UNIVERSIDADE FEDERAL DO PARANÁ

ANDRÉ LUÍS LOPES DA SILVA

ATIVIDADE ANTIOXIDANTE, TEOR DE QUERCETINA E DESENVOLVIMENTO DE MEIO DE CULTURA A BASE DE VINHAÇA PARA MICROPROPAGAÇÃO DE PLANTAS

CURITIBA
2013

ANDRÉ LUÍS LOPES DA SILVA

ATIVIDADE ANTIOXIDANTE, TEOR DE QUERCETINA E DESENVOLVIMENTO DE MEIO DE CULTURA A BASE DE VINHAÇA PARA MICROPROPAGAÇÃO DE PLANTAS

Tese apresentada ao Programa de Pós-Graduação em Engenharia de Bioprocessos, Setor de Tecnologia da Universidade Federal do Paraná, como requisito parcial para obtenção do título de Doutor em Engenharia de Bioprocessos e Biotecnologia.

Orientador: Dr. Carlos Ricardo Soccol

CURITIBA
2013

TERMO DE APROVAÇÃO

_____

Prof. Dr. Gilvano Ebling Brondani

Universidade Federal de Mato Grosso

_____

Prof[a]. Dr[a]. Luciana Porto de Souza Vandenberghe

Universidade Federal do Paraná

_____

Prof. Dr. Eduardo Andrea Lemus Erasmo

Universidade Federal do Tocantins

_____

Prof. Dr. Luiz Antonio Biasi

Universidade Federal do Paraná

_____

Orientador – Prof. Dr. Carlos Ricardo Soccol

Universidade Federal do Paraná

DEDICATÓRIA

Aos meus pais, Wimer e Arlete que sempre acreditaram em mim e pelo incondicional amor e apoio. À Ana Lygia Czap pelo apoio e compreensão durante o desenvolvimento desse trabalho.

EPÍGRAFE

“Para alguns coube o destino de Atenas, para outros o destino de Esparta!”

Frase atribuída ao General Antônio de Sousa Neto

(Herói da Revolução Farroupilha)

1803-1866

“Se não encontrarmos um caminho, abriremos um!“

Aníbal

(General cartaginês)

247-183 a.c.

“Was macht mich nicht umbringt macht mich stärker“

(Nietzsche, filósofo alemão)

1844-1900

AGRADECIMENTOS

Ao Professor Soccol pela honra de tê-lo como meu orientador e pela imensa oportunidade de crescimento e aprendizagem.

À Professora Vanete Thomaz Soccol pelos ensinamentos e amizade.

Aos professores do programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia pela colaboração e amizade.

Aos meus pais, Wimer Garcia da Silva e Arlete Maria Lopes da Silva pelo apoio e amor incondicional.

À Ana Lygia Czap por ter me apoiado durante a execução desse trabalho.

Ao Gessiel Newton Scheidt por ter me apresentado ao programa de pós-graduação em Engenharia de Bioprocessos e Biotecnologia e pela amizade e parceria desde então.

Aos colegas de laboratório que colaboraram com as pesquisas, Jefferson da Luz Costa, José Darcy dos Santos, Cristine Rodrigues, André Luiz Gollo, André Luiz de Almeida Melo, Eduardo Bittencourt Sydney e Francisco Menino Destéfanis Vítola.

À Mitiyo Fukuda Miyaoka pela amizade, atenção e disposição em ajudar.

Ao professor Luiz Antonio Biasi pela ajuda e disponibilidade do Laboratório de Micropropagação e casa de vegetação (Setor de Ciências Agrárias).

Aos estagiários que apresentaram grande dedicação durante a realização dos experimentos, Rafaela de Oliveira Penha e Helena Ross Forneck.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa.

À Deus pela sua infinita misericórdia que sempre me guiou por caminhos repletos de luz, proteção e prosperidade.

A todos os meus mentores e guias espirituais pela ajuda e superação nos momentos mais difíceis.

RESUMO

A cultura de tecidos vegetais permite a obtenção de um grande número de plantas geneticamente idênticas num curto espaço de tempo, além de permitir a produção de metabólitos secundários em ambiente controlado. Entretanto, para garantir competitividade, elevada produção e redução de custos, novas tecnologias devem ser desenvolvidas. Por essa razão os objetivos desse trabalho foram: (1) Estabelecer protocolos de micropropagação para duas espécies de bromélias (*Nidularium procerum* e *Nidularium innocentii*); (2) Desenvolver um meio de cultura usando a vinhaça (resíduo industrial) e (3) Verificar o potencial de uma nova fonte de ácido giberélico (extrato fermentado de polpa cítrica) para aumentar a atividade antioxidante e a acumulação de quercetina em duas bromélias. (1) Os protocolos de micropropagação estabelecidos, diferiram com relação à concentração de BAP usada para a multiplicação *in vitro*, sendo 4 µM de BAP (6-Benzilaminopurina) para *N. procerum* e 8 µM de BAP para *N. innocentii*, podendo ser em meio líquido ou sólido. O alongamento e enraizamento *in vitro* foram eficientemente induzidos em meio de cultura sem a adição de reguladores de crescimento. Plantas enraizadas foram satisfatoriamente aclimatizadas em substrato Plantmax HT® em casa de vegetação com nebulização intermitente resultando em 100% de sobrevivência das mudas micropropagadas. (2) Um meio de cultivo para a cultura de tecidos vegetais usando a vinhaça foi desenvolvido. A melhor diluição de vinhaça (decantada e filtrada) para a formulação dos meios é de 2,5%. Duas formulações de meio de cultura de vinhaça se destacaram sendo a KCV1 (vinhaça 2,5% suplementada com 1000 mg L^{-1} de $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} de $MnSO_4 \cdot 4H_2O$) e KCV5 (vinhaça 2,5% suplementada com 1000 mg L^{-1} de $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} de $MnSO_4 \cdot 4H_2O$ e 240 mg L^{-1} de $NaH_2PO_4 \cdot H_2O$). Todas as etapas da micropropagação foram realizadas nessas formulações a base de vinhaça, e as plantas micropropagadas foram aclimatizadas, demonstrando a eficiência do uso da vinhaça para formular meios de cultura de plantas. (3) Com relação à atividade antioxidante, a *N. innocentii* apresentou maior atividade antioxidante do que a *N. procerum*. O GA_3 pode influenciar a acumulação de metabólitos secundários em *N. innocentii*, mas não na *N.*

procerum. Compostos desconhecidos presentes no extrato fermentado de GA_3 potencializaram a atividade antioxidante e a quantidade de quercetina em *N. innocentii*. Estes resultados sugerem que a *N. innocentii* pode ser uma excelente fonte para a produção de quercetina *in vitro* usando o extrato fermentado de GA_3.

Palavras-chave: resíduo industrial, cultura de tecidos, metabólito secundário, ácido giberélico.

ABSTRACT

The plant tissue culture allows the obtaining of a large number of homogenous genetically plants in a short time, moreover to produce secondary metabolites in a controlled environment. However, to guarantee the competitiveness, high production and cost reduction, new technologies must be developed. Therefore, the aims if this study were: (1) to establish micropropagation protocols for two bromeliad species (*Nidularium procerum* and *Nidularium innocentii*); (2) to develop a new plant culture medium using vinasse (industrial residue) and (3) to verify the potential of a new source of gibberellic acid (fermented extract of citric pulp) to enhance the antioxidant activity and quercetin accumulation in two bromeliads. (1) The micropropagation protocols established differed with regards to BAP (6-Benzylaminopurine) levels used for *in vitro* multiplication, being 4 µM BAP for *N. procerum* and 8 µM BAP for *N. innocentii*, as well as in solid or liquid medium. The *in vitro* elongation and rooting was performed in medium free of plant growth regulators. Rooted plants were acclimatized in Plantmax^{TM} HT substrate in the greenhouse with intermittent nebulization, they reached 100% survival. (2) It was possible to develop a plant culture medium using vinasse that resulted in a culture medium for micropropagation. The best vinasse dilution (decanted and filtered) was 2.5%. The best formulations for vinasse culture medium were KCV1 (2.5% vinasse supplemented with 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$) and KCV5 (2.5% vinasse supplemented with 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 240 mg L^{-1} $NaH_2PO_4 \cdot H_2O$). The micropropagated plantlets in the vinasse culture medium were successfully acclimatized, demonstrating the efficacy of the use of vinasse to formulate plant culture media. (3) The *Nidularium innocentii* presents larger antioxidant activity than *Nidularium procerum*. The GA_3 can influence the accumulation of secondary metabolites in *N. innocentii*, but not in *N. procerum*. Unknown compounds present in the GA_3 fermented extract increase the antioxidant activity and quercetin content in *N. innocentii*. These results suggest that *N. innocentii* can be an excellent source to produce quercetin *in vitro* using GA_3 fermented extract.

Keywords: Industrial residue, Plant tissue culture, secondary metabolite, gibberellic acid.

SUMMARY

1 INTRODUCTION

Plant tissue culture is a technology, which involves the culture of small pieces of tissues (explants) in controlled and axenic (i.e. aseptic) environment. In these conditions, the ability of morphogenesis is controlled by cell totipotency, can be directed to organogenesis (i.e. induction of buds, shoot or roots) or embryogenesis (i.e. induction of somatic embryos). The plant tissue culture can be used for innumerous purposes: (1) massive multiplication of selected plants, (2) cell culture (i.e. suspension), (3) genetic transformation, (4) protoplast culture, (5) *in vitro* mutagenesis, (6) induction of polyploidy and haploid plants; (7) and the obtaining of identical homozygote plants via anther culture or ovule culture to produce pure lines.

The micropropagation is a technique, which utilizes the plant tissue culture and consists of different steps: (1) *in vitro* establishment (disinfection and *in vitro* culture), (2) multiplication of propagules, (3) *in vitro* elongation and rooting of shoots (4) and acclimatization of plants. The micropropagation can be used for massive multiplication of elite plants, *in vitro* conservation, obtaining of secondary metabolites and genetic transformation.

Bromeliads are plants typically of American continent, with exception of *Pitcairnia feliciana* from West Africa. Brazil has approximately 70% of biodiversity of this botany family (i.e. Bromeliaceae). However, all this biodiversity is little explored. Bioprospecting is a concept that involves the process of discovery of new products originated from biological resources (mainly by exploration of the biodiversity) for commercial purposes. However, some of these plants represent potential candidates for the development of new products and must be investigated.

The vinasse is the largest pollution source of the ethanol industry. It is a dark colored liquid wastewater with a high chemical oxygen demand that is removed from the base of distillation columns. It is generated in an average of 13 L for each liter of alcohol produced. However, this residue is rich in several mineral nutrients essential for plant growth, which can also increase crop yield. These mineral nutrients can be used to formulate plant culture medium and this process can be a useful alternative to aid to vinasse disposal.

Antioxidants are molecules that had the ability of scavenging free radicals. Currently, there are great concerns about the deleterious effects of free radicals on the healthy and each more, new antioxidant sources are necessary and used in the cosmetics and pharmaceutical industries. Quercetin is a flavonol that presents an antioxidant activity, it induces the apoptosis in tumor cells and have beneficial effects against cancer and cardiovascular diseases. Currently, quercetin is commercialized as food supplement.

The first chapter describes two micropropagation protocols of *Nidularium procerum* and *Nidularium innocentii*. These species are bromeliads and are endangered and this protocol represents a tool to research these species without to cause environmental harmful; due to the fact there are no predatory collect from its natural habitats. The second chapter approaches the vinasse use to formulate plant culture media as an alternative to reduce the cost production in the tissue culture and an alternative to vinasse disposal. The third chapter evaluates the antioxidant activity of *N. procerum* and *N. innocentii* and the use of gibberellic acid (GA_3) fermented extract obtained by solid state fermentation using *Fusarium moniliforme* as an alternative to reduce costs and enhances the accumulation of secondary metabolites that can be used for different applications.

1.1 General objective

In order to develop innovation in plant tissue culture, such as to establish new micropropagation protocols, to develop a new plant culture medium and to introduce a new source of gibberellic acid to enhance the antioxidant activity and secondary metabolites accumulation.

1.2 Specific objectives

1. In order to establish two micropropagation protocols for *Nidularium procerum* and *Nidularium innocentii*, both bromeliads in endangered;

2. In order to develop a methodology to use vinasse to formulate plant culture medium and to validate this method;
3. To evaluate the antioxidant activity and quercetin content in *Nidularium procerum* and *Nidularium innocentii*;
4. To evaluate the gibberellic acid fermented extract obtained in solid state fermentation by *Fusarium moniliforme* in plant tissue culture for enhance the antioxidant activity and quercetin accumulation.

CHAPTER I - MICROPROPAGATION OF *Nidularium innocentii* LEM. AND *Nidularium procerum* LINDM. (BROMELIACEAE)

ABSTRACT

Nidularium procerum and *Nidularium innocentii* belong to the family Bromeliaceae and have ornamental and medicinal characteristics. A procedure for the micropropagation for these bromeliads is described. Seedling were cultured on MS media (Murashige and Skoog, 1962), liquid and solid supplemented with 0, 2, 4 and 8 µM BAP (6-benzylaminopurine) and 2 µM NAA (naphthalene acetic acid). Leaf explants were cultured on MS media, liquid and solid supplemented with 0, 0.67, 1.35, 2.7 and 5.4 µM NAA (naphthalene acetic acid) and 0.55 µM BAP (6-benzylaminopurine). Isolated shoots were cultured on MS double-phase supplemented with 0, 4.1 and 8.2 µM GA_3 (gibberellic acid). Rooted plants were acclimatized in conventional and hydroponics system. *In vitro* multiplication is more suitable for seedlings than leaf explants. It recommends MS liquid or solid medium supplemented with 4 µM BAP for *Nidularium procerum* and 8 µM BAP for *Nidularium innocentii*, both media supplemented with 2 µM NAA for *in vitro* multiplication. *In vitro* elongation and rooting can be promoted efficiently on medium free of plant growth regulators. Rooted plants can be satisfactorily acclimatized in Plantmax^{TM} HT substrate in the greenhouse with intermittent nebulization.

Keywords: Bromeliads; *in vitro* propagation; hydroponics; liquid culture.

1 INTRODUCTION

Nidularium procerum Lindm. and *Nidularium innocentii* Lem. belong to the family Bromeliaceae and have ornamental and medicinal characteristics. Leaves of *N. procerum* has potent analgesic (Amendoeira *et al*., 2005a) and anti-inflammatory activities (Amendoeira *et al*., 2005b), this species presents also potent anti-eosinophil activity (i.e. anti-allergic properties) (Vieira-de-Abreu *et al*.,

2005). *N. innocentii* is used in popular medicine as anti-inflammatory, analgesic, anti-helmintic and diuretic (Chedier *et al*., 2000). *N. procerum* and *N. innocentii* are threatened and facing extinction due to predatory collect of its habitats. Micropropagation can supply enough plantlets for research, conservation, medicinal and ornamental purposes.

Micropropagation of bromeliads is already established for many species. However, *Nidularium* genus had few reports. *In vitro* growth of *Nidularium fulgens* was evaluated on MS and Knudson media (Paiva *et al*., 2006) and a protocol for *in vitro* propagation of *Nidularium fulgens* was established (Paiva *et al*., 2009). Several explants can be used for *in vitro* multiplication of bromeliads, such as seedlings (Droste *et al*., 2005; Lopes da Silva *et al*., 2007a; Lopes da Silva *et al*., 2008; Paiva *et al*., 2009; Lopes da Silva *et al*., 2009), nodal buds from crowns of young fruits (Khan *et al*., 2004) and leaves (Mercier and Kerbauy, 1992; Rech Filho *et al*., 2009; Lopes da Silva *et al*., 2009). This paper describes a protocol for micropropagation of *N. innocentii* and *N. procerum* through shoot and leaf explants.

2 MATERIALS AND METHODS

2.1 Culture establishment

Two species of bromeliads, *Nidularium procerum* Lindm. and *Nidularium innocentii* Lem. were *in vitro* established. Disinfection consisted of seeds immersion in 70% ethanol during one min, followed by immersion in commercial bleach (1% NaOCl) for 20 min, and rinsed three times with distilled sterilized water. The germination medium was MS (Murashige and Skoog, 1962), with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar.

2.2 *In vitro* multiplication

In order to induce *in vitro* multiplication, a three-way (2x2x4) ANOVA (analysis of variance) experiment was carry out, factor A consisted of two species (*N. procerum* and *N. innocentii*), factor B consisted of two consistency of medium

(solid and liquid) and factor C consisted of BAP (6-benzylaminopurine) levels which were 0, 2, 4 or 8 µM. Basal medium was MS with 30 $g.L^{-1}$ sucrose and 2 µM NAA (naphthalene acetic acid). The solid media experiment was solidified with 6 $g.L^{-1}$ agar and liquid media experiment was shaking at 75 RPM. Shoot number per explant and shoot percentage (i.e. Evaluated the explants with new shoots formed) were evaluated after 120 days of *in vitro* culture.

2.3 Shoot regeneration from leaves

Leaves were removed with tongs and cut at the half; two pieces were cut, a proximal and distal part from the leaf base. In order to induce *in vitro* multiplication from leaf explants, two experiments were carried out, one using proximal part of leaves and other using distal part of leaves. These experiments were organized in a three-way ANOVA (2 x 2 x 5) and the factor A consisted of two species (*N. procerum* and *N. innocentii*), factor B consisted of two consistency of medium (solid and liquid) and factor C consisted of NAA levels, which were 0, 0.67, 1.35, 2.7 or 5.4 µM. Basal medium was composed by MS with 30 $g.L^{-1}$ sucrose and 0.55 µM BAP. Solid media were solidified with 7 $g.L^{-1}$ agar and liquid media were shaking at 75 rpm. Flasks were covered with polypropylene lids. Number and percentage of shoots, and number and percentage of roots were evaluated after 60 days of *in vitro* culture.

2.4 *In vitro* elongation and rooting

Shoots (2 cm height) from clusters cultured *in vitro* were used as explants. Double-phase medium consisted of the same medium for solid and liquid phase with some differences. Basal medium was MS with 30 $g.L^{-1}$ sucrose. For the solid phase was used 30 mL of medium solidified with 7 $g.L^{-1}$ agar and 4 mL of medium for the liquid phase. This experiment were organized in a two-way ANOVA (2 x 3) and the factor A consisted of both species (*N. procerum* and *N. innocentii*) and factor B consisted of GA_3 (gibberellic acid) levels which were 0, 4.1 and 8.2 µM. Solid media phase supplemented with GA_3 had this plant growth regulator autoclavated and liquid media phase supplemented with GA_3 had this

growth regulator microfiltered (0.22 µm) and placed in media after autoclaving. Root number, rooting percentage, height of the aerial part (cm), leaf number, lateral shoot number, fresh weight (g) and lateral shoot percentage were evaluated after 50 days of *in vitro* culture.

2.5 Acclimatization

Plantlets from elongation media (0, 4.1 and 8.2 µM GA_3) was removed from flasks and their roots were washed with tap water (faucet). Two acclimatization system were tested, a conventional and hydroponics system. Conventional acclimatization consisted on the culture of the plantlets in substrate Plantmax^{TM} HT in the greenhouse with intermittent nebulization during 15 days. Hydroponics acclimatization consisted on the culture of the plantlets an alveolated tray with thick sand as a substrate (≥ 1 mm). This tray stayed on a nutritive solution composed with half strength MS medium salts (macro and micronutrients) (Fig. 1-d). Myo-inositol and vitamins were not added. This solution stayed inside a basin and pH was adjusted to 5.8 each 5 days, and solution level was adjusted to one liter with distilled water. Hydroponic solution was oxygenated with aid the air compressor (1.5 $L.min^{-1}$), which resulted in 202 $L.day^{-1}$. Basin was covered with transparent plastic perforate (5 cm^2 each hole) during 10 days of hydroponic culture, and these plantlets were cultured more five days without covered, the period for hydroponic culture was 15 days. Hydroponic culture remained at a growth room with a temperature at 25 ± 2^oC and 16 h of photoperiod, under light intensity of 30 µM m^{-2} s^{-1} obtained by white fluorescent lamps. At the end of 15 days of acclimatization, plantlets were cultured in a substrate plantmax^{TM} HT in the greenhouse with manual irrigation.

This experiment was organized in a three-way ANOVA (2 x 2 x 3) and the factor A consisted of two acclimatization systems (conventional and hydroponics), factor B consisted of two species (*N. procerum* and *N. innocentii*) and factor C consisted of *in vitro* elongation and rooting media where plantlets were produced, these media were supplemented with GA_3 (gibberellic acid) levels which were 0, 4.1 and 8.2 µM. Survival percentage was evaluated after 15

days of culture in acclimatization systems and 15 days of culture in the greenhouse without intermittent nebulization.

2.6 Culture conditions and statistical analysis

All media had the pH adjusted to 5.8 and were autoclaved at 1.5 kgf/cm^2 and 121º C for 20 min. The cultures were kept at 25 ± 2º C under white fluorescent light (28 µM m^{-2} s^{-1}) with a 16 h photoperiod. All experiments used culture flasks with 5 cm diameter and 8.5 cm height and with 30 mL culture medium. The experimental design was completely randomized in a factorial arrangement with five replicates of five explants. The data was submitted in a normality analysis for the Bartlett´s test and, followed by analysis of variance (ANOVA) followed by Duncan´s test, both at a $p<0.01$ and 0.05. Variables from counting were transformed to $\sqrt{x+0.5}$ and variables from percentage were transformed to arcsin $\sqrt{x/100}$. All statistical analyses were done following the procedures of the software GENES (Cruz, 2001).

3 RESULTS AND DISCUSSION

3.1 *In vitro* multiplication from seedlings

For shoot number per explant there were statistical differences for factors, type of species (*N. procerum* and *N. innocentii*), BAP levels (0, 2, 4 and 8 µM) and also for interaction between these factors. *N. procerum* presented higher multiplication rate than *N. innocentii* (Table 1). Solid and liquid media did not show statistical differences and there were not interaction between consistency of medium and BAP levels. The best BAP level for *N. procerum* was 4 µM which obtained 14.9 shoot per explant and for *N. innocentii* was 8 µM which resulted 2.8 shoot per explant (Table 1). In *Nidularium fulgens*, the best result was 5.75 shoot per explant using 4.44 µM BAP and 0.54 µM NAA (Paiva *et al*., 2009).

Table 1. Shoot number per explant of *Nidularium innocentii* and *Nidularium procerum* on liquid and solid media supplemented with different BAP (6-Benzylaminopurine) levels after 120 days of *in vitro* culture. All media was supplemented with 2 µM naphthalene acetic acid (NAA).

	N. procerum			***N. innocentii***		
BAP (µM)	**Liquid**	**Solid**	**Mean**	**Liquid**	**Solid**	**Mean**
0	4.3	5.2	4.7 Ac[1]	2.5	2.4	2.4 Bab
2	15.1	12.9	14 Ab	2.4	2.1	2.2 Bb
4	13.9	15.9	14.9 Aa	2.7	2.4	2.5 Bab
8	16.9	11.7	14.3 Ab	3.3	2.3	2.8 Ba
CV (%)			16.6			

[1]Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at p<0.05 by Duncan's test.

Similar results for *in vitro* multiplication rate of *N. innocentii* was found in *Vriesea scalaris* which reached 2 shoot per explant, however instead of BAP, it was used KIN (6-furfurylaminopurine or kinetin) (Lopes da Silva *et al*., 2009). It is probable that other cytokinins can improve rate proliferation in *N. innocentii*, as in *Dyckia maritima* which KIN was more efficient than BAP (Lopes da Silva *et al*., 2008). Nevertheless, different species has different rate multiplication, what is a genetic characteristic of the species.

Synergic effect between BAP and NAA have been demonstrated to several bromeliad species for adventitious shoot proliferation, as demonstrated in *V. fosteriana* cultivated in KC medium and supplemented with 8.9 µM BAP and 2.7µM NAA which produced approximately 22 explants per seedling (Mercier and Kerbauy, 1992). In *Nidularium fulgens*, the best result was 5.75 shoot per explant using 4.44 µM BAP and 0.54 µM NAA (Paiva *et al*., 2009), in *Vriesea gigantea* and *V. philippocoburgii* were used 8.9 µM BAP and 2.7 µM NAA (Droste *et al*., 2005) and in *Vriesea reitzii*, the highest rate of shoot multiplication was with 2-4 µM BAP and 1-2 µM NAA (Rech-Filho *et al*., 2005).

For shoot percentage statistical differences were observed for type of species only. *N. procerum* was superior to *N. innocentii* for this variable. However, there were not significance for BAP levels, consistency of media and interaction. *N. procerum* vary from 92 to 100% and *N. innocentii* from 55 to 80% for shoot percentage (Table 2). These results can be due to juvenility explant; finally it was used shoot as explants on the contrary seeds. When bromeliad seeds are used

as explants, it results in 100% shoot formation (Pompelli and Guerra, 2005) and when seedlings (shoot from seeds) are used it results in minor shoot formation rate (Lopes da Silva *et al*., 2008).

Table 2. Shoot percentage of *Nidularium innocentii* and *Nidularium procerum* on liquid and solid media supplemented with different BAP (6-Benzylaminopurine) levels after 120 days of culture. All media was supplemented with 2µM naphthalene acetic acid (NAA).

	N. procerum		***N. innocentii***	
BAP (µM)	**Liquid**	**Solid**	**Liquid**	**Solid**
0	93.3[1]	96	55	60
2	100	96	60	55
4	95	100	76.6	61
8	100	92	80	65
CV (%)	17.7			

[1]No statistical differences among treatments.

There was no hyperhydricity occurrence in these species (*N. innocentii* and *N. procerum*); when cultivated in the liquid media, whereas the most common cause of hyperhydricity is the liquid culture (Scheidt *et al*., 2011). Moreover, we did not observe hyperhydricity in these species in stationary cultures (Data not shown). Similar results were found in *Melaleuca alternifolia* cultured in stationary liquid media (Oliveira *et al*., 2010). This capacity to tolerate the stationary culture is very important to reduce production costs, whereas the use of agar and electric energy to move shakers is not necessary.

3.2 *In vitro* multiplication from leaf explants

Leaf explants presented different morphogenic answers, distal parts from the leaf base does not show visual effects and the most part died, instead of proximal parts from the leaf base formed roots and shoots in some NAA levels, these results were observed for both species. Similar results were found in *Vriesea scalaris* which shoots were formed at the base from leaves (Lopes da Silva *et al*., 2009). Another similar result with morphogenic capacities of proximal part from the leaf base was found in *Cryptanthus*, which calli were formed at the base from leaf explant (Koh and Davies, 1998). Calli were not observed in leaf

explants cultivated with NAA. Probably the endogenous level of cytokinins in leaves is higher than the NAA level used, whereas calli normally are induced with auxin level larger than cytokinin level.

There were statistical differences ($p<0.01$) for factors (type of species, consistency of the medium and NAA levels) and for all interactions in the variables shoot number, shoot percentage, root number and root percentage. However, exceptions were found for factor consistency of the medium and for the interaction among species and consistency of medium in the shoot percentage that had no statistical differences.

Shoot percentage in leaf explants using NAA was low; the best NAA level was 1.35 µM for *N. procerum* in the liquid medium (40% shoot) (Table 3 and Fig. 1a) and 2.7µM for *N. innocentii* in the solid medium (20% shoot) (Table 3). For *N. procerum* the most suitable NAA level (1.35 µM) in the liquid medium produced 2.1 shoots per explant, and the best NAA level (2.7 µM) in the solid medium produced 4 shoots per explant. For *N. innocentii*, the most suitable NAA level (5.4 µM) in the liquid medium produced 1.3 shoots per explant, and the best NAA level (2.7 µM) in the solid medium produced 2 shoots per explant (Table 4).

Table 3. Shoot percentage of *Nidularium procerum* and *Nidularium innocentii* from leaf explants (proximal part from leaf base) in solid and liquid media with different naphthalene acetic acid (NAA) levels after 60 days of *in vitro* culture. All media was supplemented with 0.55 µM 6-benzylaminopurine (BAP).

	N. procerum		***N. innocentii***	
NAA (µM)	**Liquid**	**Solid**	**Liquid**	**Solid**
0.00	0 Ab[1]	12 Aa	0 Aa	0 Ab
0.67	0 Ab	0 Aa	0 Aa	0 Ab
1.35	40 Aa	4 Ba	0 Ba	0 Bb
2.70	0 Bb	8 ABa	0 Ba	20 Aa
5.40	0 Ab	4 Aa	10 Aa	0 Ab
CV (%)	69.1			

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's test.

Table 4. Shoot number of *Nidularium procerum* and *Nidularium innocentii* from leaf explants (proximal piece) in solid and liquid media with different naphthalene acetic acid (NAA) levels. All media was supplemented with 0.55 µM 6-benzylaminopurine (BAP).

	N. procerum		***N. innocentii***	
NAA (µM)	**Liquid**	**Solid**	**Liquid**	**Solid**
0.00	0 Bb	2.0 Ab	0 Bb	0 Bb
0.67	0 Ab	0 Ad	0 Ab	0 Ab
1.35	2.1 Aa	1.3 Bc	0 Cb	0 Cb
2.70	0 Cb	4.0 Aa	0 Cb	2.0 Ba
5.40	0 Bb	1.1 Ac	1.3 Aa	0 Bb
CV (%)	17.3			

[1]Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's test.

Comparing these data obtained with leaf explants with those obtained with seedlings (Table 1 and 2), it was observed which seedlings have more competence for multiplication rate; of course that plant growth regulators used for these explants were different. Nevertheless, similar results were found in *Vriesea fosteriana*, which frequency of regeneration in leaf explants and shoot number formed per explant were lower than in seedling explants (Mercier and Kerbauy, 1992).

Leaf explants (whole leaves) of *Vriesea scalaris* cultivated on MS medium supplemented with 0.4 µM BAP, 0.5 µM NAA and 0.06 $g.L^{-1}$ NH_4NO_3 produced approximately 8 shoots per explant after 60 days of *in vitro* culture (Lopes da Silva *et al*., 2009). In *Neoregelia cruenta* it was observed direct shoot formation from whole leaves (at the base) cultivated on MS medium supplemented with 22 µM BAP and 2.5 µM NAA, which produced 50.3 shoots per explant after eight months of *in vitro* culture (Carneiro *et al*., 1999).

Roots were formed at the leaf base. Rooting in *N. procerum* occurred with 1.35 µM NAA in solid medium only, resulting in one root per explant and 4% of rooting. On the contrary, *N. innocentii* had higher rooting rate in liquid medium, the most suitable result was with 5.4 µM NAA (70%), and with 0.67, 1.35, 2.7 µM NAA (40%). Root number varied from 7 to 10.5 roots per explant in *N. innocentii* (data not shown). Similar results were found in *Vriesea fosteriana*, which shoots

were rooted with 1.1 μM NAA and after 60 days of culture every shoot formed 3-4 roots (Mercier and Kerbauy, 1992).

3.3 *In vitro* elongation and rooting

There were no statistical differences in the factors, species and GA_3 levels and there was no interaction among these factors for root percentage and leaf number variables. Factor type of species was significant for fresh mass ($p<0.01$) and for percentage of lateral shoots ($p<0.05$), nevertheless the factor GA_3 levels and interaction among factors type of species and GA_3 levels was not significant. There were no statistical differences for height of the aerial part among species and there was not interaction, although GA_3 levels were significant ($p<0.01$) (Fig. 1b). For the variable number of lateral shoots there were no statistical differences for factors type of species and GA_3 levels, however, this interaction was significant ($p<0.05$). The variable root number showed statistical differences for factors type of species and GA_3 levels ($p<0.01$), however, their interaction was not significant (Table 5).

Root number was favored with absence of GA_3. Similar result was found in *Vriesea scalaris* which culture free of plant growth regulators promoted best results (Lopes da Silva *et al*., 2009). *N. innocentii* formed more roots than *N. procerum*, 8.5 and 3.4 roots per shoot, respectively. Root percentage varied from 96 to 100% for both species and GA_3 levels (Table 6). The most suitable level of GA_3 was 8.2μM for variable height of the aerial part, resulting in 7.1cm against 4.4cm at absence of GA_3. Similar result was observed in *Dyckia maritima* cultivated on MS medium, the most suitable level of GA_3 for height of the aerial part of multiple shoots was 7.13 μM, and also observed that increases of GA_3 levels decreases number of lateral shoots (Lopes da Silva *et al*., 2004).

Table 5. Summary of two-way ANOVA (analysis of variance). Height of the aerial part (AP cm), number of lateral shoots (BL), percentage of lateral shoots (BL %), root number per explant (NR), percentage of rooting (E %), fresh weight (FW g), leaf number per explant (NF) observed in shoots of *Nidularium innocentii* and *Nidularium procerum* after 50 days of *in vitro* culture on double-phase media with different levels of gibberellic acid (0, 4.1 and 8.2 μM GA_3).

	Mean Square							
Source	**d.f.**	**AP cm**	**BL**	**BL %**	**NR**	**E %**	**FW g**	**NF**
A[1]	1	1.260 ^{ns}	0.05270 ^{ns}	2452.2 **	7.7516 *	94.09 ^{ns}	0.738 *	0.289 ^{ns}
B[2]	2	17.566 *	0.2194 ^{ns}	814.54 ^{ns}	0.5289 *	23.52 ^{ns}	0.035 ^{ns}	0.838 ^{ns}
A x B	2	2.951 ^{ns}	0.7231 **	960.18 ^{ns}	0.1282 ^{ns}	23.52 ^{ns}	0.024 ^{ns}	0.840 ^{ns}
Residual	24	0.9829	0.1505	319.50	0.0747	47.046	0.0143	0.362
CV(%)		17.1	24.1	28.8	10.9	7.7	40.0	13.2

[1] Species (*Nidularium innocentii* and *Nidularium procerum*), [2] GA_3 levels (0, 4.1 and 8.2μM), * $p < 0.01$, ** $p < 0.05$, ^{ns} Not significant.

Number of leaves varied from 15.7 to 25.7 leaves per shoot. *N. procerum* presented more percentage of lateral shoots than *N. innocentii*, 80 and 61.3%, respectively. Number of lateral shoots was higher with 8.2 μM GA_3 in *N. innocentti* and higher at the absence of GA_3 in *N. procerum*, 3.4 shoots per explant in both species. *N. innocentii* showed higher fresh weight than *N. procerum*, 0.455 and 0.142 g, respectively, although fresh mass do not show statistical difference for GA_3 levels, results of the fresh mass found in shoots cultivated with GA_3 was lower than shoots cultivated in the absence of GA_3 (Table 6). Similar result was found in *Vriesea friburgensis* cultivated on MS medium, the increase of GA_3 levels resulted in decrease of the fresh mass after 60 days of culture (Alves and Guerra, 2001).

Table 6. Characteristics observed in *N. innocentii* and *N. procerum* cultivated on double-phase media supplemented with different levels of gibberellic acid (GA_3) after 50 days of *in vitro* culture.

	Root number			**Rooting (%)**		
GA_3	**Species**			**Species**		
(µM)	***N. innocentii***	***N. procerum***	**Mean**	***N. innocentii***	***N. procerum***	**Mean**
0.0	10.8	4.1	7.4 a[1]	100[2]	96	98
4.1	7.6	3.6	5.6 b	100	100	100
8.2	7.3	2.7	5.0 b	100	96	98
Mean	8.5 a	3.4 b		100	97.3	
	Heigth of the aerial part (cm)			**Lateral shoots (%)**		
GA_3	**Species**			**Species**		
(µM)	***N. innocentii***	***N. procerum***	**Mean**	***N. innocentii***	***N. procerum***	**Mean**
0.0	5.2	3.7	4.4 c	64	100	82
4.1	5.4	6.1	5.7 b	48	72	60
8.2	7.3	6.9	7.1 a	72	68	70
Mean	5.9	5.5		61.3 b	80 a	
	Number of lateral shoots			**Fresh mass (g)**		
GA_3	**Species**			**Species**		
(µM)	***N. innocentii***	***N. procerum***	**Mean**	***N. innocentii***	***N. procerum***	**Mean**
0.0	1.9 Bb	3.4 Aa	2.6	0.5677	0.1528	0.3602
4.1	1.2 Bc	2.1 Ab	1.6	0.3485	0.1331	0.2408
8.2	3.4 Aa	1.4 Bc	2.4	0.4513	0.1401	0.2957
Mean	2.2	2.3		0.4558 a	0.1420 b	

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's test.

[2] Means without letters do not have statistical differences.

3.4 Acclimatization

There were no statistical differences after 15 days of acclimatization for factors, type of species, GA_3 levels and type of acclimatization, the survival rate varied from 91.6 to 100% (data not shown). However, after 30 days of acclimatization, there were statistical differences for factors, type of species and type of acclimatization method and for interaction triple. *N. procerum* cultivated on hydroponics presented lower survival rate than *N. innocentii*, although these species was not statistically different when cultivated in conventional

acclimatization. Survival rate was lower using hydroponics acclimatization than conventional and the GA_3 levels do not had statistical differences (Table 7). The most suitable result for acclimatization of micropropagated plants of *N. procerum* and *N. innocentii* was acquired with conventional acclimatization (Table 7 and Fig. 1c). Similar result was found in micropropagated plants of *Nidularium fulgens* cultivated in greenhouse irrigated with a nebulization system during 60 days, which survival rate was 100% (Paiva *et al*., 2009).

Micropropagated plants of *Dyckia maritima* were acclimatized using hydroponics, 80% of survival rate was reached when plants were cultivated on coconut fiber, and 60% when cultivated on worm humus and a mixture of coconut fiber: worm humus (1:1 v/v) (Lopes da Silva *et al*., 2007b), the acclimatization using hydroponics was more fast than the conventional for this species (Lopes da Silva et al., 2006). However, it is probable that the results of this present study can be more elevated using other substrates for hydroponics acclimatization. Although, acclimatization using hydroponics is efficient for the hydrophilic plants (Nhut *et al*., 2004). Nevertheless, acclimatization using hydroponics is an alternative for laboratories that do not have greenhouse with intermittent nebulization.

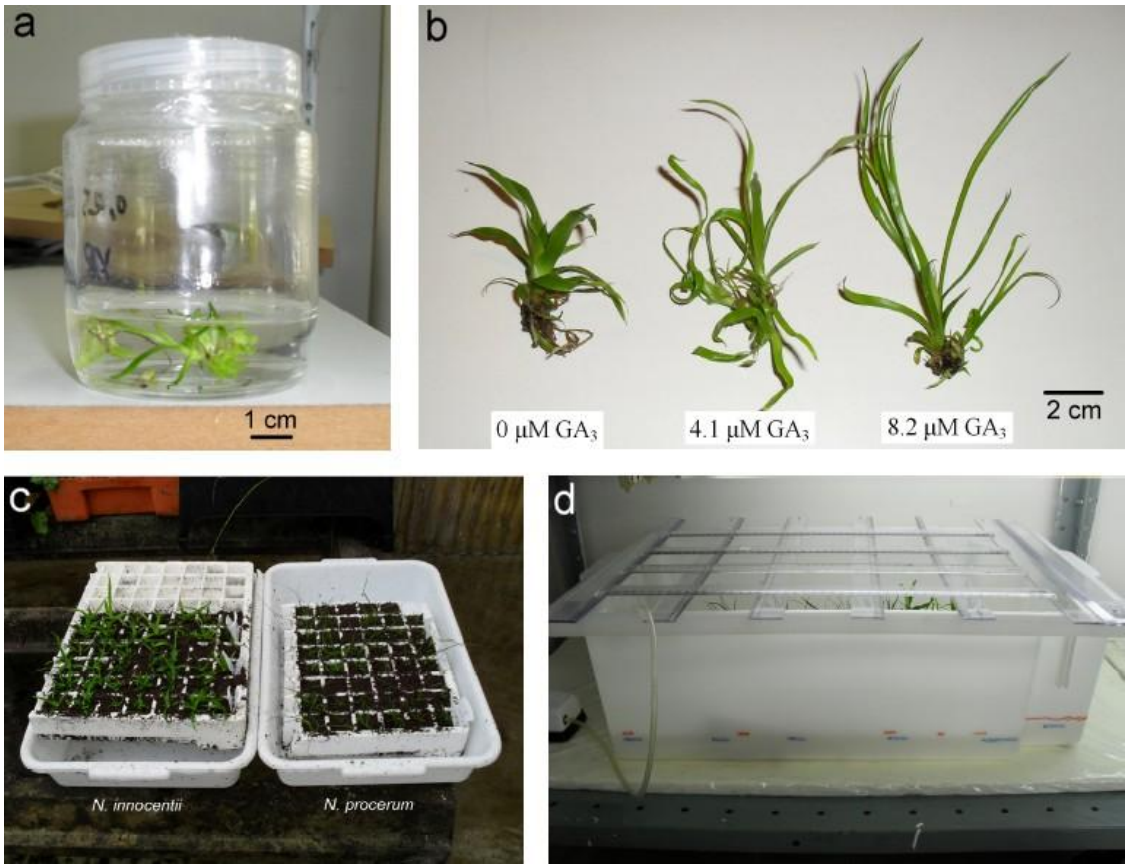

Fig. 1. (a) Multiple shoots formed from base at the leaf of *Nidularium procerum* cultivated *in vitro* on liquid medium supplemented with 1.35 µM NAA and 0.55 µM BAP, (b) *Nidularium innocentii* cultivated *in vitro* at the different levels of gibberellic acid on double-phase medium, (c) *Nidularium procerum* and *Nidularium innocentii* acclimatized on Plantmax^{TM} HT substrate and (d) Hydroponics system for plant acclimatization.

Table 7. Survival percentage of micropropagated plants of *Nidularium procerum* and *Nidularium innocentii* on conventional and hydroponics acclimatization after 30 days of *ex vitro* culture.

GA_3	**Conventional**		**Hydroponics**	
(µM)	***N. procerum***	***N. innocentii***	***N. procerum***	***N. innocentii***
0.0	100 aA[1]	100 aA	50 aC	83.3 aB
4.1	91.6 aA	100 aA	66.6 aC	83.3 aB
8.2	91.6 aA	100 aA	58.3 aC	83.3 aB
CV(%)	6.7			

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's test.

4 CONCLUSIONS

In vitro multiplication is more suitable with seedlings than leaf explants. It recommends MS liquid or solid medium supplemented with 4 µM BAP for *Nidularium procerum* and 8 µM BAP for *Nidularium innocentii*, both media supplemented with 2 µM NAA for *in vitro* multiplication. *In vitro* elongation and rooting can be promoted efficiently on medium free of plant growth regulators. Rooted plants can be satisfactorily acclimatized in a conventional acclimatization, cultivated in Plantmax^{TM} HT substrate in the greenhouse with intermittent nebulization.

REFERENCES

ALVES, G. M.; GUERRA, M. P. Micropropagation for mass propagation and conservation of *Vriesea friburgensis* var. paludosa from microbuds. **Journal of the Bromeliad Society**, v. 51, n. 5, p. 202-212, 2001.

AMENDOEIRA, F. C.; FRUTUOSO, V. S.; CHEDIER, L. M.; PEARMAN, A. T.; FIGUEIREDO, M. R.; KAPLAN, M. A.; PRESCOTT, S. M.; CASTRO-FARIA-NETO, H. C. Antinociceptive effect of *Nidularium procerum*: a Bromeliaceae from the Brazilian coastal rain forest. **Phytomedicine**, v. 12, n. 1-2, p. 78-87, 2005a.

AMENDOEIRA, F. C.; FRUTUOSO, V. S.; ZANON, C.; CHEDIER, L. M.; FIGUEIREDO, M. R.; KAPLAN, M. A. C.; MELO, C. B.; BOZZA, P. T.; CASTRO-FARIA-NETO, H. C. Anti-inflammatory Activity in the Aqueous Crude Extract of the Leaves of *Nidularium procerum*: A Bromeliaceae from the Brazilian Coastal Rain Forest. **Biological and Pharmaceutical Bulletin**, v. 28, n. 6, p. 1010-1015, 2005b.

CARNEIRO, L. A.; ARAÚJO, R. F. G.; BRITO, G. J. M.; FONSECA, M. H. P. B.; COSTA, A.; CROCOMO, O. J.; MANSUR, E. *In vitro* regeneration from leaf explants of *Neoregelia cruenta* (R. Graham) L.B. Smith, an endemic bromeliad from eastern Brazil. **Plant Cell, Tissue and Organ Culture**, v. 55, n. 2, p. 79-83, 1999.

CHEDIER, L. M.; FIGUEIREDO, M. R.; KAPLAN, M. A. C. Chemical and biological investigations on *Nidularium innocentii* Lemaire. **Anais da Academia Brasileira de Ciências,** v. 72, n. 2, p. 295-295, 2000.

CRUZ, C. D. **Programa Genes: versão Windows; aplicativo computacional em genética e estatística.** Viçosa, MG: UFV, Imprensa Universitária, 2001, 648p.

DROSTE, A.; SILVA, A. M.; MATOS, A. V.; ALMEIDA, J. W. *In vitro* culture of *Vriesea gigantea* and *Vriesea philippocoburgii*: Two vulnerable bromeliads native to southern Brazil. **Brazilian Archives of Biology and Technology**, v. 48, n. 5, p. 717-722, 2005.

KHAN, S.; NASIB, A.; SAEED, B. A. Employment of *in vitro* technology for large scale multiplication of pineapples (*Ananas comosos*). **Pakistan Journal of Botany**, v. 36, n. 3, p. 611-615, 2004.

KOH, Y. C.; DAVIES, F. T. J. Micropropagation of *Cryptanthus* with leaf explants with attached intercalary meristems excised from greenhouse stock plants. **Scientia Horticulturae**, v. 70, n. 4, p. 301-307, 1997.

LOPES DA SILVA, A. L.; FRANCO, E. T. H.; BISOGNIN, D. A.; DORNELLES, E. B.; WALTER, J. M. Ensaios sobre o alongamento de brotos laterais de *Dyckia maritima* Baker dispostos em agrupamentos - Bromeliaceae. **Caderno de Pesquisa Série Biologia**, v. 16, n. 2, p. 37-46 2004.

LOPES DA SILVA, A. L.; FRANCO, E. T. H.; WALTER, J. M.; BISOGNIN, D. A.; SPANHOLI, N. C. Aclimatização de clones de *Dyckia maritima* em diferentes substratos - Bromeliaceae. **Revista Brasileira de Agrociencia**, v. 12, n. 4, p. 495-498, 2006.

LOPES DA SILVA, A. L.; DORNELLES, E. B.; BISOGNIN, D. A.; FRANCO, E. T. H.; HORBACH, M. A. Micropropagation of *Dyckia agudensis* Irgang & Sobral an exctinction threatened bromeliad. **Iheringia Série Botânica**, v. 62, n. 1-2, p. 39-43, 2007a.

LOPES DA SILVA, A. L.; FRANCO, E. T. H.; HORBACH, M. A.; BISOGNIN, D. A.; QUOIRIN, M. Acclimatization of *Dyckia maritima* Baker using hydroponics (Bromeliaceae). **Caderno de Pesquisa Série Biologia**, v. 19, n. 3, p. 16-23, 2007b.

LOPES DA SILVA, A. L.; FRANCO, E. T. H.; DORNELLES, E. B.; GESING, J. P. A. Micropropagation of *Dyckia maritima* Baker - Bromeliaceae. **Iheringia Série Botânica**, v. 63, n. 1, p. 135-138, 2008.

LOPES DA SILVA, A. L.; FRANCO, E. T. H.; DORNELLES, E. B.; REICHERT BORTOLI, C. L.; QUOIRIN, M. *In vitro* multiplication of *Vriesia scalaris* E. Morrem (Bromeliaceae). **Iheringia Série Botânica**, v. 64, n. 2, p. 151-156, 2009.

MERCIER, H.; KERBAUY, G. B. *In vitro* multiplication of *Vriesea fosteriana*. **Plant Cell, Tissue and Organ Culture**, v. 30, n. 3, p. 247-249, 1992.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v. 15, n. 3, p. 473-497, 1962.

NHUT, D. T.; HUONG, N. T. D.; VAN-KHIEM, D. Direct microtuber formation and enhanced growth in the acclimatization of *in vitro* plantlets of taro (*Colocasia esculenta* spp.) using hydroponics. **Scientia Horticulturae**, v. 101, n. 1-2, p. 207-212, 2004.

OLIVEIRA, Y.; PINTO, F.; LOPES DA SILVA, A. L.; GUEDES, I.; BIASI, L. A.; QUOIRIN, M. An efficient protocol for micropropagation of *Melaleuca alternifolia* Cheel. **In Vitro Cellular & Developmental Biology - Plant**, v. 46, n. 2, p. 192-197, 2010.

PAIVA, P. D. O.; COELHO-NAVES, V.; FERREIRA-DUTRA, L.; PAIVA, R.; PASQUAL, M. *In vitro* propagation of *Nidularium fulgens* lem. **Interciencia**, v. 34, n. 8, p. 593-596, 2009.

PAIVA, P. D. O.; NAVES, V. C.; PAIVA, R.; PASQUAL, M. Avaliação de diferentes formulações de sais minerais para a micropropagação de *Nidularium fulgens* Lem.. **Plant Cell Culture and Micropropagation**, v. 2, n. 1, p. 9-14, 2006.

POMPELLI, M. F.; GUERRA, M. P. Micropropagation enables the mass propagation and conservation of *Dyckia distachya* Hassler. **Crop Breeding and Applied Biotechnology**, v. 5, n. 1, p. 117-124, 2005.

RECH FILHO, A.; DAL VESCO, L. L.; GUERRA, M. P. Adventitious shoots from nodule cluster cultures of *Vriesea reitzii*: an endemic and endangered bromeliad from atlantic forest. **Ciência Rural**, v. 39, n. 3, p. 909-912, 2009.

RECH FILHO, A.; DAL VESCO, L. L.; NODARI, R. O.; LISCHKA, R. W.; MÜLLER, C. V.; GUERRA, M. P. Tissue culture for the conservation and mass propagation of *Vriesea reitzii* Leme and Costa, a bromeliad threatened of extinction from the Brazilian Atlantic Forest. **Biodiversity and Conservation**, v. 14, n. 8, p. 1799-1808, 2005.

SCHEIDT, G. N.; LOPES DA SILVA, A. L.; OLIVEIRA, Y.; COSTA, J. L.; BIASI, L. A.; SOCCOL, C. R. *In vitro* growth of *Melaleuca alternifolia* Cheel in bioreactor of immersion by bubbles. **Pakistan Journal of Botany**, v. 43, n. 69, p. 2937-2939, 2011.

VIEIRA-DE-ABREU, A.; AMENDOEIRA, F. C.; GOMES, G. S.; ZANON, C.; CHEDIER, L. M.; FIGUEIREDO, M. R.; KAPLAN, M. A.; FRUTUOSO, V. S.; CASTRO-FARIA-NETO, H. C.; WELLER, P. F.; BANDEIRA-MELO, C.; BOZZA, P. T. Anti-allergic properties of the bromeliaceae *Nidularium procerum*: inhibition of eosinophil activation and influx. **International Immunopharmacology**, v. 5, n. 1966-1974, 2005.

CHAPTER II - VINASSE: A NEW SOURCE OF NUTRIENTS TO FORMULATE A CULTURE MEDIUM FOR PLANT TISSUE CULTURE

ABSTRACT

Vinasse is the main pollutant (effluent) obtained from the distillation of sugarcane in the production of fuel alcohol. However, this residue is rich in nutrients that are required by plants. A new culture medium using vinasse for the *in vitro* propagation of an orchid was developed. The vinasse was treated (decanted and filtered), and the nutrients were determined and quantified. Different formulations using vinasse were tested for an *in vitro* culture. The vinasse dilutions demonstrated a good buffering effect. The ideal vinasse dilution for media formulation was 2.5%. The best KC formulations with vinasse were KCV1 and KCV5. Compared to KC medium, these formulations demonstrated similar results for *in vitro* multiplication, with the exception of protocorm-like body number, which was inferior in the vinasse formulations. Conversely, for *in vitro* elongation and rooting, these vinasse media were superior to KC medium. KC medium promotes a low rooting rate (8%) compared to 68 and 100% obtained by KCV1 and KCV5, respectively. Moreover, plantlets cultured on KC medium become protocorm-like body clusters, which impeded the acclimatization of these explants. Plantlets elongated and rooted on KCV1 and KCV5 were successfully acclimatized with a 91% survival rate for both KC vinasse formulations. This study shows the great potential of this technology as a rational alternative to vinasse disposal and adds value to what is currently considered a waste product.

Keywords: industrial residue; *in vitro* culture; biofactory; orchid; micropropagation; vinasse disposal.

1 INTRODUCTION

Vinasse is the main effluent obtained from the distillation of the fermented sugarcane juices used to produce fuel alcohol and presents high pollutant potential. For each liter of fuel alcohol produced, approximately 10 to 18 liters of vinasse are produced, depending of the technological level of the distillery (Da Silva et al., 2007). The large volume of this residue that is produced, billions of liters per year, is of a great environmental concern. Vinasse disposal has a great environmental impact on lakes, rivers, streams and underground waters due to high BOD (biochemical oxygen demand) and COD (chemical oxygen demand), which results in the suffocation of aquatic fauna and flora, resulting in the death of aquatic life. However, this residue is rich in several mineral nutrients that are required for plant growth, and its application on crop fields increases yield (Almeida, 1952). Nevertheless, the constant application of vinasse on the soil increases soil salinity due to the high content of potassium, which is retained in the soil when the water evaporates.

Tissue culture constitutes a way to maintain available competent explants (plant parts) free from contamination for use in the *in vitro* propagation (micropropagation), genetic transformation and *in vitro* conservation (Machado et al., 2011). Tissue culture is the only methodology that can produce a large quantity of clonal plants in a short time with high phytosanitary quality. The development of new cheaper methods for *in vitro* cultures is necessary. Some progress has been made, including automated methods of *in vitro* culture using bioreactors, which reduce costs due to the use of a liquid culture medium that eliminates the need of agar that is very expensive. Moreover, the culture, using natural light and macro and micronutrients as precursors for plant growth regulators, has contributed to decreased costs.

The use of nutrients from vinasse to develop a culture medium for the *in vitro* propagation of plants can decrease the environmental impact caused by this residue and, moreover, it decreases plant production costs. Currently, plant production using *in vitro* propagation is becoming more common. Biofactories are in constant expansion in many industrialized countries. In this context, it was developed a plant culture medium using vinasse. The aim of this research was to

demonstrate the efficiency of vinasse when used as the base for a culture medium as an alternative to vinasse disposal. This study is part of a patented process (Soccol et al., 2008) that was developed in our laboratory and is the first report on the use of vinasse in plant tissue culture.

2 MATERIAL AND METHODS

2.1 Vinasse source and preparation

Vinasse was collected in artificial ponds made of impermeable material. The vinasse stored in these ponds originated from the distillation of alcohol that was obtained from sugarcane juice. The vinasse was decanted and filtered, according to Santos et al., (2013). After the pretreatment of vinasse, the biochemical oxygen demand (BOD) was determined by a five-day incubation, and the chemical oxygen demand (COD) was assessed by the open reflux method, both following the methodologies proposed by the APHA (Eaton et al., 2005).

2.2 Vinasse culture media

In order to develop a vinasse culture medium, treated vinasse (decanted and filtered) was analyzed for the sulfate, chloride, phosphate, nitrate, ammonium, calcium, magnesium, sodium, potassium, manganese, zinc and total iron content. The sulfate content was quantified by the turbidimetric method, the chloride content was quantified by the titulometric method with mercury nitrate, the phosphate content was quantified by the colorimetric method (ascorbic acid) and the nitrogen content, as nitrate and ammonium, was quantified by the reduction method with cadmium and the phenate method, respectively. The calcium and magnesium contents were obtained by titulometry with EDTA (ethylenediamine tetraacetic acid), and the sodium and potassium contents were quantified by flame photometry (Clesceri et al., 1998). The total iron content was quantified by the phenanthroline method (Saywell and Cunningham, 1937). The micronutrients, such as zinc and manganese, were quantified by atomic

absorption spectroscopy (Clesceri et al., 1998). The vinasse medium was determined on the quantity of ions contained in the KC medium (Knudson, 1946) (Table 1).

Table 1. Ions contained in the KC medium (Knudson, 1946), in the KC vinasse media and in the 2.5% treated vinasse. KCV1 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$, KCV2 = 2.5% vinasse and 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, KCV3 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 170.6 mg L^{-1} $Ca_3(PO_4)_2$, KCV4 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 120 mg L^{-1} $NaH_2PO_4 \cdot H_2O$, KCV5 = 2.5% vinasse, 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 6.5 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 240 mg L^{-1} $NaH_2PO_4 \cdot H_2O$. All vinasse used for media formulation was pretreated (decanted and filtered).

Ion ($mg.L^{-1}$)	KC	KCV1	KCV2	KCV3	KCV4	KCV5	2.5% treated vinasse[1]
Ca	169.72	210.77	210.77	276.90	210.77	210.77	41.05
NO_3	525.12	525.14	525.14	525.14	525.14	525.14	0.0165
NH_4	136.51	1.1912	1.1912	1.1912	1.1912	1.1912	1.1912
SO_4	472.76	39.25	36.45	39.25	39.25	39.25	36.45
Mg	24.65	2.545	2.545	2.545	2.545	2.545	2.545
Fe	5.02	0.698	0.698	0.698	0.698	0.698	0.698
Mn	1.85	1.67	0.072	1.67	1.67	1.67	0.072
K	71.83	44.0	44.0	44.0	44.0	44.0	44.0
PO_4	174.47	9.5	9.5	113.97	92.08	174.68	9.5
Na	-	0.175	0.175	0.175	20.17	40.16	0.175
Cl	-	0.927	0.927	0.927	0.927	0.927	0.927
Zn	-	0.019	0.019	0.019	0.019	0.019	0.019

[1] Adapted from Santos et al., (2013).

2.3 Buffer effect in vinasse

The vinasse buffer effect was evaluated by using it as a culture medium. Treated vinasse was diluted at 5, 10 and 15% and was supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP (6-Benzylaminopurine). The pH was measured and reported every seven days for 28 days.

2.4 Culture establishment

Oncidium leucochilum Batem. Ex Lindl. was used to test the vinasse culture media. Closed capsules of *O. leuchochilum* were disinfected by immersion in 70% ethanol for 1 min followed by immersion in NaOCl (2.5%) for 20 min. The capsules were then washed three times with distilled and autoclaved water and were then opened inside a laminar flow chamber, where the seeds were inoculated on half-strength MS salts (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose and solidified with 6 g L^{-1} agar (Vetec®). The seedlings were *in vitro* propagated using the same medium supplemented with 0.3 mg L^{-1} BAP (Scheidt et al., 2009).

2.4.1 *In vitro* culture on vinasse dilutions

To evaluate the effects of the dilution of vinasse, shoots (1 cm aerial part height) were cultured in liquid vinasse media at a 0, 2.5, 5 and 10% dilution. MS medium was used as a control to compare the vinasse dilutions. All media were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP. The leaf number, lateral shoot number, root number, height of the aerial part (cm), fresh mass (g), protuberance number, shoot percentage, rooting percentage, percentage of explants with some necrosis point and survival percentage were evaluated after 60 days of *in vitro* culture.

2.4.2 *In vitro* culture on MS medium with substitution of iron and calcium by vinasse ions

To substitute the iron and calcium of the MS medium with the vinasse ions, $CaCl_2 \cdot 2H_2O$, $Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ were removed from the MS medium, which was then supplemented with 0, 2.5, 5 and 10% treated vinasse. Complete MS medium was used as control. All media were liquid and were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP. Shoots with 1.5 cm height were used as explants. All variables described above were analyzed after

60 days of *in vitro* culture, with exception of the percentage of explants with some necrosis point which was substituted by the hyperhydricity percentage.

2.4.3 *In vitro* multiplication on different KC vinasse media

To induce multiple shoots in explants, different KC vinasse media were tested. The media were: KC (control), KCV1, KCV2, KCV3, KCV4 and KCV5 (Table 1). All media were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP and were solidified with 6 g L^{-1} agar. KC medium was used as a control. All variables described above were analyzed after 60 days of *in vitro* culture.

2.4.4 *In vitro* elongation and rooting on different KC vinasse media

Shoots (1 cm aerial part height) were cultured in three media: KC (control), KCV1 and KCV5. All media were supplemented with 30 g L^{-1} sucrose and 1 g L^{-1} activated charcoal and were solidified with 6 g L^{-1} agar. The shoot number, root number, height of the aerial part (cm), fresh mass (g), shoot percentage, root percentage, protuberance percentage and survival percentage were evaluated after seven months of *in vitro* culture.

2.5 Acclimatization

Plantlets from the elongation and rooting media were removed from the flasks, and their roots were washed with tap water (faucet). Complete plantlets were obtained in KCV1 and KCV5 only; therefore, only plantlets originating from these two media were acclimatized. The plantlets were cultured in substrate coconut fiber in the greenhouse with intermittent nebulization for 14 days. After two weeks of intermittent nebulization, the plantlets were transferred to a greenhouse with manual irrigation. Leaf fertilizer was applied every week. This fertilizer consisted of NPK (06-06-08) + micronutrients, Ca, S, Mg, B, Cu, Zn (01-0.5-0.5-0.03-0.1-0.1). The survival percentage was evaluated after 28 days of *ex vitro* culture.

2.6 Culture conditions and statistical analysis

All media had their pH adjusted to 5.7 and were autoclaved at 1.5 kgf/cm^2 and 121 ºC for 20 min. The cultures were kept at 25 ± 2 ºC under white fluorescent light (28 µM m^{-2} s^{-1}) with a 16 h photoperiod. All experiments used culture flasks with 5 cm diameter and 8.5 cm height and with 30 mL culture medium. The experimental design was completely randomized with five replicates of ten explants. The data were submitted to a normality analysis using the Lilliefor's test followed by an analysis of variance (ANOVA) and Duncan´s test, both at a $p<0.05$. The counting variables were transformed to $\sqrt{x+0.5}$, and the percentage variables were transformed to $\arcsin\sqrt{x/100}$. All statistical analyses were performed with the GENES software (Cruz, 2001).

2.7 Cost analysis of KC formulations

The Prices and the amount of the commercialized reagents were acquired in the local trade and calculated for prepare of 1000 liters of culture medium. The cost reduction was expressed in percentage compared to the KC medium. The values were just calculated for the KCV1 and KCV5 media.

3 RESULTS AND DISCUSSION

3.1 Nutrient determination, biochemical oxygen demand (BOD) and chemical oxygen demand (COD)

All nutrients present in KC medium were found in the treated vinasse (Table 1). Moreover, several nutrients such as Na, Cl and Zn that were not present in KC medium were found in treated vinasse (Table 1). The treated vinasse had a greater quantity of nutrients than the KC medium (Santos et al., 2013); therefore, it must be diluted prior to use. A 10% vinasse dilution was used for the establishment of a hydroponics solution, which produced results similar to a commercial solution (Santos et al., 2013). This result suggests that a high vinasse dilution was suitable for plant growth. The nutrients found in a greater

amount in the vinasse dilution were Ca, K and SO_4 (Table 1). Similar results were found in vinasse from sugarcane, where a high content of calcium and potassium was observed (Almeida, 1952; Glória, 1976).

The BOD is the oxygen amount consumed during the degradation of organic matter by a biological process. This measure is associated with the toxicity of the waste to aquatic life; high levels of BOD cause suffocation of aquatic fauna and flora resulting in the death of aquatic life. The BOD limit level for effluents before they are dispersed into the environment is 50 mg L^{-1} (WHO, 1989). The BOD of pure vinasse is between 20,000 and 35,000 mg L^{-1}, meaning that this waste is extremely harmful to the fauna of lakes and rivers (Da Silva et al., 2007); however, the treated vinasse (decanted and filtered) used in the present study has a lower, but still high, BOD of 6,524.6 mg L^{-1}.

The COD is the amount of oxygen consumed using an oxidizing agent under controlled conditions; nevertheless, environmentally, COD represents an indirect measure that indicates the amount of organic compounds in water. Normally the COD is higher than the BOD because the oxidizing agents are more aggressive under the controlled conditions. The acceptable COD level of an effluent to be dispersed in the environment is 1000 mg L^{-1} (WHO, 1989). Pure vinasse is between 50,000 and 150,000 mg L^{-1} COD (Pant and Adholeya, 2007). The treated vinasse had a COD of 7808.6 mg L^{-1}, which was still far over the limit. The vinasse will be well diluted prior to use, below 10%, which will further reduce the COD value.

3.2 Buffer effect in vinasse

After seven days, the vinasse pH decreased from 5.7 to approximately 5.4; at days 14 and 21 the pH increased, and at 28 days the pH was almost stable in the 5 and 10% vinasse, whereas the pH in the 15% vinasse had decreased (Fig. 1). Different vinasse dilutions had similar behaviors, but smaller vinasse dilutions showed a reduced trend toward acidification (Fig. 1). These results are similar to those observed during the application of the pure vinasse in the field; initially the soil is acidified, and after some time the pH returns to near the initial value (Almeida, 1952).

The appropriate range of pH for the *in vitro* germination of orchids is from 4.5 to 5.5 (Knudson, 1946). However, for the *in vitro* culture of different orchid explants, a pH of up to 5.8 has been used (Knapp et al., 2000; Chapla et al., 2009). Hence, the buffer effect of the culture medium must be stable, allowing little variation. Different vinasse dilutions presented relative buffer effects, which did not vary outside of the range appropriate for orchid explant culture.

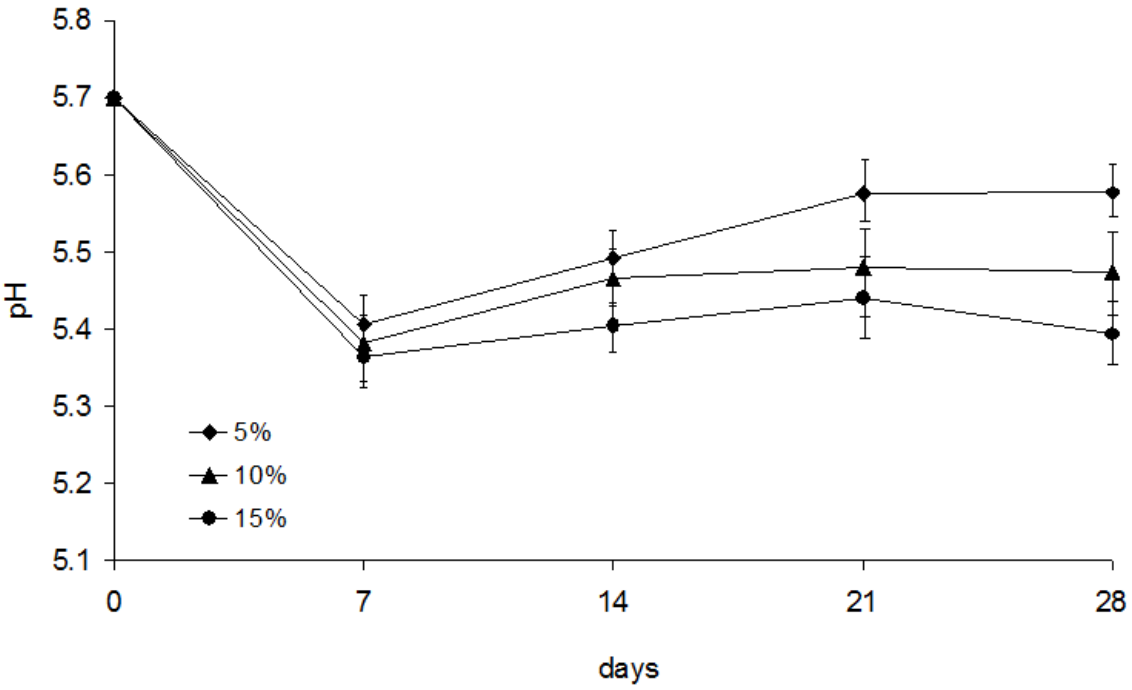

Fig. 1. Buffer effect in liquid media composed of different vinasse dilutions (5, 10 and 15%). The pH was measured each week until 28 days. Error bars correspond to the standard deviation of the mean.

3.3 Culture on vinasse dilutions

The survival percentage of explants were not significantly different among the MS (control) and 0 and 2.5% vinasse, varying from 95.8 to 100%; however, the 5 and 10% vinasse dilutions were significantly different from the control, varying from 83.3 and 50% (Table 2). The vinasse dilution is an important variable in formulating culture medium due its direct influence on the explant survival. Smaller vinasse dilutions (5 and 10%) possessed a significant phytotoxic effect on explants.

The absence of vinasse decreased the percentage of leaves with several necroses, suggesting that this orchid is sensitive to the presence of inorganic salts that were added to the culture medium (Table 2). The MS medium used as a control showed the best results for shoot number, protuberance number and fresh mass (Table 2); nevertheless, the medium with 2.5% vinasse presented results for shoot height and shoot percentage similar to those found with the MS medium. Moreover, the medium with 2.5% vinasse showed superior results for leaf number, root number and rooting percentage (Table 2). The 2.5% vinasse dilution was ideal for plant tissue culture and did not affect explant survival. This dilution also provided the best results for some of the growth parameters.

Table 2. *In vitro* multiplication of *Oncidium leucochilum* in different vinasse dilutions. Leaf number (LN), shoot number (NB), root number (RN), shoot height (SH cm), fresh mass (FM g), protuberance number (PN), shoot percentage (SP %), rooting percentage (R %), percentage of explants with some necrosed region (N %) and survival percentage (S %) are shown. All media were liquid, not shaken and supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP (6-benzylaminopurine).

Treatment	**LN**	**NB**	**RN**	**SH cm**	**FM mg**
MS (control)	7.46 b	1.37 a	1.00 ab	2.66 a	320.5 a
0% vinasse	4.75 c	0.15 c	0.23 c	1.97 b	127.7 b
2.5% vinasse	10.06 a	0.54 b	1.27 a	2.45 a	150.2 b
5% vinasse	5.82 c	0.33 b	0.40 bc	1.93 b	109.6 b
10% vinasse	5.44 c	0.40 b	0.28 c	1.95 b	109.8 b
CV(%)	5.8	5.6	17.0	7.9	17.1
Treatment	**PN**	**SP %**	**R %**	**N %**	**S %**
MS (control)	16.6 a	45.8 a	27.1 ab	91.6 b	100 a
0% vinasse	0.6 b	20.8 b	18.7 b	66.6 a	95.8 a
2.5% vinasse	0.2 b	41.6 a	45.8 a	100 c	100 a
5% vinasse	0.3 b	27.5 b	26.6 ab	100 c	83.3 b
10% vinasse	0.0 b	20.8 b	12.5 b	100 c	52.1 c
CV(%)	22.7	9.3	26.8	7.4	7.4

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test.

3.4 *In vitro* culture on the MS medium with the substitution of iron and calcium with vinasse ions

In this experiment, there were no significant differences for leaf number and root percentage (Table 3). The MS medium (control) had a greater shoot number, but the MS (control) and the 2.5% and 5% vinasse did not show a significant difference for root number, shoot height or fresh mass. For *in vitro* multiplication, the shoot number (propagules) was inferior to the control, which is not useful for shoot proliferation. Even so, this medium could be used to promote the elongation and rooting of the explants because the same results for the number or roots, shoot height and fresh mass were obtained for the control and the 2.5 and 5% vinasse.

Shoot and survival percentage did not show a significant difference for the MS (control) and the MS supplemented with 0, 2.5 and 5% vinasse but was significantly greater than the 10%vinasse (Table 3). Hyperhydricity was observed in the absence of iron and calcium (0% vinasse) in approximately 60% of the explants. Liquid culture is the most common cause of hyperhydricity in plant tissue culture (Scheidt et al., 2011). Some species are more resistant than others; nevertheless, *O. leucochilum* can be cultivated *in vitro* in liquid medium without the occurrence of hyperhydricity. This result was most likely caused by an iron and calcium deficiency; in studies on calcium deficiency in tissue cultures, it was shown that an increase in calcium in the medium reduced hyperhydricity and tip necrosis (Sha et al., 1985). Moreover, increases in calcium have been effective in eliminating hyperhydricity in quince (*Cydonia oblonga*) cultures (Singha et al., 1990). Increasing the concentration of iron in a medium with 0.7–0.8% agar during micropropagation of carnation (*Dianthus caryophyllus*) is reported to reduce hyperhydricity in the cultures (Yadav et al., 2002).

Protuberances were observed in all treatments and were highest in the MS (control) and the 0 and 2.5% vinasse (Table 3). The vinasse might possess a substance that inhibits the formation of protuberances because these structures are produced in larger number in the largest vinasse dilutions and decrease in number in the lowest vinasse dilutions. Moreover, the inhibitory effect of the vinasse on protuberance induction might be associated with the presence of

ethanol residues in vinasse because ethanol is an inhibitor of somatic embryogenesis (Caldas et al., 1998), and the protuberances of orchids are similar to somatic embryos, as suggested by the name protocorm-like bodies.

The possibility of an *in vitro* culture in liquid media is advantageous due to the production cost reduction by removing the need for agar (i.e., the most expensive component of culture media) and the process automation by using bioreactors. Other advantages of this experiment are that it was conducted in stationary culture, which also reduced production costs because the electricity that is required to move shakers is not necessary (Lopes da Silva et al., 2011).

Table 3. *In vitro* multiplication of *Oncidium leucochilum* in different vinasse dilutions supplemented with MS medium (Murashige and Skoog, 1962) without $CaCl_2 \cdot 2H_2O$, $Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ after 60 days of culture. The MS medium (control) was used complete. Leaf number (LN), shoot number (SN), root number (RN), shoot height (SH cm), fresh mass (FM g), protuberance number (PN), shoot percentage (SP %), root percentage (R %), hyperhydricity percentage (H %) and survival percentage (S %) are shown.

Treatment	LN	SN	RN	SH cm	FM g
MS (control)	12.1 a[1]	3.8 a	7.1 ab	3.4 ab	1.038 a
0% vinasse	10.3 a	2.0 b	3.2 c	2.4 c	0.628 bc
2.5% vinasse	11.1 a	2.0 b	7.6 a	3.8 a	0.847 ab
5% vinasse	15.5 a	2.1 b	8.2 a	3.8 a	0.877 ab
10% vinasse	12.7 a	1.4 b	4.1 bc	2.8 bc	0.413 c
CV(%)	12.0	17.5	21.4	17.1	12.3
Treatment	**PN**	**SP %**	**R %**	**H %**	**S %**
MS (control)	21.2 a	75.0 a	87.5 a	0.0 b	100 a
0% vinasse	19.9 a	86.6 a	58.3 a	59.2 a	100 a
2.5% vinasse	16.3 a	83.0 a	90.0 a	0.0 b	100 a
5% vinasse	3.6 b	91.6 a	90.0 a	0.0 b	100 a
10% vinasse	1.3 b	35.0 b	77.0 a	0.0 b	60 b
CV(%)	31.9	24.1	27.4	23.2	12.3

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test.

3.5 *In vitro* multiplication on different KC vinasse media

There were no significant differences for shoot number, root number, rooting percentage and survival percentage (Table 4). The KC medium (control) was superior for protuberance number when compared to other KC formulations. KCV2 and KCV3 presented higher rates of leaves with necrosis, 87.5 and 62.5%, respectively (Table 4). KCV2 had a lower amount of manganese (0.072 mg L^{-1} Mn) compared to the other formulations, KC (1.85 mg L^{-1} Mn) and KCV1, KCV3, KCV4 and KCV5 (1.67 mg L^{-1} Mn). The main symptom of manganese deficiency is internerval chlorosis associated with small necrosed points (Taiz and Zeiger, 2004). The higher rate of leaves with necrosis in the KCV3 formulation was not caused by a manganese deficiency, nevertheless it is possible that it could be provoked by the use of $Ca_3(PO_4)_2$ as the source of phosphate. Similar results were found in the protuberances of *Encyclia boothiana* (orchid), where $Ca_3(PO_4)_2$ promoted phytotoxicity leading to death after 120 days of *in vitro* culture (Stenberg and Kane, 1998). The lowest rate of shoot percentage was found in KCV2. This may also be attributed to the low amount of manganese in this formulation because manganese is responsible for enzyme activation of the tricarboxylic acid cycle, which is associated with energy production in plant cells (Taiz and Zeiger, 2004).

The best result for shoot height was found in KC (control); nevertheless, it was not significantly different from KCV1, KCV2, KCV4 and KCV5 (Table 4). The reduced shoot height observed in the explants of KCV3 was caused by the use of $Ca_3(PO_4)_2$, which likely influenced the higher rate of leaves with necrosis, as discussed above. The best results for leaf number were found in KC (control), KCV4 and KCV5, most likely due to a higher amount of phosphate present in these formulations, 174.47, 92.08 and 174.68 mg L^{-1} PO_4, respectively. Although KCV3 has 113.97 mg L^{-1} PO_4, this source of phosphate has low solubility and the precipitated phosphate is unavailable to the explants. This fact can also be used to explain why the explants cultivated in the KCV3 medium presented low fresh mass compared to KC and KCV5 (Table 4). The best results for fresh mass were obtained in KC (control) and KCV5, which were significantly different than the other KC formulations. The KC and KCV5 possessed the same level of

phosphate, which suggested the influence of this ion in increasing the fresh mass. This ion is needed by plants in large quantities. In this study, the levels of phosphate used in all of the KC formulations were not harmful to the development of the explants. Some KC formulations just decreased some of the parameters of growth.

Table 4. *In vitro* multiplication of *Oncidium leucochilum* on KC medium (Knudson, 1946) and on two KC formulations composed of 2.5% treated vinasse (decanted and filtered), (KCV1 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$), (KCV2 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$), (KCV3 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 170.6 mg L^{-1} $Ca_3(PO_4)_2$), (KCV4 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 120 mg L^{-1} $NaH_2PO_4 \cdot H_2O$) and (KCV5 = 1000 mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 65 mg L^{-1} $MnSO_4 \cdot 4H_2O$ and 240 mg L^{-1} $NaH_2PO_4 \cdot H_2O$) after 60 days of culture. Leaf number (LN), shoot number (SN), root number (RN), shoot height (SH cm), fresh mass (FM g), protuberance number (PN), shoot percentage (SP %), rooting percentage (R %), percentage of explants with some necrosed region (N %) and survival percentage (S %) are shown. All media were supplemented with 30 g L^{-1} sucrose and 0.3 mg L^{-1} BAP (6-benzylaminopurine) and were solidified with 6 g L^{-1} agar.

Treatment	LN	SN	RN	SH cm	FM g
KC (control)[2]	16.0 ab[1]	3.7 a	0.6 a	2.8 a	0.266 a
KCV1 vinasse	13.1 bc	3.3 a	0.7 a	2.7 ab	0.129 b
KCV2 vinasse	12.4 c	2.4 a	0.7 a	2.5 abc	0.131 b
KCV3 vinasse	13.0 bc	2.3 a	0.5 a	2.4 bc	0.124 b
KCV4 vinasse	17.5 a	4.4 a	0.5 a	2.5 abc	0.102 b
KCV5 vinasse	15.1 abc	3.6 a	0.5 a	2.6 ab	0.224 a
CV(%)	5.6	12.3	19.4	6.4	17.3
Treatment	**PN**	**N %**	**R %**	**SP %**	**S %**
KC (control)	18.6 a	0 d	37.5 a	100 a	100 a
KCV1 vinasse	0 b	37.5 bc	50 a	100 a	100 a
KCV2 vinasse	0 b	87.5 a	50 a	87.5 b	100 a
KCV3 vinasse	0 b	62.5 ab	50 a	100 a	100 a
KCV4 vinasse	1.5 b	12.5 dc	25 a	100 a	100 a
KCV5 vinasse	0.4 b	37.5 bc	50 a	100 a	100 a
CV(%)	22.9	44.7	53.7	7.2	0

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test.

3.6 *In vitro* elongation and rooting on different KC vinasse media

The vinasse media were superior to the KC medium in promoting *in vitro* elongation and rooting (Fig. 2). The KC medium promoted a low rooting rate (8%) compared to the 68 and 100% obtained by KCV1 and KCV5, respectively (Table 5). Moreover, plantlets cultured on the KC medium only became protocorm-like body clusters (Fig. 2), making it impossible to acclimatize these explants. The result for root number was also higher in vinasse media than KC (control), with KCV5 (3.8 roots per explant) being the greatest followed by KCV1 (1.8 roots per explant). The KC (control) presented the lowest root number (0.1 root per explant). The KC promoted the highest number of protocorm-like bodies, which impeded root formation and elongation in plants (Fig. 2). The vinasse media presented inhibition toward the formation of protocorm-like bodies. The KCV1 and KCV5 did not show statistically different values for shoot height (Table 5). It is common for the explants that were removed from the multiplication medium to continue to proliferate due to the endogenous presence of the cytokinins used to induce the *in vitro* multiplication of buds, protuberances and shoots, as demonstrated in several reports. For example, in *Dyckia maritima,* it was suggested that prior to a phase of rooting, submitting the clusters of shoots to a shoot elongation phase would avoid new shoot formation (Lopes da Silva et al., 2004). When explants cultivated on *in vitro* rooting media are difficult to root, an *in vitro* elongation medium can be used prior to transfer to the *in vitro* rooting medium. The phase of *in vitro* elongation and rooting can be conducted simultaneously (with one subculture, one medium and by using isolated shoots) or separately (with two subcultures and two different or the same media, using the first to subculture shoot or bud clusters and the second to subculture isolated shoots). The prior elongation of shoot clusters is necessary when explants continue to multiply and the rooting is not suitable. However, in *Vriesea fosteriana,* the supplementation of 0.54 µM NAA (1-naphthaleneacetic acid) was necessary to stop these lateral shoot proliferations as well as to return apical growth of the shoots. In this way, rooting was easily induced (Mercier and Kerbauy, 1992). However, the vinasse appears to possess a compound that can avoid protocorm-like body proliferation, which would be useful for plant tissue

culture and must be further investigated. The vinasse media decreased the shoot percentage but did not affect the shoot number, which was not significantly different than in the KC medium. This result is suitable for the induction of in vitro rooting as discussed above. The survival percentage was 100% in all treatments (Table 5).

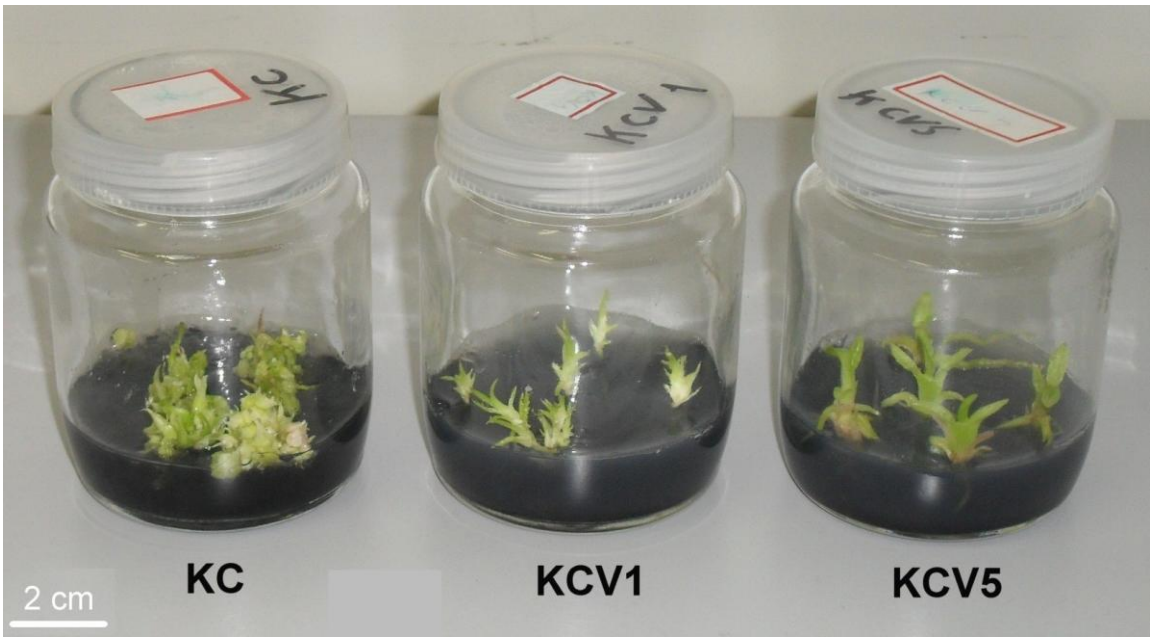

Fig. 2. *In vitro* elongation and rooting of *Oncidium leucochilum* on KC medium (control) and two KC formulations with 2.5% vinasse after six months. Media are free of plant growth regulators.

Table 5. *In vitro* elongation and rooting of *Oncidium leucochilum* cultured on vinasse media after eight months. Rooting (R %), root number (RN), height of the aerial part (HPA cm), survival (S %), fresh mass (FM g), protuberances (P %), shoot (Sh %) and shoot number (SN) are shown.

Treatment	**R %**	**RN**	**HPA cm**	**S %**
KC (control)	8 c[1]	0.1 c	0.87 b	100 a
KCV1	68 b	1.8 b	1.58 a	100 a
KCV5	100 a	3.8 a	1.99 a	100 a
CV(%)	17.8	12.6	21.3	0
Treatment	**FM g**	**P %**	**Sh %**	**SN**
KC (control)	0.399 a	100 a	100 a	2.2 a
KCV1	0.114 b	8 b	72 b	2.6 a
KCV5	0.171 b	0 b	64 b	2.6 a
CV(%)	56.9	31.0	29.8	15.3

[1] Means within a column followed by the same letter for each parameter are not different at $p<0.05$ by Duncan's test.

3.7 Acclimatization

At least one elongated shoot is necessary for the acclimatization of micropropagated plants. The KC medium did not produce suitable elongated shoots, which impeded the attempt to acclimatize these explants (Table 5 and Fig. 2). Different culture media can influence plant survival during acclimatization, as demonstrated in microshoots of *Melaleuca alternifolia* that were cultivated in half-strength MS and full-strength MS, resulting in 80 and 100% survival, respectively (Oliveira et al., 2010). Plantlets elongated and rooted on KCV1 and KCV5 media were successfully acclimatized with a 91% survival rate for both KC vinasse formulations. This result indicated that vinasse could be used successfully to develop plant tissue culture. A complete protocol for the micropropagation of *O. leucochilum* was established with high rates of plant survival. Acclimatization protocols with survival rates above 90% are suitable for all plant species. In this phase, a great number of plants did not survive due to hard stress that was caused by the transfer from *in vitro* conditions to *ex vitro* conditions (Schuck et al., 2012). Low survival rates limit the commercial use of micropropagation. This protocol for micropropagation, using vinasse media, can be adapted for other plant species, as observed in our laboratory with bromeliads, which were also successfully micropropagated.

3.8 Cost analysis of the KC formulations

The KC formulations using vinasse presented a cost reduction of 32.7 and 18.8% compared with KC medium (Knudson, 1946) for KCV1 and KCV5, respectively (Table 6). The component more expansive of these formulations was the nitrogen source ($Ca(NO_3)_2.4H_2O$), representing 66% of KC medium value. For the KC formulations using vinasse this nitrogen source represents 98.1% and 81.4% of cost for KCV1 and KCV5, respectively. The use of an alternative source of nitrogen could reduce drastically the cost of these vinasse culture media.

Table 6. Costs involved in the production of culture media for orchid plants: KC (1946), KCV1 and KCV5. Values (prices) obtained in September, 2010.

Reagents	Amount used in medium ($g.L^{-1}$)	Commercialized amount (g)	Value of the flask (R$)	1000 L culture medium		
				Cost of KC medium (R$)	Cost of KCV1 medium (R$)	Cost of KCV5 medium (R$)
$Ca(NO_3)_2.4H_2O$	1.0000	1000	55,00	55,00	55,00	55,00
$FeSO_4.7H_2O$	0.0250	500	20,00	1,00	-	-
KH_2PO_4	0.2500	1000	23,00	5,75	-	-
$MgSO_4.7H_2O$	0.2500	500	22,00	11,00	-	-
$(NH_4)_2SO_4$	0.5000	1000	20,00	10,00	-	-
$MnSO_4.4H_2O$	0.0075	500	37,00	0,55	0,481	0,481
$NaH_2PO_4.H_2O$	0.2400	500	24,00	-	-	11,52
2.5% Vinasse	-	-	0,57[1]	-	0,57	0,57
Total cost of culture medium (R$)				83,30	56,05	67,57
Cost reduction in percentage				-	32.7	18.8

[1] Cost of the decantation treatment for 25 liters of vinasse (using the reagents $Ca(OH)_2$ and $Al_2(SO_4)_3$). The cost of the vinasse transport was inconsiderate due to differences among the distances of the ethanol industries.

4 CONCLUSIONS

It was possible to develop a plant culture medium using vinasse that resulted in a suitable *in vitro* culture medium for *Oncidium leucochilum* (orchid). The best vinasse dilution (decanted and filtered) was 2.5%. The vinasse dilutions demonstrated a good buffer effect. The best KC formulations with vinasse were KCV1 and KCV5, and the KCV5 was superior to KCV1 for *in vitro* rooting. For *in vitro* multiplication, the results were similar among the vinasse formulations (KCV1 and KCV5) and the KC medium (control), with the exception of protocorm-like body number, which was smaller in the vinasse formulations. For *in vitro* elongation and rooting, the vinasse formulations were superior to the KC medium. The micropropagated plantlets that were obtained were successfully acclimatized, demonstrating the efficacy of the use of vinasse to formulate plant culture media. This shows the great potential of this technology as a rational alternative to vinasse disposal.

REFERENCES

ALMEIDA, J. R. **O problema da vinhaça em São Paulo**. Piracicaba: Instituto Zimotécnico da ESALQ, p. 24, (Boletim - IZ-ESALQ, 3), 1952.

CALDAS, L. S.; HARIDASAN, P.; FERREIRA, M. E. Meios nutritivos. In: TORRES, A. C.; CALDAS, L. S.; BUSO, J. A. (Eds.) **Cultura de tecidos e transformação genética de plantas**. Brasília: ABCTP/EMBRAPA-CNPH, 1998. p. 87-132.

CHAPLA, P. I.; BESSON, C.; OLIVEIRA, L. K.; SILVA, J. M.; ROCHA, A. C. D. S.; STEFANELLO, S. pH, activated charcoal and gelling agents of the culture medium on the *in vitro* growth of *Miltonia flavescens* Lindl. **Plant Cell Culture and Micropropagation**, v. 5, n. 2, p. 87-93, 2009.

CLESCERI, L. S.; GREENBERG, A. E.; EATOM, A. D. **Standard methods for the examination of water and wastewater**. APHA: Washington, 1220 p. 1998.

CRUZ, C. D. **Programa Genes: versão Windows; aplicativo computacional em genética e estatística.** Viçosa, MG: UFV, Imprensa Universitária, 2001, 648p.

DA SILVA, M. A. S.; GRIEBELER, N. P.; BORGES, L. C. Use of stillage and its impact on soil properties and groundwater. **Revista Brasileira de Engenharia Agrícola e Ambiental**, v. 11, n. 1, p. 108-114, 2007.

EATON, A. D.; CLESCERI, L. S.; RICE, E. W.; GREENBERG, A. E. **Standard methods for the examination of water and wastewater**. APHA: Washington, 1368p. 2005.

GLÓRIA, N. A. **Emprego da vinhaça para fertilização**. Piracicaba: s.i., CODISTIL, s.n.p., 1976.

KNAPP, J. E.; KAUSCH, A. P.; CHANDLEE, J. M. Transformation of three genera of orchid using the bar gene as a selectable marker. **Plant Cell Reports**, v. 19, n. 9, p. 893-898, 2000.

KNUDSON, L. A nutrient for the germination of orchid seeds. **American Orchid Society Bulletin**, v. 15, p. 214–217, 1946.

LOPES DA SILVA, A. L.; BISOGNIN, D. A.; DORNELLES, E. B.; WALTER, J. M.; FRANCO, E. T. H. Ensaios sobre o alongamento de brotos laterais de *Dyckia maritima* Baker dispostos em agrupamentos - Bromeliaceae. **Caderno de Pesquisa Série Biologia**, v. 16, n. 2, p. 37-46, 2004.

LOPES DA SILVA, A. L.; COSTA, J. L.; ALCANTARA, G. B.; CARVALHO, D. C.; SCHUCK, M. R.; BIASI, L. A.; SCHEIDT, G. N.; SOCCOL, C. R.

Micropropagation of *Nidularium innocentii* Lem. and *Nidularium procerum* Lindm. (Bromeliaceae). **Pakistan Journal of Botany**, v. 44, n. 3, p. 1095-1101, 2012.

MACHADO, M. P.; LOPES DA SILVA, A. L.; BIASI, L. A. Effect of plant growth regulators on *in vitro* regeneration of *Lavandula dentata* L. shoot tips. **Journal of Biotechnology and Biodiversity**, v. 2, n. 3, p. 28-31, 2011.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v. 15, n. 3, p. 473-497, 1962.

MERCIER, H.; KERBAUY, G. B. *In vitro* multiplication of *Vriesea fosteriana*. **Plant Cell, Tissue and Organ Culture**, v. 30, n. 3, p. 247-249, 1992.

OLIVEIRA, Y.; PINTO, F.; LOPES DA SILVA, A. L.; GUEDES, I.; BIASI, L. A.; QUOIRIN, M. An efficient protocol for micropropagation of *Melaleuca alternifolia* Cheel. **In Vitro Cellular & Developmental Biology - Plant**, v. 46, n. 2, p. 192-197, 2010.

PANT, D.; ADHOLEYA, A. Biological approaches for treatment of distillery wastewater: a review. **Bioresource Technology**, v. 98, n. 12, p. 2321–2334, 2007.

SANTOS, J. D.; LOPES DA SILVA, A. L.; COSTA, J. L.; SCHEIDT, G. N.; NOVAK, A. C.; SYDNEY, E. B.; SOCCOL, C. R. Development of a vinasse nutritive solution for hydroponics. **Journal of Environmental Management**, v. 114, p. 8-12, 2013.

SAYWELL, L. G.; CUNNINGHAM, B. B. Determination of Iron: Colorimetric o-Phenanthroline Method. **Industrial & Engineering Chemistry Analytical Edition**, v. 9, n. 2, p. 67-69, 1937.

SCHEIDT, G. N.; LOPES DA SILVA, A.L.; DRONK, A. G.; BIASI, L. A.; ARAKAKI, A. H.; SOCCOL, C. R. *In vitro* multiplication of *Oncidium leucochilum* (Orchidaceae) in different culture system. **Biociências**, v. 17, n. 1, p. 82-85, 2009.

SCHEIDT, G. N.; LOPES DA SILVA, A. L.; OLIVEIRA, Y.; COSTA, J. L.; BIASI, L. A.; SOCCOL, C. R. *In vitro* growth of *Melaleuca alternifolia* Cheel in bioreactor of immersion by bubbles. **Pakistan Journal of Botany**, v. 43, n. 6, p. 2937-2939, 2011.

SCHUCK, M. R.; LIPSKI, B.; LOPES DA SILVA, A. L.; CARVALHO, D. C.; BIASI, L. A. Acclimatization of micropropagated plants of fox grape cv. Bordô (*Vitis labrusca* L.) in different substrates. **Journal of Biotechnology and Biodiversity**, v. 3, n. 4, p. 206-212, 2012.

SHA, L.; MCCOWN, B. H.; PETERSON, L. A. Occurrence and cause of shoot-tip necrosis in shoot cultures. **Journal of the American Society for Horticultural Science**, v. 110, n. 5, p. 631-634, 1985.

SINGHA, S.; TOWNSEND, E. C.; OBERLY, G. H. Relationship between calcium and agar on vitrification and shoot-tip necrosis of quince (*Cydonia oblonga* Mill.) shoots *in vitro*. **Plant Cell, Tissue and Organ Culture**, v. 23, n. 2, p. 135-142, 1990.

SOCCOL, C. R.; SANTOS, J. D.; SCHEIDT, G. N. Desenvolvimento de uma solução nutritiva preparada a base de vinhaça. Universidade Federal do Paraná. Patente (**DEPR. 01508000078**). 2008.

STENBERG, M. L.; KANE, M. E. *In vitro* seed germination and greenhouse cultivation of *Encyclia boothiana* var. erythronioides, an endangered Florida orchid. **Lindleyana**, v. 13, p. 101-112, 1998.

TAIZ, L.; ZEIGER, E. **Fisiologia vegetal**. 3. ed. Porto Alegre: Artmed, 2004. 719p.

YADAV, M. K.; GAUR, A. K.; GARG, G. K. Development of suitable protocol to overcome hyperhydricity in carnation during micropropagation. **Plant Cell, Tissue and Organ Culture**, v. 72, n. 2, p. 153-156, 2003.

WHO - WORLD HEALTH ORGANIZATION. **Health guidelines for the use of wastewater in agriculture and aquaculture**. Geneva, 1989. 74p. (Technical Report Series, 778).

CHAPTER III - ANTIOXIDANT ACTIVITY OF *Nidularium innocentii* LEM. AND *Nidularium procerum* LINDM. CULTURED ON *IN VITRO* WITH DIFFERENT SOURCES AND LEVELS OF GIBBERELLIC ACID

ABSTRACT

Nidularium procerum and *Nidularium innocentii* (Bromeliaceae) were cultivated *in vitro* on media supplemented with different sources and levels of GA_3 (Gibberellic acid), these sources were the commercial powder (analytical degree) and fermented extract by *Fusarium moniliforme* obtained via solid state fermentation. Crude methanolic extracts of these plants (fresh mass) were prepared after 90 days of *in vitro* culture and used to determine the total phenolic, total flavonoids and quercetin content, as well as the antioxidant activity measured by free radical scavenging activity of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ABTS and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The best results for quercetin content was obtained in *N. innocentii* cultivated on 8.2 µM GA_3 fermented extract (76.4 mg/100g fresh mass) and 8.2 µM GA_3 analytical degree (43.7 mg/100g fresh mass), being observed approximately 2.1 and 1.2 times higher than observed in the control, respectively. The GA_3 had increased the quercetin content in *N. innocentii* and not in *N. procerum*. Moreover, the GA_3 effects are maximized by substances present in fermented extract. The largest antioxidant activity was obtained in *N. innocentii* cultivated on was 8.2 µM GA_3 fermented extract being 1.5 and 1.8 times higher than the control as observed by ABTS and DPPH assays. These results suggest that *N. innocentii* can be an excellent source to produce quercetin *in vitro* using GA_3 fermented extract.

Keywords: Quercetin; DPPH; ABTS; Solid State Fermentation; Bromeliad; *Fusarium moniliforme*.

1 INTRODUCTION

The plants *Nidularium procerum* Lindm., and *Nidularium innocentii* Lem., are bromeliads and have ornamental and medicinal characteristics. The *N. procerum* leaves has a potent analgesic (Amendoeira et al., 2005a), anti-inflammatory activity (Amendoeira et al., 2005b) and potent antieosinophil activity (i.e. Anti-allergic properties) (Vieira de-Abreu et al., 2005). *N. innocentii* is used in popular medicine as anti-inflammatory, analgesic, anti-helmintic and diuretic (Chedier et al., 2000). The bromeliads has attracted substantial interest in recent years as it is the candidates to develop new medicaments and as source of several secondary metabolites and raw material, such as the fibers produced by *Neoglaziovia variegata* (Arr. Cam.) Mez., used in the textile industry (Silveira et al., 2009), cysteine proteases like the penduliflorain I obtained from *Hohenbergia penduliflora* (A. Rich.) Mez. (Pérez et al., 2010) and bromelain from *Ananas comosus* (L.) Merr. (Ngampanya and Phongtongpasuk, 2006), both used in medicine and the food industry. Antibacterial activity and potent antioxidant activity was demonstrated in leaf extracts of *Encholirium spectabile* (Santana et al., 2012). Moreover, other studies have been demonstrated the great potential of these plants for innumerous applications.

The antioxidant activity of plants can be due to various types of molecules, like enzymes (e.g. superoxide dismutase, catalase and peroxiredoxins), carotenoids, tocopherols, ascorbic acid, polyphenolic compounds and others. Among polyphenolic compounds is the flavonoids and among these is the flavonols (e.g. quercetin). Quercetin (also named as 3,3',4',5,7-pentahydroxy flavone) is one of most widely distributed flavonols in plants, it is presents in most vegetables and fruits and also forms the chemical backbone for other flavonoids. Quercetin accumulating in higher plants forms glycosides with a large variety of sugars (Wittig et al., 2001). The intake of quercetin contained in food and herbal medicinal products has been associated with various health benefits and therapeutic or protective effects which are believed to be based on its antioxidant activity (Middleton and Kandaswami, 1994). Quercetin offers several potential therapeutic uses in the prevention of cardio vascular diseases, cancer, cataract,

schizophrenia and prostatitis. Quercetin inhibits the growth of malignant cells, nevertheless the exact mechanisms responsible for the antitumor effect of quercetin, however, is not thoroughly under stood yet (Wei et al., 1994). However, due to the various preventive effects in human healthy, quercetin is already used as a food supplement available in the commerce.

Gibberellic acid (GA_3) is a plant hormone that belongs to the group of gibberellins. Plants produce low amounts of GA_3. Therefore, this hormone is produced by microorganisms. Nowadays, it is industrially produced by submerged fermentation, but this process results in low yield with high production costs. One alternative to reduce the costs of GA_3 production is the solid-state fermentation (SSF) that allows the use of agro-industrial residues (Lopes da Silva et al., 2013). SSF was carried out using *Fusarium moniliforme* and citric pulp (CP) extract giving 5.9 g $GA_3.kg^{-1}$ dry CP after 3 days of fermentation (Rodrigues et al., 2009). Nodal segments of *Lavandula angustifolia* Mill. cultivated in different GA_3 levels and sources (analytical degree and fermented extract of citric pulp) did not present significant differences for the most characteristics evaluated, this study suggests the efficient use of GA_3 fermented extract in tissue culture (Lopes da Silva et al., 2013). The GA_3 used in plant tissue culture influences the physiology of explants; nevertheless diverse effects can be produced depending of type of explant, concentration, culture medium, species and interactions with diverse factors. However, secondary metabolites accumulation can be increased with GA_3 application, as observed in *Lathyrus sativus* L. wherein the neurotoxin content in leaves was increased (Bano and Sanaullah, 1995) and also have been increased cichoric acid, caftaric acid and chlorogenic acid, as well as increased the free radical scavenging activity in *Echinacea purpurea* L. (Abbasi et al., 2012).

Bromeliads showed vegetative growth very slow, this can limit their use to produce secondary metabolites; nevertheless the tissue culture can overcome this problem using bioreactors to promote fast multiplication of shoots, calli proliferation or cell suspension. Bioreactors produce larger biomass and multiplication rate than the conventional flask culture (Scheidt et al., 2011). Moreover, in tissue culture several methodologies to increase secondary metabolites can be performed.

The aim of this research was to evaluate the effects of fermented extract of GA_3 obtained by Solid State Fermentation and comparing with a commercial source of GA_3 (Analytical degree) on *in vitro* culture of two bromeliads: *Nidularium innocentii* and *Nidularium procerum* for antioxidant activity, total phenol and total flavonoids content and quercetin content.

2 MATERIAL AND METHODS

2.1 Chemical reagents

The chemical reagent ABTS [2,2´-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] was purchased from CALBIOCHEM (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Gallic acid monohydrate was purchased from Vetec (Rio de Janeiro, Brazil). All other chemicals used were of analytical and HPLC grade and obtained from Sigma Co. (St. Louis, MO).

2.2 Gibberellic acid sources

The pure GA_3 was a commercial powder (analytical degree) dissolved with 1N NaOH and solubilized with distilled water. The GA_3 extract was a fermented extract obtained by Solid-State Fermentation using *Fusarium moniliforme* LPB 03 in citric pulp extract supplemented with sucrose, it was used two columns with forced aeration (30 $mL.min^{-1}$) under 70% humidity during 5 days, the GA_3 was extracted with sodium phosphate buffer (pH 7.4) and filtered, this material was precipitated with 20% ethanol, this fermented extract was not purified, it was used crude (Rodrigues et al., 2009). Quantitative determination of GA_3 in fermented extract was performed by spectrophotometry with acidification of fermented extract with 30% HCl during 60 to 80 min at 20°C and 254nm absorbance (Lu et al., 1995).

2.2.1 *In vitro* establishment of bromeliads

The seed disinfection of *Nidularium procerum* and *Nidularium innocentii* was performed as proposed by Lopes da Silva et al. (2012). This process consisted of the seed immersion in 70% ethanol (v/v) during one minute, followed by immersion in commercial bleach (1% active chlorine) for 20 min, and rinsed three times with distilled sterilized water. The germination medium was MS (Murashige and Skoog, 1962), with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar. Seedlings were *in vitro* multiplicated on MS medium supplemented with 30 $g.L^{-1}$ sucrose, 2 µM NAA (naphthalene acetic acid), 4 µM BAP (6-benzylaminopurine) and solidified with 6 $g.L^{-1}$ agar.

2.2.2 *In vitro* culture of shoots on GA_3 sources and levels

Shoots (2 cm height) from clusters propagated *in vitro* were used as explants. Basal medium was MS with 30 $g.L^{-1}$ sucrose and solidified with 7 g L^{-1} agar. This experiment were organized in a three way ANOVA (2 x 2 x 3) and the factor A consisted of two species (*N. procerum* and *N. innocentii*), the factor B consisted of two sources of GA_3 (analytical degree and fermented extract) and the factor C consisted of GA_3 levels which were 0, 4.1 and 8.2 µM. The amounts of fermented extract used were 0.5 and 1 $mL.L^{-1}$ for the obtaining of 4.1 and 8.2 µM GA_3, respectively. The total phenol, total flavonoids and quercetin content, as well as the free radical scavenging activity measured by ABTS and DPPH were evaluated after 90 days of *in vitro* culture. All GA_3 solutions were added to media and after autoclaved.

2.3 Methanol extract

Micropropagated plant fresh mass (1 g) were macerated and extracted in 10 mL methanol (1:10 w/v) during 24 h at room temperature under 80 rpm agitation (in the dark), after they were filtered with Whatman No. 1 filter paper. The extracts were stored at -20^{o} C.

2.4 Total phenol assay

The total phenolic content was determined by using Folin-Ciocalteu (Singleton and Rossi, 1965) modified assay. To 500 µL of each sample (three replicates), 2.5 mL Folin-Ciocalteu solution (1:10 v/v) and after five minutes was added 2 mL Na_2CO_3 (7.5% w/v) were added and stored at room temperature for 60 min. The absorbance of all samples was measured at 740 nm using a SP2000-UV spectrophotometer. The standard curve was determinate using the levels of 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 $\mu g.mL^{-1}$ gallic acid. Methanol was used as blank. Results were expressed as milligrammes of gallic acid equivalent per gramme of 100 g^{-1} fresh weight (mg GAE/100 g^{-1} fresh weight).

2.5 Total flavonoid content

Total flavonoid content was determined by a colorimetric method (Bao et al., 2005) with modifications. Aliquots (0.5 mL) of the diluted extracts or standard solutions were pipetted into 15 mL falcon tubes containing 2 mL distilled H_2O and mixed with 0.15 mL 5% $NaNO_2$. After 5 min, 0.15 mL 10% $AlCl_3.6H_2O$ solution was added, and the mixture remained for another 5 min, and then 1 mL 1 M NaOH was added. The reaction solution was well mixed, maintained for 15 min and the absorbance of all samples was measured at 415 nm using a SP2000-UV spectrophotometer. Total flavonoid content was estimated using the standard quercetin curve; the standard quercetin curve was determinate using the levels of 6.25, 12.5, 25, 50, 100, 150 and 200 $\mu g.mL^{-1}$ quercetin. Methanol was used as blank. Results were expressed as milligrammes of quercetin equivalent per gramme of 100 g^{-1} fresh weight (mg QE/100 g^{-1} fresh weight).

2.6 Quercetin content determination

The analyses of quercetin content were performed on an HPLC Varian ProStar using an Ultraviolet (UV) detector with a 250 nm wavelength. A Microsorb C18 reverse phase column (4.6 x 250 mm) was used, with 1 mL min^{-1} flow. The mobile phase solvent was methanol and water (50:50 v/v), and the

injected volume for each run was 20 µL. The run time was 15 min. In order to obtain a calibration curve, quercetin with 98% purity (Sigma Aldrich, EUA) was used at concentrations of 5, 10, 12.5, 25 and 50 µg L^{-1} in methanol. The resulting chromatogram values were graphed and a linear equation was used to calculate the quercetin content of the samples. Samples were microfiltered in hydrophilic membrane GV (durapore) of PVDF (polyvinylidene difluoride), pore size 0.22 µm.

2.7 ABTS assay

Trolox Equivalent Antioxidant Capacity (TEAC) of bromeliads extracts was determined, using the method reported by Re et al. (1999), adapted for 96-well microplates: 200 µL of $ABTS^{+}$ solution were added to 50 µL of properly diluted sample/standard. Absorbances of reactions products were determined with a microplate reader, at 734 nm. The standard curve was linear between 25 and 800 mM Trolox. Results are expressed in µM Trolox Equivalent/g fresh mass.

2.8 DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay

Total antioxidant activity of samples was determined by a method originally described by Brand-Williams et al. (1995) and adapted by Rufino et al. (2007) and Fukumoto and Mazza (2000), with some modifications and adapted for 96-well microplates. The working solution was prepared by dissolving 2.4 mg DPPH with 100mL methanol and used in the same day. Three different concentrations of each bromeliads extract (10 µL) were allowed to react with 290 µL of the DPPH solution, until stabilization (5 minutes). Then the absorbance was measured at 515 nm by microplate reader. These absorbance results were plotted against the extracts concentrations and the linear regression of data was used to estimate the IC_{50} (concentration of extract that reduces the concentration of DPPH to 50% of the initial concentration). The standard curve was linear between 0 and 60 µM DPPH. Additional dilution was applied for those samples which presented absorbance values out of the linear range of the standard curve.

2.9 Culture conditions and statistical analysis

All media had their pH adjusted to 5.8 and were autoclaved at 1.5 kgf/cm^2 and 121 ºC for 20 min. The cultures were kept at 25 ± 2º C under white fluorescent light (28 µM m^{-2} s^{-1}) with a 16 h photoperiod. All experiments used culture flasks with 5 cm diameter and 8.5 cm height and with 30 mL culture medium. The experimental design was completely randomized in a factorial arrangement (three-way) with five replicates of five explants. The data was submitted in a normality analysis for the Lilliefors´s test, and submitted to the analysis of variance (ANOVA) followed by Duncan's test at a $p<0.05$. All statistical analyses were done following the procedures of the software SOC (Embrapa, 1990).

3 RESULTS AND DISCUSSION

3.1 Total phenol assay

The best result for the total phenol content was found in *N. innocentii* cultivated on 8.2 µM GA_3 fermented extract (162.3 mg GAE.100 g^{-1} fresh mass), followed by 8.2 µM GA_3 analytical degree (112.6 mg GAE.100 g^{-1} fresh mass). The influence of GA_3 sources in *N. innocentii*, fermented extract and analytical degree (8.2 µM GA_3) were approximately 2.2 and 1.5 times the value obtained in control (0 µM GA_3), respectively. The best result found in *N. procerum* was 64.9 mg GAE.100 g^{-1} fresh mass found in the control (Table 1).

The *N. innocentii* presented larger total phenol content than *N. procerum*, with exception of those plants cultivated on 4.1 µM GA_3 (analytical degree) that there was no statistical difference with *N. innocentii* cultivated in this same treatment (Table 1). Total phenol content in *N. innocentii* was influenced by GA_3 independent of the source used (analytical degree and fermented extract), nevertheless the fermented extract was more efficient than the analytical degree. On the contrary, *N. procerum* practically was not influenced by the any GA_3 source and level, with exception in 4.1 µM GA_3 fermented extract that was significant minor than the control (GA_3 absense) (Table 1).

The total phenol content is an indirect measure of the antioxidant activity of an extract; whereas it does not evaluate the ability of these phenolic compounds in scavenging free radicals, in spite of that, it is normally correlated with antioxidant activity. Moreover, it is very important to evaluate the quality of the extraction process.

There are no much reports about total phenolic content in bromeliads. Nevertheless, some studies were found in fresh fruits of *Bromelia antiacantha* Bertol. (Santos et al., 2008), in dried leaves of *Encholirium spectabile* Mart. ex Schult. f. (Santana et al., 2012) and *Neoglaziovia variegata* (Arruda) Mez, (Oliveira Junior et al., 2013). However, these plants were not cultivated *in vitro* and were used dried leaves, fruit and others solvents which becomes hard to compare with the results of the present study. Nevertheless, the methanolic extract of fresh fruits of *Bromelia antiacantha* had approximately 50 mg GAE.100 g^{-1} (Santos et al., 2008); being close to results found in methanolic extract of fresh leaves of *N. procerum* cultivated *in vitro* (Table 1). On the other hand, the most significant total phenolic content found in *Encholirium spectabile* was 206.4 mg GAE.g^{-1} for ethyl acetate extract (Santana et al., 2012) and 608.50 mg GAE.g^{-1} for ethyl acetate extract of dried leaves of *Neoglaziovia variegata* (Oliveira Junior et al., 2013). Of course, dried leaf extracts presented more elevate content than fresh extracts, however, in these reports, others solvents were used, such as chloroform, ethanol and hexane showing lower values than the obtained in ethyl acetate extract, which suggests that total phenol extraction in *N. procerum* and *N. innocentii* could be more efficient with the use of the ethyl acetate. Moreover, it suggests that the potential of these plants for antioxidant activity can be yet larger than the observed in the present study.

Table 1. Total phenolic content in methanolic extracts of *Nidularium procerum* and *Nidularium innocentii* cultivated *in vitro* on different sources and levels of gibberellic acid after 90 days. It was expressed in mg gallic acid equivalent/100 g^{-1} fresh mass.

GA_3	*Nidularium procerum*		*Nidularium innocentii*	
(µM)	**Fermented extract**	**Analytical degree**	**Fermented extract**	**Analytical degree**
0.0	64.9 Ba[1]	64.9 Ba	74.2 Ac	74.2 Ab
4.1	47.9 Cb	60.0 Ba	83.6 Ab	66.3 Bb
8.2	64.9 Ca	57.9 Ca	162.3 Aa	112.6 Ba
CV(%)		6.3		

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's Test.

3.2 Total flavonoid content

The presence of the two GA_3 sources on culture media had allowed an increase in the total flavonoid content in both species, nevertheless in some levels; however, there was a significant interaction between the species, GA_3 sources and GA_3 levels. The best results for total flavonoid content were reached on 4.1 µM GA_3 analytical degree for *N. procerum* (214.3 mg QE/100 g^{-1} fresh mass) and on 4.1 (204.8 mg QE/100 g^{-1} fresh mass) and 8.2 (215.8 mg QE/100 g^{-1} fresh mass) µM GA_3 fermented extract for *N. innocentii*, there was not statistical differences between these treatments (Table 2).

The total flavonoids is not completely associated with the total phenolic content in extracts of *N. procerum*, considering that the total flavonoids had increased according to the GA_3 levels in the treatments of fermented extract and the total phenolic content did not followed this tendency (Table 1 and 2), this is observed also in treatment with GA_3 analytical degree in which there was statistical differences between the levels while there was not statistical differences for the total phenolic content (Table 1 and 2). However in *N. innocentii* the results obtained for total flavonoids had an association with the total phenolic content, the total phenolic content increases according the GA_3 level and the total flavonoids too. Similar results with *N. procerum* were found in several *Citrus* species where no correlation was found between these total

phenolic and flavonoids content (Ghasemi et al., 2009). The total flavoinoids is also an indirect measure of antioxidant activity and therefore not always is correlated with it or with the total phenol content. Nevertheless, it is very used to evaluate the extraction process efficiency.

Table 2. Total flavonoid content in methanolic extracts of *Nidularium procerum* and *Nidularium innocentii* cultivated *in vitro* on different sources and levels of gibberellic acid after 90 days. It was expressed in mg quercetin equivalent/100 g^{-1} fresh mass.

GA_3	***Nidularium procerum***		***Nidularium innocentii***	
(µM)	**Fermented extract**	**Analytical degree**	**Fermented extract**	**Analytical degree**
0.0	107.5 Ac[1]	107.5 Ab	87.5 Bb	87.5 Bc
4.1	125.8 Cb	214.3 Aa	204.8 Aa	79.0 Cb
8.2	172.5 Ba	87.2 Cc	215.8 Aa	137.3 Bb
CV(%)		8.2		

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's Test.

3.3 Quercetin content determination

The best results for quercetin content was obtained in *N. innocentii* cultivated on 8.2 µM GA_3 fermented extract (76.4 mg/100g fresh mass) and 8.2 µM GA_3 analytical degree (43.7 mg/100g fresh mass), being observed approximately 2.1 and 1.2 times higher than observed in the control, respectively (Table 3). The GA_3 had increased the quercetin content in *N. innocentii* and not in *N. procerum* (Table 3). It demonstrates that quercetin accumulation influenced by GA_3 can be species dependent. Moreover, the compounds present in fermented extract had a benefic influence for the quercetin accumulation in *N. innocentii*, what demonstrate an excellent result for this extract. The GA_3 purification elevates the product cost and may have consequences to make unfeasible the bioprocess for plant secondary metabolites that request the use of GA_3.

Several fruits and vegetables had their hydrolysed samples examined for quercetin content and found the highest concentration of quercetin in onions (28.4-48.6 mg/100g), kale (11 mg/100g), broccoli (3 mg/100g) and tomatoes (0.8

mg/100g). Among the fruit examined, the quercetin concentration averaged 1.5 mg/100g, being the apples the fruit with the highest concentration (2.1-7.2 mg/100g) (Hertog et al., 1992), according this another study the *N. procerum* and *N. innocentii* are excellent source of quercetin and may be explored commercially being produced *in vitro*.

Table 3. Quercetin content in methanolic extracts of *Nidularium procerum* and *Nidularium innocentii* cultivated *in vitro* on different sources and levels of gibberellic acid after 90 days. It was expressed in mg/100 g^{-1} fresh mass.

GA_3	***Nidularium procerum***		***Nidularium innocentii***	
(µM)	**Fermented extract**	**Analytical degree**	**Fermented extract**	**Analytical degree**
0.0	33.6 Aa[1]	33.6 Aa	36.8 Ac	36.8 Ab
4.1	12.0 Db	26.2 Ca	47.3 Ab	39.3 Bab
8.2	34.3 Ca	31.4 Ca	76.4 Aa	43.7 Ba
CV(%)		5.2		

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's Test.

3.4 Antioxidant activity

3.4.1 ABTS assay

The best result was found in extracts of *N. innocentii* cultivated on 8.2 µM GA_3 fermented extract that was observed an antioxidant activity of 2.73 µM Trolox Equivalent/g^{-1} fresh mass, being 1.5 times higher than the control (1.83 µM Trolox Equivalent/g^{-1} fresh mass) (Table 4). There is significant interaction among the source and levels of GA_3 in *N. innocentii*, the GA_3 fermented extract was superior to induce antioxidant activity than the GA_3 analytical degree. The level of 4.1 µM GA_3 promoted a decrease in the antioxidant activity independent of GA_3 source used. The level of 8.2 µM GA_3 presented differences among the GA_3 sources, being superior just in fermented extract, the analytical degree showed an inferior value compared to the control (Table 4).

N. innocentii had demonstrated high antioxidant activity than *N. procerum*, in the absence of GA_3 (control). Nevertheless, some treatments applied to *N.*

innocentii presented inferior results to *N. procerum*, these results were observed in 4.1 and 8.2 GA_3 analytical degree (Table 4). On the other hand, the GA_3, as well as the fermented extract or analytical degree had decreased the antioxidant activity in *N. procerum* (Table 4).

The antioxidant activity found in *N. innocentii* cultivated on 8.2 μM GA_3 fermented extract (2.73 μM Trolox Equivalent/g^{-1} fresh mass) can be considered relatively high, when compared with the TEAC observed in some fruits, such as apple (1.60), apricot (1.39) and peach (1.22), and similar with kiwi (2.72), these analyzed extracts were prepared of similar form with the extracts used in the present study, being the solvent used the 80% methanol (1:10 w/v) and same unit of TEAC (μM Trolox Equivalent/g^{-1} fresh mass) (Scalzo et al., 2005).

Table 4. Antioxidant activity of methanolic extracts of *Nidularium procerum* and *Nidularium innocentii* cultivated *in vitro* on different sources and levels of gibberellic acid after 90 days. Antioxidant activity was determined by the ABTS assay and the data were expressed in μM Trolox Equivalent/g^{-1} fresh mass.

GA_3	***Nidularium procerum***		***Nidularium innocentii***	
(μM)	**Fermented extract**	**Analytical degree**	**Fermented extract**	**Analytical degree**
0.0	1.76 Ba	1.76 Ba	1.83 Ab	1.83 Aa
4.1	1.34 Bb	1.36 Bb	1.79 Ac	1.37 Bc
8.2	1.31 Cb	1.20 Cc	2.73 Aa	1.69 Bb
CV(%)		5.8		

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's Test.

3.4.2 DPPH assay

The best result for antioxidant activity measured by DPPH scavenging was obtained in extracts of *N. innocentii* cultivated on 8.2 μM GA_3 fermented extract; which was observed an IC_{50} of 84.8 μM, followed by GA_3 analytical degree (IC_{50} = 95.7 μM) (Table 5). These results represent approximately 1.8 and 1.6 times more than the control, for 8.2 μM GA_3 fermented extract and GA_3 analytical degree, respectively.

Ethyl acetate fractionated extract from dried leaves of *Neoglaziovia variegata* presented the best antioxidant activity (IC_{50} 5.08 μg/ml) for DPPH

scavenging, which is equivalent to 12.7 µM, on the other hand, the ethanol extract showed and antioxidant activity of 243.7 µg/ml (i.e. equivalent to IC_{50} = 609.2 µM), this result is very inferior when compared with the results of the present study (Table 5), whereas the ethanol is the most similar solvent to the methanol according to polarity (Lima-Saraiva et al., 2012). These results suggest that *N. procerum* and *N. innocentii* can be an antioxidant activity yet larger than the obtained in this study, being necessary to test other solvents and extraction process.

The antioxidant activity measured by DPPH scavenging in *N. procerum* showed different results compared with total phenolic, quercetin content and ABTS assay (Table 1, 3, 4 and 5). Therefore, varied methodologies must be used to avoid false positives, whereas in most cases, extracts presented substances that can interfere in the methodology, the DPPH is a simple method and is more indicate to evaluate the antioxidant activity of synthetic compounds and/or purified compounds. The DPPH is a colorimetric method and therefore is not very applied for colored substances due to interference caused by pigments (Chandrasekar et al., 2006). This result is coherent with the amount of pigmentation of *N. procerum* that had much more pigmentation (probable chlorophyll) than *N. innocentii*, how can be verified visually, *N. procerum* present a colored green much intense (dark green) and *N. innocentii* shows a clear green. Nevertheless, for the best result of this study, which was found in *N. innocentii* cultivated on 8.2 µM GA_3 fermented extract, the DPPH scavenging and ABTS assay are concordant (Table 4 and Table 5).

Table 5. Antioxidant activity of methanolic extracts of *Nidularium procerum* and *Nidularium innocentii* cultivated *in vitro* on different sources and levels of gibberellic acid after 90 days. Antioxidant activity was determined by the DPPH assay and the data were expressed in IC_{50} (µM).

GA_3	*Nidularium procerum*		*Nidularium innocentii*	
(µM)	**Fermented extract**	**Analytical degree**	**Fermented extract**	**Analytical degree**
0.0	293.2 Bb	293.2 Bc	153.1 Ab	153.1 Ab
4.1	314.2 Bc	163.1 Aa	159.6 Ab	307.2 Bc
8.2	278.2 Da	178.1 Cb	84.8 Aa	95.7 Ba
CV(%)	7.4			

[1] Means within a column followed by the same lower case letter and means within a line followed by the same capital letter for each parameter are not different at $p<0.05$ by Duncan's Test.

4 CONCLUSIONS

The *Nidularium innocentii* presents larger antioxidant activity than *Nidularium procerum*. The *N. procerum* does not be analyzed by DPPH assay due to interference caused by pigmentation excess. The GA_3 can influence the accumulation of secondary metabolites in *N. innocentii*, but not in *N. procerum*. Unknown compounds present in the GA_3 fermented extract increase the antioxidant activity and quercetin content in *N. innocentii*. These results suggest that *N. innocentii* can be an excellent source to produce quercetin *in vitro* using GA_3 fermented extract.

REFERENCES

ABBASI, B. H.; STILES, A. R.; SAXENA, P. K.; LIU, C-Z. Gibberellic acid increases secondary metabolite production in *Echinacea purpure* hairy roots. **Applied Biochemistry and Biotechnology**, v. 168, n. 7, p. 2057-2066, 2012.

AMENDOEIRA, F. C.; FRUTUOSO, V. S.; CHEDIER, L. M.; PEARMAN, A. T.; FIGUEIREDO, M. R.; KAPLAN, M. A.; PRESCOTT, S. M.; CASTRO-FARIA-NETO, H. C. Antinociceptive effect of *Nidularium procerum*: a Bromeliaceae from the Brazilian coastal rain forest. **Phytomedicine**, v. 12, n. 1-2, p. 78-87, 2005a.

AMENDOEIRA, F. C.; FRUTUOSO, V. S.; ZANON, C.; CHEDIER, L. M.; FIGUEIREDO, M. R.; KAPLAN, M. A. C.; MELO, C. B.; BOZZA, P. T.; CASTRO-FARIA-NETO, H. C. Anti-inflammatory Activity in the Aqueous Crude Extract of the Leaves of *Nidularium procerum*: A Bromeliaceae from the Brazilian Coastal Rain Forest. **Biological and Pharmaceutical Bulletin**, v. 28, n. 6, p. 1010-1015, 2005b.

BANO, A.; SANAULLAH, M. Effects of plant growth regulators on neurotoxin content in leaves of *Lathyrus sativus*. **Pakistan Journal of Botany**, v. 27, n. 1, p. 139-141, 1995.

BAO, J. S.; CAI, Y.; SUN, M.; WANG, G. Y.; CORKE, H. Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. **Journal of Agricultural and Food Chemistry**, v. 53, n. 6, p. 2327-2332, 2005.

BRAND-WILLIAMS, W.; CUVELIER, M. E.; BERSET, C. Use of a free radical method to evaluate antioxidant activity. **Lebensmittel-Wissenschaft & Technologie**, v. 28, n. 1, p. 25-30, 1995.

CHANDRASEKAR, D.; MADHUSUDHANA, K.; RAMAKRISHNA, S.; DIWAN, P. V. Determination of DPPH free radical scavenging activity by reversed-phase HPLC: A sensitive screening method for polyherbal formulations. **Journal of Pharmaceutical and Biomedical Analysis**, v. 40, n. 2, p. 460-464, 2006.

CHEDIER, L. M.; FIGUEIREDO, M. R.; KAPLAN, M. A. C. Chemical and biological investigations on *Nidularium innocentii* Lemaire. **Anais da Academia Brasileira de Ciências,** v. 72, n. 2, p. 295-295, 2000.

EMBRAPA - Núcleo Tecnológico para Informática. **SOC - Software Científico**. Campinas, São Paulo. 1990.

FUKUMOTO, L. R.; MAZZA, G. Assessing Antioxidant and Prooxidant Activities of Phenolic Compounds. **Journal of Agricultural and Food Chemistry**, v. 48, n. 8, p. 3597-3604, 2000.

GHASEMI, K.; GHASEMI, Y.; EBRAHIMZADEH, M. A. Antioxidant activity, phenol and flavonoid contents of 13 Citrus species peels and tissues. **Pakistan Journal of Pharmaceutical Sciences**, v. 22, n. 3, p. 277-281, 2009.

HERTOG, M. G. L.; HOLLMAN, P. C. H.; KATAN, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. **Journal of Agricultural and Food Chemistry**, v. 40, n. 12, p. 2379-2383, 1992.

LOPES DA SILVA, A. L.; COSTA, J. L.; ALCANTARA, G. B.; CARVALHO, D. C.; SCHUCK, M. R.; BIASI, L. A.; SCHEIDT, G. N.; SOCCOL, C. R. Micropropagation of *Nidularium innocentii* Lem. and *Nidularium procerum* Lindm. (Bromeliaceae). **Pakistan Journal of Botany**, v. 44, n. 3, p. 1095-1101, 2012.

LOPES DA SILVA, A. L.; RODRIGUES, C.; COSTA, J. L.; MACHADO, M. P.; PENHA, R. O.; BIASI, L. A.; VANDENBERGHE, L. P. S.; SOCCOL, C. R. Gibberellic acid fermented extract obtained by solid-state fermentation using citric pulp by *Fusarium moniliforme*: Influence on *Lavandula angustifolia* Mill. cultivated *in vitro*. **Pakistan Journal of Botany**, 2013. (no prelo)

LIMA-SARAIVA, S. R. G.; GUIMARÃES, A. L.; OLIVEIRA, A. P.; SARAIVA, H. C. C.; OLIVEIRA-JÚNIOR, R. G.; BARROS, V. R. P.; MENEZES, V. G.; OLIVEIRA, R. A.; SILVA, F. S.; LIMA, R. S.; MATOS, M. H. T.; AMORIM, E. L. C.; ALMEIDA, J. R. G. S. Antioxidant activity and acute toxicity of *Neoglaziovia variegata* (Bromeliaceae). **African Journal of Biotechnology**, v. 11, n. 75, p. 13998-14006, 2012.

LU, Z. X.; XIE, Z. C.; KUMAKURA, M. Production of gibberellic acid in *Gibberella fujikuroi* adhered onto polymeric fibrous carriers. **Process Biochemistry**, v. 30, n. 7, p. 661-665, 1995.

MIDDLETON JR, E.; KANDASWAMI, C. The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: Harborne, J. B. (Ed.), **The flavonoids: Advances in Research Since 1986**. : London: Chapman & Hall, 1994. p. 619-652.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, v. 15, n. 3, p. 473-497, 1962.

NGAMPANYA, B.; PHONGTONGPASUK, S. Effects of sucrose concentration on crude bromelain production of *in vitro* culture of pineapple (*Ananas comosus* var. 'Pattavia'). **Kasetsart Journal: Natural Science**, v. 40, n. 5 (suplemento), p. 129-134, 2006.

OLIVEIRA JUNIOR, R. G.; ARAÚJO, C. S.; SOUZA, G. R.; GUIMARÃES, A. L.; OLIVEIRA, A. P.; LIMA-SARAIVA, S. R. G.; MORAIS, A. C. S.; SANTOS, J. S. R.; ALMEIDA, J. R. G. S. *In vitro* antioxidant and photoprotective activities of dried extracts from *Neoglaziovia variegata* (Bromeliaceae). **Journal of Applied Pharmaceutical Science**, v. 3, n. 1, p. 122-127, 2013.

PÉREZ, A.; CARVAJAL, C.; TREJO, S.; TORRES, M. J.; MARTIN, M. I.; LORENZO, J. C.; NATALUCCI, C. L.; HERNÁNDEZ, M. Penduliflorain I: a cysteine protease isolated from *Hohenbergia penduliflora* (A. Rich.) Mez (Bromeliaceae). **The protein journal**, v. 29, n. 4, p. 225-233, 2010.

RE, R.; PELLEGRINI, N.; PROTEGGENTE, A.; PANNALA, A.; YANG, M.; RICE-EVANS, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology & Medicine**, v. 26, n. 9-10, p. 1231-1237, 1999.

RODRIGUES C.; VANDENBERGHE, L. P. S.; TEODORO, J.; OSS, J. F.; PANDEY, A.; SOCCOL, C. R. A new alternative to produce gibberellic acid by solid state fermentation. **Brazilian Archives of Biology and Technology**, v. 52, n. especial, p. 181-188, 2009.

RUFINO, M. S. M.; ALVES, R. E.; BRITO, E. S.; MORAIS, S. M.; SAMPAIO, C. G.; PÉREZ-JIMÉNES, J.; SAURA-CALIXTO, F. D. **Metodologia Científica:** Determinação da Atividade Antioxidante Total em Frutas pela Captura do Radical Livre DPPH. Embrapa Agroindústria Tropical, 2007. 4p. (Embrapa Agroindústria Tropical. ComunicadoTécnico, n. 127).

SANTANA, C. R. R.; OLIVEIRA-JÚNIOR, R. G.; ARAÚJO, C. S.; SOUZA, G. R.; LIMA-SARAIVA, S. R. G.; GUIMARÃES, A. L.; OLIVEIRA, A. P.; SIQUEIRA FILHO, J. A.; PACHECO, A. G. M.; ALMEIDA, J. R. G. S. Phytochemical Screening, Antioxidant and Antibacterial Activity of *Encholirium spectabile* (Bromeliaceae). **International Journal of Sciences**, v. 1, p. 1-19, 2012.

SANTOS, V. N. C.; FREITAS, R. A.; DESCHAMPS, F. C.; BIAVATTI, M. W. Ripe fruits of *Bromelia antiacantha*: investigations on the chemical and bioactivity profile. **Revista Brasileira de Farmacognosia**, v. 19, n. 2A, p. 358-365, 2009.

SCALZO, J.; POLITI, A.; PELLEGRINI, N.; MEZZETTI, B.; BATTINO, M. Plant genotype affects total antioxidant capacity and phenolic contents in fruit. **Nutrition**, v. 21, n. 2, p. 207-213, 2005.

SCHEIDT, G. N.; LOPES DA SILVA, A. L.; OLIVEIRA, Y.; COSTA, J. L.; BIASI, L. A.; SOCCOL, C. R. *In vitro* growth of *Melaleuca alternifolia* Cheel in bioreactor of immersion by bubbles. **Pakistan Journal of Botany**, v. 43, n. 69, p. 2937-2939, 2011.

SINGLETON, V. L.; ROSSI Jr, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. **American Journal of Enology and Viticulture**, v. 16, n. 3, p. 144-158, 1965.

SILVEIRA, D. G.; SOUZA, F. V. D.; PELACCANI, C. R.; SOUZA, A. S.; LEDO, C. A. S.; SANTANA, J. R. F. Micropropagation and *in vitro* conservation of *Neoglaziovia variegata* (Arr. Cam.) Mez, a fiber producted bromeliad from Brazil. **Brazilian Archives of Biology and Technology**, v. 52, n. 4, p. 923-932, 2009.

VIEIRA-DE-ABREU, A.; AMENDOEIRA, F. C.; GOMES, G. S.; ZANON, C.; CHEDIER, L. M.; FIGUEIREDO, M. R.; KAPLAN, M. A.; FRUTUOSO, V. S.; CASTRO-FARIA-NETO, H. C.; WELLER, P. F.; BANDEIRA-MELO, C.; BOZZA, P. T. Anti-allergic properties of the bromeliaceae *Nidularium procerum*: inhibition of eosinophil activation and influx. **International Immunopharmacology**, v. 5, n. 1966-1974, 2005.

WEI, Y. Q.; ZHAO, X.; KARIYA, Y.; FUKATA, H.; TESHIGAWARA, K.; UCHIDA, A. Induction of apoptosis by quercetin: involvement of heat shock protein. **Cancer Research**, v. 54, n. 18, p. 4952-4957, 1994.

WITTIG, J.; HERDERICH, M.; GRAEFE, E. U.; VEIT, M. Identification of quercetin glucuronides in human plasma by high-performance liquid chromatography–tandem mass spectrometry. **Journal of Chromatography B: Biomedical Sciences and Applications**, v. 753, n. 2, p. 237-243, 2011.

5 GENERAL CONCLUSIONS

- Efficient micropropagation protocols were established for *Nidularium procerum* and *Nidularium innocentii*; These protocols can be used to aid the conservation of these species endangered, to produce massive propagation of homogenous elite plants and to promote bioprospecting studies;
- A new technology for vinasse disposal was developed and adds value to what is currently considered a wastewater product. We developed a plant culture medium for tissue culture and an efficient protocol for micropropagation was established for *Oncidium leucochilum* (Orchidaceae) and the micropropagated plants were successfully acclimatized, demonstrating the efficacy of the use of vinasse to formulate plant culture media;
- *Nidularium innocentii* is the most suitable species to explore the antioxidant activity and quercetin production, and the GA_3 fermented extract of *Fusarium moniliforme* obtained via solid state fermentation is a great candidate to increase the antioxidant activity, quercetin content and other secondary metabolites in plant tissue culture, moreover, the GA_3 fermented extract can be an alternative to reduce the production costs of secondary metabolites.

6 SUGGESTIONS FOR FUTURE RESEARCH

- The micropropagation protocol of *Nidularium innocentii* must be explored using shoots etiolated as explants, to optimize the rate multiplication;
- The micropropagation protocol of *Nidularium innocentii* could be optimized using others cytokinins and culture medium supplementation;
- Others plant culture medium can be formulated to extend the application of vinasse culture medium to other plants and increase its utilization to vinasse disposal;
- Extraction methods including dried leaves and different solvents could result in higher antioxidant activity and quercetin content in *Nidularium* species;
- Elicitors can be used to enhance the accumulation of quercetin and increase in antioxidant activity.

7 **PAPERS PUBLISHED**

SANTOS, J. D.; LOPES DA SILVA, A. L.; COSTA, J. L.; SCHEIDT, G. N.; NOVAK, A. C.; SYDNEY, E. B.; SOCCOL, C. R. Development of a vinasse nutritive solution for hydroponics. **Journal of Environmental Management**, v. 114, p. 8-12, 2013.

MELO, A. L. A.; LOPES DA SILVA, A. L.; COSTA-RIBEIRO, M. C. V.; MIYAOKA, M. F.; THOMAS-SOCCOL, V.; SOCCOL, C. R. Selection of *Bacillus thuringiensis* Berliner strains to control *Aedes aegypti* Linnaeus. **Journal of Biotechnology and Biodiversity**, v. 4, p. 78-83, 2013.

LOPES DA SILVA, A. L.; COSTA, J. L.; ALCANTARA, G. B.; CARVALHO, D. C.; SCHUCK, M. R.; BIASI, L. A.; SCHEIDT, G. N.; SOCCOL, C. R. Micropropagation of *Nidularium innocentii* Lem. and *Nidularium procerum* Lindm. (Bromeliaceae). **Pakistan Journal of Botany**, v. 44, p. 1095-1101, 2012.

SCHEIDT, G. N.; LOPES DA SILVA, A. L.; OLIVEIRA, Y.; COSTA, J. L.; BIASI, L. A.; SOCCOL, C. R. *In vitro* growth of *Melaleuca alternifolia* Cheel in bioreactor of immersion by bubbles. **Pakistan Journal of Botany**, v. 43, p. 2937-2939, 2011.

COSTA, J. L.; LOPES DA SILVA, A. L.; SCHEIDT, G. N.; LEMUS, E. A. E.; SOCCOL, C. R. Estabelecimento *in vitro* de sementes de pinhão manso (*Jatropha curcas* L.) - Euphorbiaceae. **Caderno de Pesquisa Série Biologia**, v. 22, p. 5-12, 2010.

SCHEIDT, G. N.; LOPES DA SILVA, A. L.; DRONK, A. G.; BIASI, L. A.; ARAKAKI, A. H.; SOCCOL, C. R. Multiplicação *in vitro* de *Oncidium leucochilum* (Orchidaceae) em diferentes sistemas de cultivo. **Biociências**, v. 17, p. 82-85, 2009.

Papers in press

LOPES DA SILVA, A. L.; RODRIGUES, C.; COSTA, J. L.; MACHADO, M. P.; PENHA, R. O.; BIASI, L. A.; VANDENBERGHE, L. P. S.; SOCCOL, C. R. Gibberellic acid fermented extract obtained by solid-state fermentation using citric pulp by *Fusarium moniliforme*: Influence on *Lavandula angustifolia* Mill. cultivated *in vitro*. **Pakistan Journal of Botany**, 2013.