IN VITRO PROPAGATION OF *Nidularium fulgens* Lem.

Patrícia Duarte de Oliveira Paiva, Vanessa Coelho Naves, Leonardo Ferreira Dutra, Renato Paiva and Moacir Pasqual

SUMMARY

Nidularium fulgens *Lem. is a native bromeliad species of the Brazilian Atlantic Forest, used in gardens and vases. Like other bromeliad species, plants are extracted from their environment in order to be commercialized. This study tested different concentrations of growth regulators on the in vitro propagation of* N. fulgens*. Pre-established in vitro seedlings were inoculated in MS culture medium with BA (6-benzylaminopurine) added at the concentrations of 0, 0.5, 1.0, 2.0 and 4.0mg·l^{-1} in combination with NAA (naphthaleneacetic acid) at 0, 0.1, 0.5, 1.0mg·l^{-1} (experiment 1), and with TDZ (thidiazuron) at 0, 0.01, 0.02, 0.05 and 0.1mg·l^{-1} in combination with NAA at 0, 0.1, 0.5 and 1.0mg·l^{-1} (experiment 2). The combination of auxins and cytokinins, especially TDZ, is important for micropro-pagation of* N. fulgens*. Higher numbers of shoots were obtained when 0.02mg·l^{-1} TDZ plus 0.5 or 0.1mg·l^{-1} NAA or MS medium was supplemented with 1.0mg·l^{-1} BA and 0.1mg·l^{-1} NAA. The occurrence of shoots longer than 1.0cm was optimized when MS medium was supplemented with 0.02mg·l^{-1} TDZ and 0.5mg·l^{-1} NAA. Roots were not observed when BA or TDZ were added to the MS culture medium. Plants over 2.0cm were acclimatized, resulting in a 100% survival rate. The use of the produced plants is suggested for in vitro preservation programs of endangered populations.*

Introduction

Bromeliacea are plants with impressive exotic forms, a wide color range and a variety of flowers and leaves. They have an important function in the ecology of various environments, serving as food and shelter for many animals, acting as water reservoirs in dry environments, and improving soil fertility by the decomposition of their leaves, making it viable for the development and survival of other plant species (Leme and Marigo, 1993).

The lack of data about propagation and cultivation techniques has discouraged bromeliad production, and often species are commercialized that have been extracted from their environment (Melo, 1996). In many cases, these species are removed from populations with few representatives, as is the case of *Nidularium fulgens* Lem., a species found in the Brazilian Atlantic Forest in the states of Rio de Janeiro and São Paulo.

Bromeliads are propagated by seeds both in nature and in commercial cultivation (Rauh, 1990; Landgraf and Paiva, 2009) but this process is very slow. Furthermore, depending on the species and/or environmental conditions, the plant flowers and produces only once in its life time (Cândido, 1995, 1996), while *in vitro* germination can reach 100% (Mercier and Nievola, 2003; Naves *et al*., 2003). Another form of propagation common among bromeliads is by separating side shoots, offshoots or "pups" (Cândido, 1996). However, few shoots are usually produced per plant and they are not enough to supply the growing market (Kämpf, 1992).

The use of tissue culture techniques is an important form of propagation for bromeliads, permitting large scale production of high quality plants and also for preservation (Melo, 1996; Naves *et al*., 2003, 2004; Rech Filho *et al*., 2005; Sarasan *et al*., 2006). Micropropagation protocols have been studied for various bromeliad species (Mekers, 1977; Hosoki and Asahira, 1980; Mathews and Rao, 1982; Pierik and Steegmans, 1984; Pierik *et al*., 1984; Pierik and Sprenkels, 1988; Van Dijck *et al*., 1988; Mercier and Kerbauy, 1992, 1993, 1994; Vinterhalter and Vinterhalter, 1994; Naves, 2001; Arrabal *et al*., 2002; 2002; Rech Filho *et al*., 2005; Pickens *et al*., 2006).

To develop a protocol for *in vitro* propagation, it is essential to establish the multiplication process, determining the type and concentration of growth regulators. Their addition to the culture medium controls growth and morphogenesis, and there is also interaction with the endogenous growth substances (George, 1996).

According to Grattapaglia and Machado (1998) BA (6-benzilaminopurine) is the cytokine that provides best results on *in vitro* aerial parts multiplication and adventitious buds induction. Carneiro *et al.* (1998) cultivated *Cryptanthus sinuosus* leaf explants from *in vitro* germinated seed in solid culture medium supplemented with 2.2mg·l^{-1} BA and 0.25mg·l^{-1} NAA (naphthaleneacetic acid). Studies by Mekers (1977) showed that the presence of NAA or GA_3 (gibberellic acid) at concentrations of 1.0mg·l^{-1} in Knudson culture medium, promoted the germination of *Vriesea splendens* at a higher rate than in the control plant and the auxin NAA also stimulated earlier seedling development.

KEYWORDS / Bromeliad / Growth Regulators / Micro-propagation / Ornamental Plants / Tissue Culture /

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Patrícia Duarte de Oliveira Paiva. Doctor in Agronomy, Universidade Federal de Lavras (UFLA), Brazil. Professor, UFLA, Brazil. Address: Departamento de Agricultura, UFLA, CEP 37.200-000, Lavras-MG, Brazil. e-mail: pdolivei@ufla.br

Vanessa Coelho Naves. M.Sc., UFLA, Brazil. Agronomist, IGAM-Instituto Mineiro de Gestão das Águas, Brazil. e-mail: vcnaves@yahoo.com.br

Leonardo Ferreira Dutra. Doctor in Agronomy, Universidade Federal de Pelotas, Brazil. Researcher, EMBRAPA, Brazil. e-mail: leo@cpact.embrapa.br

Renato Paiva. Ph.D. in Agronomy, University of Illinois, USA. Professor, UFLA, Brazil. e-mail: renpaiva@ufla.br

Moacir Pasqual. Doctor in Genetics and Plant Breeding, ESALQ, USP. Professor, UFLA, Brazil. e-mail: mpasqual@ufla.br

PROPAGACIÒN *IN VITRO* DE *Nidularium fulgens* Lem.

Patrícia Duarte de Oliveira Paiva, Vanessa Coelho Naves, Leonardo Ferreira Dutra, Renato Paiva y Moacir Pasqual

RESUMEN

Nidularium fulgens *Lem. es una especie de bromelia nativa del Bosque Atlántico Brasilero, utilizada en jardines y floreros. Al igual que con otras especies de bromelias, las plantas son extraídas de su hábitat para ser comercializadas. En este estudio se ensayó el efecto de diferentes concentraciones de reguladores del crecimiento en la propagación de* N. fulgens*. Plántulas preestablecidas in vitro fueron inoculadas en medio de cultivo MS con BA (6-benzilaminopurina) en concentraciones de 0; 0,5; 1,0; 2,0 y 4,0mg·l^{-1} en combinación con NAA (ácido naftaleneacético) 0; 0,1; 0,5 y 1,0mg·l^{-1} (experimento 1), o inoculadas con TDZ (thidiazuron) 0; 0,01; 0,02; 0,05 y 0,1mg·l^{-1} en combinación con NAA 0; 0,1; 0,5 y 1,0mg·l^{-1} (experimento 2). La combinación de auxinas y citoquinas, especialmente TDZ, es importante para la micropropagación de* N. fulgens*. Se obtuvo más vástagos cuando se empleó 0,02mg·l^{-1} de TDZ con 0,5 o 0,1mg·l^{-1} NAA, o cuando el medio MS fue suplementado con 1,0mg·l^{-1} BA y 0,1mg·l^{-1} NAA. La ocurrencia de vástagos de más de 1,0cm de largo fue optimizada cuando el medio se suplementó con 0,02mg·l^{-1} TDZ y 0,5mg·l^{-1} NAA. No se observaron raíces cuando se añadió BA o TDZ al medio de cultivo. Se aclimatizaron plantas con más de 2,0cm resultando en un 100% de supervivencia. Se sugiere el uso de las plantas producidas para programas de preservación in vitro de poblaciones en peligro de extinción.*

PROPAGAÇÃO *IN VITRO* DE *Nidularium fulgens* Lem.

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RESUMO

Nidularium fulgens *Lem. é uma espécie de bromélia nativa do Bosque Atlântico Brasileiro, utilizada em jardins e vasos. Igualmente que com outras espécies de bromélias, as plantas são extraídas de seu hábitat para ser comercializadas. Neste estudo se ensaiou o efeito de diferentes concentrações de reguladores do crescimento na propagação de* N. fulgens*. Plântulas preestabelecidas in vitro foram inoculadas em meio de cultivo MS (MURASHIGE & SKOOG, 1962) com BAP (6-benzilaminopurina) em concentrações de 0; 0,5; 1,0; 2,0 e 4,0mg·l^{-1} em combinação com ANA (ácido naftaleneacético) 0; 0,1; 0,5 e 1,0mg·l^{-1} (experimento 1), ou inoculadas com TDZ (thidiazuron) 0; 0,01; 0,02; 0,05 e 0,1mg·l^{-1} em combinação com ANA 0; 0,1; 0,5 e 1,0mg·l^{-1} (experimento 2). A combinação de auxinas e citoquinas, especialmente TDZ, é importante para a micropropagação de* N. fulgens*. Obteve-se maior número de germinações quando se empregou 0,02mg·l^{-1} de TDZ com 0,5 ou 0,1mg·l^{-1} ANA, ou quando o meio MS foi suplementado com 1,0mg·l^{-1} BAP e 0,1mg·l^{-1} ANA. A ocorrência de partes aéreas com mais de 1,0cm de comprimento foi optimizada quando o meio foi suplementado com 0,02mg·l^{-1} TDZ e 0,5mg·l^{-1} ANA. Não foram observadas raízes quando acrescentado BAP ou TDZ ao meio de cultivo. Aclimatizaram-se plantas com mais de 2,0cm resultando em um 100% de sobrevivência. Sugere-se o uso das plantas produzidas para programas de preservação in vitro de populações em perigo de extinção.*

Analyzing the influence of the auxins on seed germination and later seedling growth of three different species of bromeliad, Pierik *et al*. (1984) reported that NAA added to MS culture medium at concentrations between 0.5 and 0.8mg·l^{-1} was efficient to promote root and shoot growth.

The objective of the present study was to analyze the effects of growth regulator types and concentrations on *in vitro* propagation of *Nidularium fulgens*, in order to produce shoots for species preservation.

Material and Methods

Nidularium fulgens Lem. seedlings were used as explants, obtained from *in vitro* seed germination in MS culture medium (Murashige and Skoog, 1962) without growth regulators and supplemented with 7% agar and 0.3% sucrose (Paiva *et al*., 2006).

Eight weeks after germination, the explants were inoculated in MS culture medium supplemented with 7% agar and 0.3% sucrose (Paiva *et al*., 2006) and subjected to two experiments. The first one (experiment 1) tested the effect of BA (0.0, 0.5, 1.0, 2.0 and 4.0mg·l^{-1}) combined with NAA (0.0, 0.1, 0.5 and 1.0mg·l^{-1}). The second one (experiment 2) tested the effect of TDZ (0.0, 0.01, 0.02, 0.05 and 0.1mg·l^{-1}) combined with NAA (0.0, 0.1, 0.5 and 1.0mg·l^{-1}). The pH was adjusted to 5.8 before autoclaving at 121°C and 1.1kg·cm^{-2} for 20min. A 15ml volume of MS culture medium was placed in 25×150ml test tubes, in which the explants were inoculated individually and later transferred to a growth chamber with a 16h light period, light intensity of 3000 lux at 26 ±1°C, for 120 days.

A complete randomized block design was used for the two experiments in a 5×4 factorial design with four replicates, four test tubes per plot.

Plants higher than 2.0cm (plants smaller than this size were not used due to separation difficulties) were acclimatized in foam trays containing the commercial substrate Plantimax® (Ferreira *et al*., 2007) and maintained in greenhouse with 50% shade, irrigated by a nebulization system. After 60 days, plants could be transferred to vases (Naves *et al*., 2004).

Results and Discussion

There was an effect of the treatments tested on the number and size of shoots formed and rooting percentage, and there was interaction between the growth regulators tested, both in experiment 1 (BA×NAA) and in experiment 2 (TDZ×NAA).

Experiment 1

Most shoots (5.75) were obtained with the combination of 1mg·l^{-1} BA and 0.1mg·l^{-1} NAA (Figure 1). Lower values (3.25) were obtained using 0.5mg·l^{-1} BA in the absence of NAA or in combination of 0.5 or 1.0mg·l^{-1} NAA (2.86 and 2.07

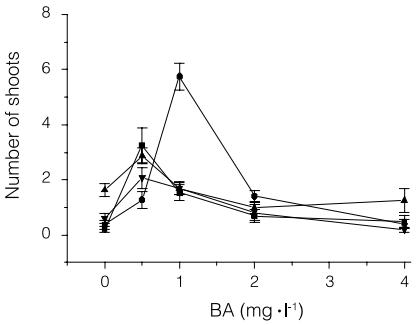

Figure 1. Number of shoots in *N. fulgens* seedlings cultivated in MS culture medium containing different NAA and BA concentrations. NAA concentrations were 0.0 (■), 0.1 (◆), 0.5 (▲), and 1.0 (▼) mg·l^{-1}. Each point is the mean ±SE of four replicates.

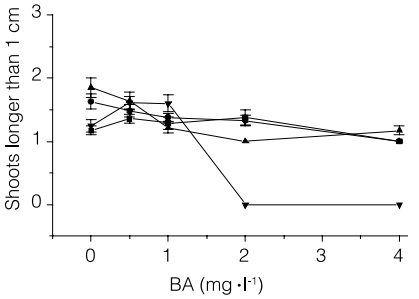

Figure 2. Shoots longer than 1.0cm in *N. fulgens* seedlings cultivated in MS culture medium containing different NAA and BA concentrations. NAA concentrations were 0.0 (■), 0.1 (◆), 0.5 (▲), and 1.0 (▼) mg·l^{-1}. Each point is the mean ±SE of four replicates.

shoots/explant, respectively). Mercier and Kerbauy (1992, 1994) micropropagated *Vriesea hieroglyphica* and *V. forsteriana*, and reported greater shoot induction in Knudson culture medium with the addition of 2.0mg·l^{-1} BA and 0.5mg·l^{-1} NAA. The value of 1.63 shoots obtained with 0.5 mg·l^{-1} NAA in the absence of BA, is in line with results by Hosoki and Asahira (1980), who induced shoots in *Quesnelia quesneliana* only by adding NAA to the MS culture medium.

Generally, absence or higher concentrations (2.0 and 4.0mg·l^{-1}) of BA gave the worst responses. Lower numbers of shoots (0.21 and 0.19) were obtained when no growth regulators were used or when higher concentrations, 1.0mg were used l^{-1} NAA and 4mg were used l^{-1} BA, respectively, were used.

However, these results are lower than those obtained by Carneiro *et al*. (1998) with *Cryptanthus sinuosus*, who obtained induction of 41.29 shoots. As this is a different specie, it was to be expected that there would also be different responses regarding treatment with growth regulators.

Formation of shoots longer than 1.0cm was not greatly influenced by the treatments tested. The largest shoot length detected was 1.85cm when 0.5mg·l^{-1} NAA was used in the absence of BA (Figure 2). Pierik *et al*. (1984) also reported the efficiency of NAA in promoting bromeliad shoot growth.

Combinations of 0.1mg·l^{-1} NAA in the absence of BA or in the combinations of 0.5mg·l^{-1} BA with 0.5 or 1.0mg·l^{-1} NAA; and 1mg·l^{-1} NAA with 1mg·l^{-1} BA, also promoted the formation of longer shoots (1.63, 1.64, 1.61 and 1.60cm, respectively).

The cytokinins induce the breakdown of apical dormancy and proliferation of auxiliary shoots (George, 1996). However, as the number of shoots increases, their size is normally reduced, starting at certain concentrations. This was observed in studies with *Alcantarea imperialis* (Naves, 2001).

None of the explants cultivated in MS culture medium with the addition of BA formed roots. However, when this growth regulator was not added, high rooting percentages (98.21%) were obtained, regardless of the NAA concentrations. Generally, the exogenous cytokines inhibit rooting, but at very low concentrations and in some species, they can present a promoting effect (Assis and Teixeira, 1998).

Experiment 2

The highest number of shoots (12.13) was obtained with the combination of 0.5mg·l^{-1} NAA and 0.02mg·l^{-1} TDZ (Figure 3). With almost seven shoots per explant, the next was a combination of 0.1mg·l^{-1} NAA and 0.02mg·l^{-1}TDZ. The 0.5mg·l^{-1} NAA concentrations provided the greater number of shoots in all the combinations with TDZ. Similarly to this variable in the previous experiment (NAA×BA), the lowest and highest growth regulator concentrations tended to induce the worst responses.

Shoots longer than 1.0cm were also optimized with NAA at 0.5mg·l^{-1} combined with 0.02mg·l^{-1} TDZ (Figure 4). Lower values, but with the possibility of immediate use for subcultures were obtained by combining 1.0mg·l^{-1} NAA and 0.02mg·l^{-1} TDZ (5.27), 0.5mg·l^{-1} NAA and 0.01mg·l^{-1} TDZ (4.96), 0.1mg·l^{-1} NAA and 0.02mg·l^{-1} TDZ (3.67), 1.0mg·l^{-1} NAA and 0.01mg·l^{-1} TDZ (3.32), or 0.5mg·l^{-1} NAA without TDZ (3.04).

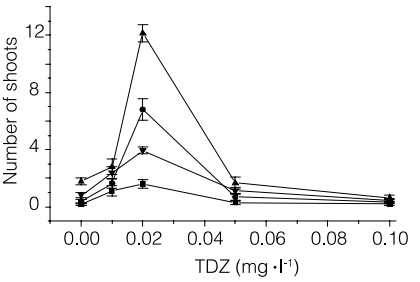

Figure 3. Number of shoots in *N. fulgens* seedlings, cultivated in MS culture medium containing different NAA and TDZ concentrations. NAA concentrations were 0.0 (■), 0.1 (◆), 0.5 (▲), and 1.0 (▼) mg·l^{-1}. Each point is the mean ±SE of four replicates.

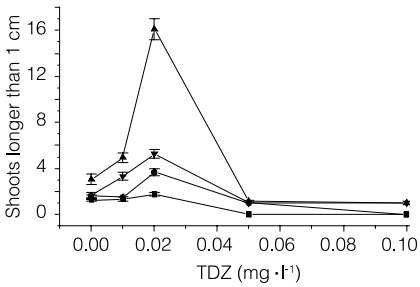

Figure 4. Shoots longer than 1cm in *N. fulgens* seedlings, cultivated in MS culture medium containing different NAA and TDZ concentrations. NAA concentrations were 0.0 (■), 0.1 (◆), 0.5 (▲), and 1.0 (▼) mg·l^{-1}. Each point is the mean ±SE of four replicates.

The explants did not survive at the highest TDZ concentrations (0.05 and 0.1mg·l^{-1}) in the absence or at low NAA concentrations (0.1mg·l^{-1}), probably due to the phytotoxic effect of TDZ.

Generally, the best results for shoot number and length were obtained with the use of TDZ compared to BA. A similar result was reported by Naves *et al.* (2004) on in vitro cultivation of *Alcantarea imperialis.*

TDZ has been described as a substance with a potent cytokine effect and the capacity to induce multiple shoots on plants, and thus can be used at lower concentrations. Several studies have been carried out using TDZ to induce auxiliary shoots (Fellman *et al*., 1987; Huetteman and Preece, 1993). The use of TDZ has led to better results in inducing and multiplying shoots in various species, as compared to other cytokines.

Similarly to the rooting percentage of the explants submitted to BA, when treated with TDZ the explants presented the same performance. There was no root emission in the explants cultivated in MS culture medium containing TDZ (data not shown). On the other hand, when this growth regulator was not present, regardless of the NAA concentration, 100% of the explants rooted, confirming the report by Assis and Teixeira (1998) that, in general, the exogenous cytokines inhibited rooting.

Plants over 2.0cm in length were acclimatized and resulted in a 100% survival rate. The use of the plants thus produced is suggested for *in vitro* preservation programs of endangered populations.

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