação de *Desmodium incanum*. Com estas informações pode-se confirmar a importância do estado nutricional das plantas durante o cultivo *in vitro* para a fase de aclimatação.

Palavras-chave: Pastagens. Bioma Pampa. Aclimatização. Exigências nutricionais.

Introduction

Desmodium incanum DC. is the most abundant native legume in grasslands of Rio Grande do Sul, Brazil, showing a widely spread (Medeiros *et al*., 2006, Maldaner *et al*., 2014). It's a perennial and summer crop species popularly known as "pega-pega" (Oliveira, 1983), it has good characteristics as forage, moderately palatable and persistent (when grazing, it has strong rooting at the nodes forming stolons) and it has good acceptance by the animals (Boldrini, 1993).

The information available about the biodiversity of native grasslands is still primitive (Overbeck et al., 2007), and their forage potential is still neglected by most technicians and producers, due to an incorrect management of native pastures, combined with inadequate animal stocks, which leads to the extinction of many native species, among them the *D. incanum.* So that, strategies for conservation are needed, and the micropropagation is an efficient technique for the preservation of germplasm, where whole plants can be obtained from the cultivation of cells, tissues or plant organs (Pence, 2011).

Acclimatization is the main critical phase of micropropagation due to various factors that can impact the survival of plants (Silva *et al*., 2011). Acclimatization comprises a set of techniques and procedures which are designed to adapt plantlets to field conditions, involving the transfer of heterotrophic to autotrophic condition (Santos *et al*., 2014). According to Murashige (1974), micropropagation may consist of four distinct stages, and the acclimatization is considered the most difficult of them. The adaptation to this condition is delicate due to some variables such as temperature, light, humidity and substrate, which can take the plant to a stress condition and reduce its growth, influencing the survival and development of the acclimatized seedlings (Junghans and Souza, 2009). The transfer from protected and sterile environment, with sugars and saturated moisture to non-sterile, and a reduced humidity environment, has led to the loss of plants, low growth rate and extended period to getting acclimated plants (Lakso *et al*., 1986).

In addition, in *in vitro* propagation, the sealing of the flasks is mandatory since it prevents contamination as well as prevents dehydration of plants or the culture medium. However, in these airtight bottles, ethylene concentration is increased, and CO_2 concentration reduced concomitantly, leading restriction

on photosynthetic photon flow and gas exchange, decreasing the rate of transpiration and photosynthesis of plants. This hinders the absorption of water and nutrients, causing reduction in the growth rate of the explants that may lead to high losses during acclimatization (Zobayed, 2006; Xião *et al*., 2011).

According to Silva, *et al.* (2011) the biggest problem that restricts the use of micropropagation is the low survival rate of seedlings during *ex vitro* acclimatization, due a high loss of water by transpiration. Moreover, *in vitro* formed roots are not efficient enough in absorbing water and nutrients during transferring plants to substrate. Few studies have reported details of transplanting and acclimatization of micropropagated plantlets. Thus, the problems and the solutions found in this process become even more important in commercial production systems as well in germplasm conservation projects.

The aim of this study was to observe the development and mainly survival during the process of acclimatization of *D. incanum* plantlets *in vitro* originated under different nutritional standards.

Materials and Methods

A mixed *Desmodium incanum* seed batch, collected in the region of the campaign of Rio Grande do Sul (Black Hulha, Dom Pedrito, São Gabriel) was used for the test. Seeds were disinfected according to the methodology described by Maldaner *et al.* (2014) and then were germinated in different nutritional conditions. Treatments consisted of variations in the concentrations of the culture medium MS (Murashige and Skoog, 1962), as follow: MS complete culture medium; M/2: MS culture medium with half strength salts and Fe/2: MS culture medium containing half of the iron concentration. These treatments were chosen from preliminary tests.

After 30 days under temperature conditions of 25 ± 2 ° C, 16 hour photoperiod and light intensity of 35 µmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps in a growth chamber of the Laboratório de Insumos Biológicos do Centro de Pesquisas em Florestas, in Santa Maria, RS, the vials were opened and maintained for three days in the same room conditions (temperature and light). Then they were maintained for five days on another room with natural light and temperature.

After this period, the seedlings were transferred into plastic cups (250 ml) with Carolina Soil® commercial substrate previously sterilized. Plants were carefully wiping with drawing all culture medium excess of the root system. They stayed for seven days in a room with natural light and temperature, and then were led to greenhouse glass, where they remained for 45 days when were assessed for survival, height and number of leaves per plant.

The resulting data were subjected to analysis of variance and means were compared by Tukey range multiple test with $\alpha = 0.05$. All statistical analyzes were performed using ASSISTAT 7.7 (Silva and Azevedo, 2002) software.

Results and discussion

There was a significant variation in the percentage of survival of acclimatized plants due to the nutritional standards that were originally submitted. It was observed that almost 100% of the plants originated in MS culture medium with half the standard concentration of salts survived in acclimatization phase, while

those derived from MS complete culture medium showed a percentage survival ten times smaller, moreover, established plants in culture medium in which they halved the iron concentration had virtually 100% mortality in the acclimatization process (Figure 1). Corroborating these results, Oliveira *et al*. confirmed in 2010 that the composition of the culture medium is essential for the survival ornamental pineapples pre-acclimatization, especially with regard to rooting. Moreover, similar to our results, Caroá plants from MS culture medium modified, had its survival percentage favored by low concentrations of salts (Silveira *et al*., 2013).

Reduced survival in a complete MS medium (Figure 1) may be explained due a low nutritional requirement of this species, and might suggest that high nutrients supply may be toxic. In the same way, Monfort *et al*. (2015) observed that atroveran (*Ocimum selloi* Benth.) plants developed in complete MS medium showed lower height than those grown on MS medium with half of the concentration of salts. Our results can be attributed to the fact that *D. incanum* is a species that adapts to all kinds of soils, growing well in soils with medium acidity, may persist and vegetate in very acid soils (pH 4.5 or less) of low fertility (Coradin *et al.*, 2011).

On the other hand, the reduction of the concentration of iron to 50% appears to be excessive, indicating deficiency of this element by species, resulting in chlorotic leaves and the death of seedlings (Figure 1). Many authors relate the symptoms of chlorosis with a deficiency of micronutrients such as boron, zinc, iron and manganese (Broadley *et al*., 2007). Iron is a constituent of chromosomes and iron proteins involved in photosynthesis, N_2 fixation and respiration. To this end it is reversibly oxidized from Fe^{2+} to Fe^{3+} during electron transfer. Iron deficiency symptom is chlorosis between the veins, appearing first in young leaves because iron cannot be readily mobilized from older leaves. The leaves become chlorotic, because iron is required for the synthesis of some of the complexes consisting of chlorophyll and protein in the chloroplast (Taiz and Zeiger, 2009).

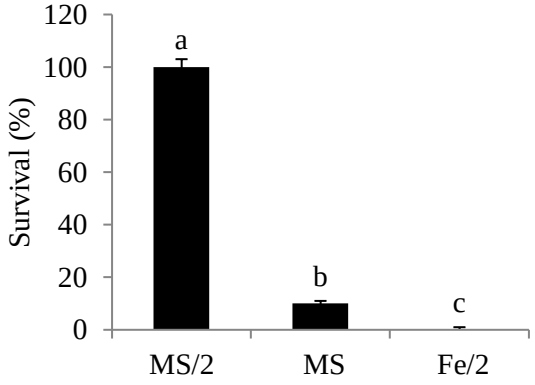

Figure 1: Survival of *Desmodium incanum* acclimatized plants under different nutritional status in *in vitro* culture. Identical letters indicate no significant differences among treatments at the same harvest time ($p<0.05$) according to Tukey's multiple range test.

One of the main causes of low survival rate of acclimatized plants is the excessive loss of water by plants (Brainerd and Fuchigami, 1992; Schuck *et al*., 2012). The reduced amount of epicuticular wax of *in vitro* originated plants is associated with increasing water loss. A higher transpiration rate was observed in

leaves of plants with low amounts of epicuticular wax, when compared with plants grown in a greenhouse or who were already acclimatized (Wardle *et al*., 1983). The mechanism of stomatal opening and closing in plants from *in vitro* culture in acclimatization stage is slower than in cultivated or maintained in greenhouse ones, causing rapid loss of water, leading to a collapse in the leaves, resulting in chlorotic plants. This is due to the variation in brightness that plants are exposed during this process.

Considering survival results, we became to work with only two treatments, MS and MS/2, for growth assessments (Figure 1).

Shoot height was significantly higher in plants grown in culture media with half strength MS salts, compared to full MS medium (Figure 2A). Similarly, the leaves number was many times greater in plants that had been grown on MS/2 medium (Figure 2B). Other authors have observed beneficial effects of reducing the concentration of the components of MS medium (Bandinelli *et al*., 2013; Russowski and Nicoloso, 2003).

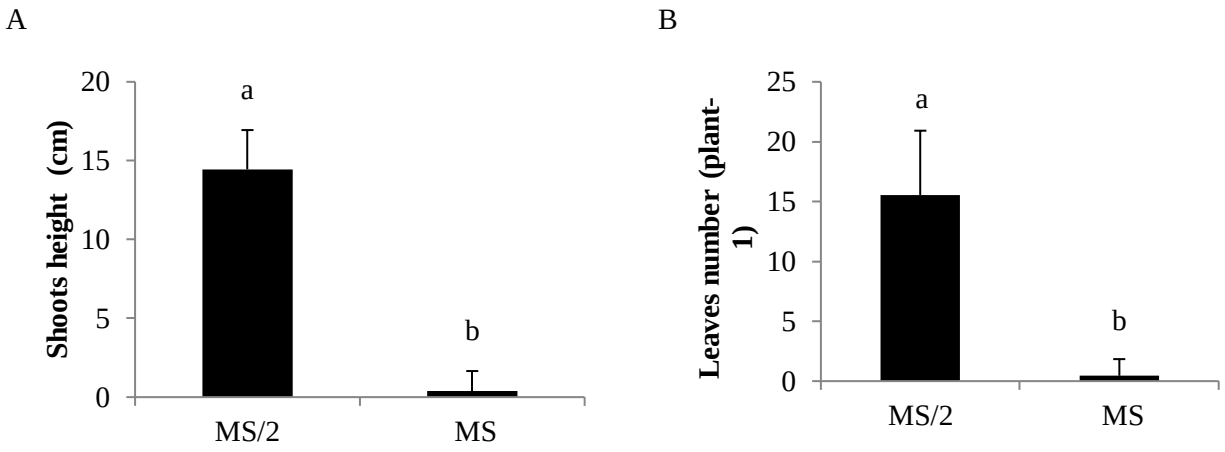

Figure 2: Height of shoots and number of leaves of *Desmodium incanum* acclimatized plants under different nutritional status in *in vitro* culture. Identical letters indicate no significant differences among treatments at the same harvest time ($p<0.05$) according to Tukey's multiple range test.

According Pasqual *et al*. (2011), during the acclimatization process changes in anatomy, morphology and physiology of plants are observed, thus influencing the growth, development and survival of plantlets. However, the original condition of the plants may have been fundamental for the differences; like reported in previous results, the culture medium MS with full concentration was not optimal for *in vitro* culture of *D. incanum* (Schwalbert *et al.*, 2014), and halving the concentration of MS medium stimulated growth in height and the number of sheets in *D. incanum*.

MS medium with its full concentration of salts (100%) reduced shoot height and caused a decrease in the number of leaves (Figure 2A, 2B) showing that *D. incanum* does not grow well in culture media with high concentration of salts. It was observed a chlorosis in plants grown in culture medium with 100% of the salts, which may be an indication of toxicity by excessive nutrients. Other authors relate the symptoms of chlorosis on leaves with toxicity caused by excess nutrients (Mascarenhas *et al*., 2013).

The study of the acclimatization process of pampa biome species, which are in constant anthropic pressure, has great importance for the conservation of this biome. This work opens perspectives for studies with other species of this and other biomes.

Conclusions

The composition of culture medium used for *in vitro* establishment is crucial in the success of the acclimatization process. The halving of salts concentration of MS medium was efficient both for *in vitro* culture and for acclimatization process of *Desmodium incanum*.

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