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DEVELOPING A PLANT CULTURE MEDIUM COMPOSED OF VINASSE ORIGINATING FROM *HAEMATOCOCCUS PLUVIALIS* CULTURE

ANDRÉ LUIZ GOLLO[1], ANDRÉ LUÍS LOPES DA SILVA[1], KHIOMARA KHÉMELI DELLANI DE LIMA[1], JEFFERSON DA LUZ COSTA[2], MARCELA CÂNDIDO CAMARA[1], LUIZ ANTONIO BIASI[3], CRISTINE RODRIGUES[1], LUCIANA PORTO DE SOUZA VANDENBERGHE[1], VANETE THOMAZ SOCCOL[1] AND CARLOS RICARDO SOCCOL[1*]

[1]*Bioprocess Engineering and Biotechnology Division, Federal University of Paraná, Brazil*
[2]*Campus Gurupi, Federal University of Tocantins, Brazil*
[3]*Laboratory of the Plant Micropropagation, Federal University of Paraná, Brazil*
[*]*Corresponding author's e-mail: soccol@ufpr.br*

Abstract

The mineral nutrients in vinasse provide support for algal and plant growth. Algal culture releases organic compounds into its liquid culture medium. These organic and inorganic substances can be useful for formulating a plant tissue culture medium, because tissue culture medium is composed of organic and inorganic components. Therefore, the aims of this study were to develop a plant culture medium by using the vinasse that is employed for *Haematococcus pluvialis* culture (algal filtrate); to investigate the possible beneficial effects of the biocompounds in the micropropagation of *Nidularium procerum* (Bromeliaceae), to evaluate quercetin content, total phenolics content in vinasse and to evaluate the cytotoxicity of the media by performing a bioassay with *Artemia salina*. The vinasse that originated from *H. pluvialis* culture can be used to formulate plant tissue culture at a 3% dilution, and its mineral nutrients can support *In vitro* plant growth, but some nutrients must be supplemented to enhance its efficiency. An efficient micropropagation protocol was developed for *N. procerum*. The micropropagated plants were suitable for transfer to the field (they were acclimatized). This culture medium provides a way to reuse wastewater, gives a rational alternative to vinasse disposal and adds value to what is currently considered to be an undesirable residue. Moreover, this process can reduce the production costs of clonal seedlings and/or bioactive compounds in biofactories. There was no apparent biostimulatory effect by the algal filtrate on morphogenesis; however, it did increase quercetin production. The *H. pluvialis* culture that was grown in the vinasse decreased the cytotoxicity and phenolic compound contents, which prevented explant tissue necrosis and represented a treatment for this residue for safer disposal in the environment.

Key words: Cytotoxicity, *Artemia salina*, Vinasse disposal, Bromeliaceae, Quercetin, *Nidularium procerum*.

Introduction

Plant culture media are composed of organic and inorganic substances including carbohydrates, vitamins and macro and micronutrients; these media can be supplemented with amino acids, plant growth regulators, complex mixtures and other substances depending of the objective. At present, plant tissue culture is used in several regions of the world in biofactories to produce clonal seedlings and bioactive compounds. However, it is necessary to develop alternatives to reduce production costs (Scheidt *et al*., 2011). Industrial wastes are a cheap source of nutrients and organic compounds and can be used to formulate plant culture medium.

Vinasse is the largest source of pollution in the ethanol industry. It is a dark-colored liquid residue with a high chemical oxygen demand, and it is removed from the base of distillation columns. Vinasse is generated at an average of 12-15 L for each liter of alcohol produced (Santos *et al*., 2013). This huge production of wastewater presents a serious environmental concern (Ahmad *et al*., 2013). However, vinasse is rich in several mineral nutrients that are necessary for plant growth. Therefore, new rational alternatives for vinasse disposal were developed to take advantage of its mineral nutrients, including a nutritive solution for hydroponics (Santos *et al*., 2013) and a plant tissue culture medium (Lopes da Silva *et al*., 2014). The mineral nutrients present in vinasse also support algal growth.

The unicellular green microalga *Haematococcus pluvialis* (Chlorophyceae) is photosynthetic and grows in fresh water, and it is one of the best sources of astaxanthin. This alga has the ability to accumulate a superior amount of astaxanthin (up to 4% of its dry weigh) relative to other sources such as *Phaffia rhodozyma* (approximately 0.04% of its dry weight) (Boussiba *et al*., 1999). The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a common pigment in marine environments where it is produced by phytoplankton and is conserved through the food chain in some marine animals, leading to prominent color formation in the flesh and shell (Lorenz & Cysewski, 2000). This pigment is used as a feed additive in aquaculture to produce salmon in confinement, where it is the responsible pigment for salmon coloration. In nature, salmon acquire their coloration through their natural diet (i.e., the pigment is present in its diet and originates from the food chain) and in confinement, this pigment must be inserted into its diet as a feed additive.

Algal filtrate and algal extracts are used as biostimulants to enhance the resistance and yield of plants. Vinasse originating from *H. pluvialis* culture (algal filtrate) is rich in mineral nutrients, and it releases several organic compounds into the culture medium; nevertheless, some of these substances are thought to be beneficial for improving plant growth and morphogenesis. Therefore, the aims of this study were to develop a plant culture medium with the vinasse used for *H. pluvialis* culture, to investigate the

possible beneficial effects of biocompounds present in algal filtrate on the micropropagation of *Nidularium procerum* (Bromeliaceae) and on quercetin content, to determine the total phenolics in vinasses and to evaluate vinasse cytotoxicity by performing a bioassay with *Artemia salina*.

Material and methods

***Haematococcus pluvialis* culture:** Mixed must vinasse from sugarcane was used as a basal medium for *Haematococcus pluvialis* culture. This crude vinasse was diluted to 3% and supplemented with 0.7% NaCl, and the pH was adjusted to 7.0. A 0.4 $g.L^{-1}$ quantity of inoculum was used for the initial culture (cells in vegetative growth). The inoculum was prepared by using the vinasse medium described above. The culture was performed with 0.5 vvm air at 25°C, and after 15 days of culture (the cells were in vegetative growth, and no cyst formation occurred), the algal biomass was separated and the resulting vinasse (algal filtrate) was used to make plant culture media.

Plant culture media composed of vinasse from *H. pluvialis* culture: To develop the vinasse culture media, vinasse originating from *H. pluvialis* culture (after 15 days of culture) was analyzed for sulfate, chloride, phosphate, nitrate, ammonium, calcium, magnesium, sodium, potassium, manganese, zinc, copper and total iron contents. These ions were determined following the methodologies proposed by the APHA (Anon., 2005). We developed the vinasse media on the basis of the ion quantity contained in KC medium (Knudson, 1946) (Table 1) and MS medium (Murashige & Skoog, 1962) (Table 2). These vinasse media formulations were performed by adding reagents of analytical grade. To prepare media by using vinasse above 3%, the vinasse was concentrated in a rotoevaporator at 55°C.

Table 1. Comparative mineral salt contents of different vinasse sources and different KC-based vinasse media that were used for the *In vitro* culture of *Nidularium procerum.* VP (3% crude vinasse), TV (3% treated vinasse (filtered and decanted)), VH (3% vinasse used from 15-day-old *Haematococcus pluvialis* culture), KC (Knudson C medium), VH1 (VH medium 1) and VH5 (VH medium 5).

Ion ($mg.L^{-1}$)	PV[1]	TV[1]	VH	KC	VH1	VH5
Ca	94.8	49.3	14.5	169.72	184.22	184.22
NO_3	-	0.02	0.5	525.12	525.62	525.62
NH_4	-	1.43	0.36	136.51	0.36	0.36
SO_4	50.4	43.7	191.2	472.76	663.96	663.96
Mg	4.9	3.0	3.89	24.65	28.54	28.54
Fe	1.3	0.84	0.12	5.02	0.12	0.12
Mn	0.15	0.086	0.14	1.85	1.99	1.99
K	48.6	52.8	26.8	71.83	26.8	26.8
PO_4	16.8	11.4	0.35	174.47	0.35	165.55
Na	0.26	0.21	141.2	-	141.2	181.1
Cl	1.8	1.1	30	-	30	30
Zn	0.036	0.022	0.009	-	0.009	0.009
Cu	-	-	0.18	-	0.18	0.18

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

Table 2. Comparative mineral salt contents of different vinasse sources and different MS-based vinasse media used for the *In vitro* culture of *Nidularium procerum*. CV (2.7% crude vinasse), TV (2.7% treated vinasse (filtered and decanted)), VH (2.7% vinasse used from a 15-day-old *Haematococcus pluvialis* culture), MS (Murashige and Skoog (1962) culture medium (MS)), (MSVH 2.7%) MSVH medium 1, (MSVH 5.4%) MSVH medium 2 and (MSVH 10.8%) MSVH medium 3.

Ion ($mg.L^{-1}$)	CV[1]	TV[1]	VH	MS	MSVH 2.7%	MSVH 5.4%	MSVH 10.8%
Ca	85.32	44.33	13.05	119.84	119.37	119.33	118.99
NO_3	-	0.018	0.45	2440.52	2440.97	2441.42	2442.32
NH_4	-	1.29	0.33	371.77	372.1	372.43	373.09
SO_4	45.36	39.36	172.08	166.19	322.67	481.11	799.9
Mg	4.38	2.75	3.5	36.46	36.04	36.09	36.19
Fe	1.21	0.75	0.11	5.42	5.53	5.64	5.83
Mn	0.13	0.07	0.13	5.5	5.63	5.76	6.02
K	43.74	47.52	24.12	783.53	807.65	831.77	880.01
PO_4	15.12	10.26	0.32	118.71	119.03	119.35	119.99
Na	0.23	0.19	127.1	4.65	131.75	258.85	513.05
Cl	1.6	1.0	27	212.72	215.1	218.9	226.2
Zn	0.033	0.020	0.0081	1.95	1.96	1.9662	1.9824
Cu	-	-	0.16	0.0063	0.1663	0.3263	0.6463
Co	-	-	-	0.0062	NA[2]	NA	NA
MoO_4	-	-	-	0.165	NA	NA	NA
B	-	-	-	1.08	NA	NA	NA

[1] Adapted from Santos *et al*. (2013), [2] None available (this figure shows MS salt levels, but the levels present in vinasse are unknown)

The *In vitro* establishment of *N. procerum*: *Nidularium procerum* seed disinfection was performed as proposed by Lopes da Silva *et al*. (2012). The seeds were immersed in 70% ethanol (v/v) for one minute, followed by immersion in commercial bleach (1% active chlorine) for 20 min, and they were then rinsed three times with distilled sterilized water. The germination medium was MS (Murashige & Skoog, 1962), which was mixed with 30 $g.L^{-1}$ sucrose and solidified with 6 $g.L^{-1}$ agar (Type I, Himedia). Seedlings were multiplied *In vitro* on MS medium supplemented with 30 $g.L^{-1}$ sucrose, 2 μM NAA (naphthalene acetic acid) and 4 μM BAP (6-benzylaminopurine) and solidified with 7 $g.L^{-1}$ agar.

***In vitro* multiplication on media composed of vinasse from *H. pluvialis* culture:** *N. procerum*-isolated shoots (2 cm height) were removed from the clusters that were propagated *In vitro*, and these shoots were used as explants. The KC-based vinasse media were as follows: (VH1) was composed of 3% vinasse supplemented with 1000 mg L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$, 5.68 mg L^{-1} $MnSO_4{\cdot}4H_2O$ and 250 mg L^{-1} $MgSO_4.7H_2O$, and (VH5) was made from 3% vinasse supplemented with 1000 mg L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$, 5.68 mg L^{-1} $MnSO_4{\cdot}4H_2O$, 250 mg L^{-1} $MgSO_4.7H_2O$ and 240 mg L^{-1} $NaH_2PO_4{\cdot}H_2O$. The MS-based vinasse media were as follows: (MSVH 2.7%) was composed of 2.7% vinasse supplemented with MS medium salts and vitamins, and its Ca and Mg sources were modified to 390 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 330 $mg.L^{-1}$ $MgSO_4.7H_2O$. (MSVH 5.4%) was made from 5.4% vinasse supplemented with MS medium salts and vitamins, and its Ca and Mg sources were modified to 342 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 295 $mg.L^{-1}$ $MgSO_4.7H_2O$. (MSVH 10.8%) was composed of 10.8% vinasse supplemented with MS medium salts and vitamins, and its Ca and Mg sources were modified to 245 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 225 $mg.L^{-1}$ $MgSO_4.7H_2O$. The KC and MS media were used as controls. All media were supplemented with 30 $g.L^{-1}$ sucrose, 2 μM NAA and 4 μM BAP and then solidified with 7 $g.L^{-1}$ agar. All the vinasse used for media formulation originated from *H. pluvialis* culture at 15 days old. The shoot height (cm), shoot number, shoot percentage, root number, root percentage, leaf number, fresh mass (mg) and survival percentage were evaluated after 60 days of *In vitro* culture.

***In vitro* rooting on media composed of vinasse from *H. pluvialis* culture:** Shoots (1 cm height) were isolated from clusters that were cultured in the multiplication media as follows: KC, VH1, VH5, MS, MSVH 2.7%, MSVH 5.4% and MSVH 10.8%. These isolated shoots were cultured on the same media. The supplementation of these media was adjusted to promote rooting. All media were supplemented with 30 $g.L^{-1}$ sucrose and 1 $g.L^{-1}$ activated charcoal free of plant growth regulators and then solidified with 7 $g.L^{-1}$ agar. The root number, shoot number, height of the aerial parts (cm), leaf number, fresh mass (g), shoot percentage, root percentage and survival percentage were evaluated after 60 days of *In vitro* culture.

Acclimatization: Plantlets from all the rooting media were removed from the flasks, and their roots were washed with tap water (faucet). The acclimatization consisted in culturing the plantlets in Plantmax HT (Buschle & Lepper S.A., Joinville-SC, Brazil) substrate in the greenhouse with intermittent nebulization over 15 days. These plantlets were removed from intermittent nebulization after 15 days of acclimatization, but they were kept inside the greenhouse with manual irrigation. Their survival percentage was evaluated after 30 days of ex vitro culture.

Quercetin content determination: Shoots that were cultured in all multiplication media (after 60 days of *In vitro* culture) were processed by having their fresh mass (1 g) macerated and extracted in 10 mL of methanol (1:10 w/v) over 24 h at room temperature under 80 rpm agitation (in the dark) after passing through Whatman No. 1 filter paper. The extracts were stored at -20°C.

Quercetin content analyses were performed on an HPLC Varian ProStar by using an Ultraviolet (UV) detector at a wavelength of 250 nm. A Microsorb C18 reverse phase column (4.6 x 250 mm) was used at a 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. To obtain a calibration curve, quercetin of 98% purity (Sigma Aldrich, EUA) was used at concentrations of 5, 10, 12.5, 25 and 50 $\mu g\ L^{-1}$ in methanol. The resulting chromatogram values were graphed, and a linear equation was used to calculate the quercetin contents of the samples. The samples were microfiltered through a hydrophilic membrane GV (Durapore) made of PVDF (polyvinylidene difluoride), with a pore size of 0.22 μm. All the assays were performed in triplicate.

An *Artemia salina* bioassay and the total phenolic contents of vinasse: A modified Solis *et al*. (1992) method was employed for the vinasse cytoxicity analysis. Brine shrimps (*Artemia salina* Leach) were hatched from 0.025 g of cysts in a plastic bottle (250 mL), which was filled with artificial sea water (as prepared from Aquasalt (41.4 $g.L^{-1}$) from Aqua One and adjusted to pH 8.5 under constant aeration for 48 h (in the light). After hatching, the active nauplii that were freed from the cysts were harvested from the brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a micropipette (10 μL) and placed in each well (24-microwell plate) containing 1 mL of sterile artificial sea water each. The vinasse dilutions were added to each well and the volume was brought to 1 mL, for a total of 2 mL per well. Three vinasse types were tested as follows: (1) crude vinasse, (2) treated vinasse (filtered and decanted) according the Santos *et al*. (2013) procedure and (3) vinasse originating from *H. pluvialis* culture (after 15 days of culture), at levels of 0, 0.5, 1.0, 2.0, 3.0, 6.0 and 9.0%. A positive control was made from potassium dichromate at 0, 1, 3.125, 6.25, 12.5, 25 and 50 $\mu g\ mL^{-1}$. After 24 h of treatment, the numbers of dead and living nauplii were counted, and these data were used to estimate the LC_{50} (lethal concentration, 50%) and 95% confidence interval (CI_{95}). All assays were performed in triplicate.

The total phenolic contents of the following three vinasse types were measured: (1) crude vinasse, (2) treated vinasse (filtered and decanted) according to Santos *et al*. (2013) procedure and (3) vinasse originating from *H. pluvialis* culture. These vinasse preparations were diluted to 3% and their total phenolic contents were determined by using a Folin-Ciocalteu (Singleton & Rossi, 1965) modified assay. To 500µL of each sample (three replicates), 2.5 mL Folin-Ciocalteu solution (1:10 v/v) was added, and after five minutes, 2mL of Na_2CO_3 (7.5% w/v) was added and the solution stored at room temperature for 60 min. The absorbance of all the samples was measured at 740 nm with a SP2000-UV spectrophotometer. The standard curve was determined by using 0.39, 3.9, 7.8, 15.6, 31.2, 62.5 and 125 $\mu g.mL^{-1}$ of gallic acid. Methanol was used as a blank. The results were expressed in $\mu g.mL^{-1}$ of gallic acid equivalent.

Culture conditions and statistical analysis: The pH of all media was adjusted to 5.8, and the media were then autoclaved at 1.5 kgf/cm^2 and 121°C for 20 min. The cultures were kept at 25 ± 2°C under white fluorescent light (28 $\mu M\ m^{-2}\ s^{-1}$) with a 16 h photoperiod. All experiments were performed in culture flasks with a 5 cm diameter and an 8.5 cm height and in 30 mL of culture medium. The experimental design was completely randomized with six replicates of five explants. The data was subjected to a normality analysis for Lilliefors's test, and it was also subjected to an analysis of variance (ANOVA) followed by Duncan's multiple range test at a $p<0.05$. All statistical analyses were performed by following SOC software procedures (Embrapa, 1990). The LC50 (lethal concentration, 50%) and 95% confidence intervals (CI_{95}) were calculated by Trimmed Spearman-Karber method by using TSK software version 1.5 (Usepa, 1990).

Results and Discussion

Vinasse originating from *H. pluvialis* culture: A chemical analysis of the vinasse originating from *H. pluvialis* culture demonstrated that the highest levels of mineral nutrients were SO_4 and Na, followed by Cl and Ca (Tables 1 and 2). A high SO_4 level is commonly found in mixed must vinasse; however, juice must vinasse has a low SO_4 content. Another study determined the composition of vinasse originating from different types of must (considering a 3% vinasse concentration); the results indicated that there were 111.9, 192.0 and 22.8 $mg.L^{-1}$ SO_4 for mixed must vinasse, molasses must vinasse and juice must vinasse, respectively (Marques, 2006). In this study, 172.1 $mg.L^{-1}$ SO_4 was determined. This variation in the SO_4 content of mixed must vinasse is related to its origin; whereas this vinasse is produced by a juice mixture, most vinasse is made of molasses must vinasse. Juice must vinasse is more suitable for developing plant culture medium because of its low SO_4 content, which can be used at a higher concentration than other types (i.e., molasses must vinasse and mixed must vinasse), allowing for larger mineral nutrient availability and helping to avoid the salt stress caused by a high SO_4 level.

The high Cl and Na contents were caused by the supplementation of vinasse with 0.7% NaCl (Tables 1 and 2), this supplementation with NaCl is used to promote stress in this alga, this stress is necessary to stimulate this alga to form cysts (i.e., the phase which the astaxanthin is accumulated). Other ion contents that can be highlighted are PO_4, Fe and NH_4, which were dramatically lower in comparison with other vinasse samples, suggesting higher consumption by alga. The first vinasse type used to formulate a plant culture medium was the juice must; therefore, it was compared with the results of the present study. However, to formulate a plant culture medium using vinasse, it is important to determine its mineral nutrient composition and to perform the necessary ionic adjustments (i.e., supplementation with reagents).

***In vitro* multiplication:** There were no significant differences for all variables evaluated in the KC-based vinasse media on seedling multiplication (Table 3). The media that included the nutrients present in vinasse for KC-based medium were suitable; however, the shoot numbers varied from 5.6 to 7.4 shoots per explant, and for the same species cultivated on MS medium supplemented with the same plant growth regulators, the number reached 14.9 shoots per explant, but in another report the explants were evaluated after 120 days (i.e., double the time) (Lopes da Silva *et al*., 2012). In comparing KC medium with MS medium, we observed a large difference in the amount and availability of ions, and KC medium does not have several ions that were present in MS medium as follows: Na, Cl, Zn, Cu, Co, MoO_4 and B. MS medium presents a higher salt concentration than KC medium for NO_3, NH_4, Mn, K, Fe and Mg. KC medium had higher levels of Ca, SO_4 and PO_4 than MS medium (Tables 1 and 2).

VH1 could be used commercially, and its primary advantages would be cost reduction (even in comparison with KC and VH5, because it employed less reagents than the other media), and it is possible that the $MgSO_4.7H_2O$ could be removed without influencing the multiplication efficacy; this consideration was based on another study in which a similar culture medium was developed, named KCV1. This medium uses 2.5% vinasse (decanted and filtered, not used to culture alga) and is supplemented with 1000 mg L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$ and 65 mg L^{-1} $MnSO_4{\cdot}4H_2O$, and it yielded suitable results for the micropropagation of *Oncidium leucochilum* (Orchidaceae) (Lopes da Silva *et al*., 2014). Moreover, the amount of Mg present in vinasse from *H. pluvialis* culture is higher (0.89 $mg.L^{-1}$ more) than that of the treated vinasse (decanted and filtered) (Table 1). The other advantage of commercial VH1 use is decreased bureaucracy. Because this medium uses $Ca(NO_3)_2{\cdot}4H_2O$ as a nitrogen source; the reagent is not controlled by the Armed Forces. However, NH_4NO_3 and KNO_3 are controlled by various safety agencies in several countries because of their possible use in explosives production.

Table 3. The *In vitro* multiplication and rooting of *Nidularium procerum* cultivated in KC-based vinasse media after 60 days. Shoot height (H cm), shoot number (SN), Shoot percentage (SP %), root number (RN), root percentage (R %), leaf number (LN), fresh mass (FM mg) and survival percentage (S %). KC (Knudson C medium), VH1 (VH medium 1) and VH5 (VH medium 5).

Medium	*In vitro* multiplication							
	H cm	SN	SP %	RN	R %	LN	FM mg	S %
KC	2.5 a[1]	7.4 a	88 a	0.16 a	8 a	25.7 a	57.9 a	100 a
VH1	2.5 a	5.6 a	96.6 a	0.06 a	6.6 a	18.8 a	47.1 a	100 a
VH5	2.6 a	6.7 a	93.3 a	0.46 a	20 a	21.4 a	62.1 a	100 a
CV(%)	7.5	17.9	17.7	19.7	17.3	14.0	31.4	0.0
Medium	***In vitro* rooting**							
	R %	RN	SP %	SN	H cm	LN	FM mg	S %
KC	60.0 a	1.4 a	86.6 a	2.4 a	3.2 a	16.2 a	55.1 a	86.6 a
VH1	80.0 a	1.5 a	73.3 a	1.8 a	3.1 a	13.2 a	36.3 a	86.6 a
VH5	86.6 a	1.5 a	80.0 a	1.9 a	2.8 a	12.0 a	34.3 a	80.0 a
CV(%)	24.7	14.6	15.7	16.4	18.3	12.1	28.2	16.8

[1] Means within a column followed by the same letter do not differ from one another according to Duncan's test at $p<0.05$

Table 4. The *In vitro* multiplication and rooting of *Nidularium procerum* as cultivated in KC-based vinasse media after 60 days. The shoot height (H cm), shoot number (SN), shoot percentage (SP %), root number (RN), root percentage (R %), leaf number (LN), fresh mass (FM mg) and survival percentage (S %). MS (Murashige & Skoog (1962) culture medium (MS)), (MSVH 2.7%) MSVH medium 1, (MSVH 5.4%) MSVH medium 2 and (MSVH 10.8%) MSVH medium 3.

Medium	*In vitro* multiplication							
	H cm	SN	SP %	RN	R %	LN	FM mg	S %
MS	3.8 a	9.4 a	96 a	0.7 a	28 a	34.4 a	199.5 a	100 a
MSVH 2.7%	3.6 a	5.4 b	96 a	0.7 a	48 a	24.1 b	87.0 b	100 a
MSVH 5.4%	3.9 a	4.5 bc	96 a	0.3 a	16 a	21.8 b	81.4 b	100 a
MSVH 10.8%	3.6 a	2.9 c	100 a	0.7 a	35 a	14.6 c	68.4 b	100 a
CV (%)	30.6	16.1	11.9	66.6	55.0	9.4	68.5	0
Medium	***In vitro* rooting**							
	R %	RN	SP %	SN	H cm	LN	FM mg	S %
MS	92 a[1]	4.0 a	100 a	4.5 a	3.4 a	26.5 a	109.4 a	96.0 a
MSVH 2.7%	64 b	1.8 b	100 a	2.6 b	2.3 b	16.5 b	54.0 b	83.2 a
MSVH 5.4%	60 b	0.9 b	100 a	2.3 b	2.6 b	15.7 b	39.9 b	96.0 a
MSVH 10.8%	50 b	1.4 b	80 b	2.1 b	2.3 b	13.8 b	36.6 b	83.2 a
CV (%)	31.8	16.6	11.7	16.2	16.6	14.1	45.2	19.7

[1] Means within a column followed by the same letter do not differ from each other according to Duncan's test at $p<0.05$

The *In vitro* multiplication on MS-based vinasse media presented significant differences in the shoot number, leaf number and fresh mass. The best multiplication result was found in MS (control), which reached 9.4 shoots per explant (Table 4). The increasing vinasse concentration in the culture medium formulations increased the sodium and sulfate contents (Table 2) and decreased the shoot number, leaf number and fresh weight (Table 4). The results found in MS-based vinasse media suggests the occurrence of salt stress, whereas the MS already presents an elevated salinity in comparison with KC medium, and when mixed with concentrated vinasse, the levels of Na and SO_4 decreased the multiplication efficacy (Tables 2 and 3). Inhibited growth and yield is caused by a reduction in the osmotic potential caused by excess salts and/or their toxicant effects. Na and SO_4 can contribute significantly to salt stress occurrence, in spite of high Na levels inhibiting the K uptake (Taiz & Zeiger, 2004), and they also reduce the efficiency of use for other nutrients (Rego *et al*., 2011), primarily by competition during the uptake process. Another finding that supported salt stress occurrence is that salinity often reduces shoot growth more than root growth (Läuchli & Epstein, 1990), as observed in the multiplication (Tables 3 and 4). There were no significant differences in the shoot height, shoot percentage, root number, root percentage and survival percentage found for the MS-based vinasse media (Table 4).

The methodology employed to develop MS-based vinasse media was different for the KC-based vinasse medium, for which the media were supplemented with reagents and vinasse was diluted to 3%. There was also a reagent supplementation for the MS-based vinasse media formulations, but the vinasse was concentrated at different levels (2.7, 5.4 and 10.8%). The aim was to identify the effects of putative bioactive compounds present in algal filtrate that could promote the growth of explants, and it was believed that these biocompounds showed this putative effect only at high concentrations. However, high Na and SO_4 levels impeded the observation of possible effects because of salt stress (Table 2). Other studies must be performed to eliminate these excessive ions and avoid negative interference in plant growth.

***In vitro* rooting:** There were no significant differences in any of the variables evaluated for the KC-based vinasse media on rooting (Table 3). The root number and root percentage varied from 1.4 to 1.5 roots per explant and 60 to 86.6% rooting (Table 3). However, no plant growth was observed and the shoot height found for explants was small (1 cm height), whereas the shoot length is often correlated with the rooting percentage (Silva *et al*., 2006; Bisognin *et al*., 2008). In another study of *N. procerum*, explants at 2 cm in height and the largest rooting rate and root number obtained was 96% and 4.1 root per explant after 50 days of culture on MS medium (which was free of plant growth regulators) in a double-phase system (i.e., a solid inferior layer and a liquid superior layer) (Lopes da Silva *et al*., 2012). Although the explant length is important to induce rooting, another condition that can favor rooting is the supplementation of the culture medium with vitamins. KC medium supplemented with MS vitamins favored root development in an orchid hybrid named *Brassocattleya* Pastoral x *Laeliocattleya* Amber Glow (Fernandes da Silva *et al*., 2009). The presence of activated charcoal was beneficial for *Orthophytum mucugense* rooting, and even in culture medium with 1.11 µM Indole-3-butyric acid (IBA) or in the absence of plant growth regulators, there was approximately 20% more rooting than there was in its absence (Cerqueira Lima *et al*., 2012).

Root formation in isolated *N. procerum* shoots occurred in culture medium that was free of plant growth regulators, and this observation is similar to the results found in *Dyckia macedoi* (Mercier & Kerbauy, 1993), *D. agudensis* (Silva *et al*., 2007), *D. maritima* (Silva *et al*., 2008), *Vriesea scalaris* (Silva *et al*., 2009) and *Orthophytum mucugense* (Cerqueira Lima *et al*., 2012). IBA did not favor rooting in *Vriesea scalaris* (Silva *et al*., 2009) and *Orthophytum mucugense* (Cerqueira Lima *et al*., 2012); however, the rooting percentage in some species can be increased by employing lower auxin concentrations, especially for NAA, with concentrations of 1.1 µM for *Vriesea fosteriana* (Mercier & Kerbauy, 1992), 0.1 $mg.L^{-1}$ for *Dyckia macedoi* (Mercier & Kerbauy, 1993) and 3.22 µM for *Hohenbergia penduliflora* (Pérez *et al*., 2013). However, if rooting can be achieved without using plant growth regulators and there is no interference in plant survival during acclimatization, this practice represents a cost reduction in the micropropagation process and suggests that these species are good candidates for establishing suitable protocols for *ex vitro* rooting due to ease of rooting.

The survival rate varied from 80 to 86.6% (Table 3), and this result is most likely associated with the explant length, whereas much smaller explants can be dehydrated depending of the osmotic potential of culture medium. If the culture medium is more hypertonic than the explant content, then water moves from the explant to the culture medium and promotes explant dehydration. Another consideration is the explant length (2 cm height) used for multiplication in this study. To overcome this problem, a prior phase for shoot elongation can help to elevate the survival rate of explants, yielding larger explants.

Lateral shoot formation also occurred in the explant base during the rooting phase, and the shoot rate and shoot number varied from 73.3 to 86.6% and 1.8 and 2.4 shoot per explant, respectively (Table 3). This morphogenetic process occurs because of the presence of endogenous cytokinin levels that are added to the culture medium for the multiplication phase (i.e., to promote multiple shoot formation). These explants originated from multiplication medium containing cytokinins, and even so, when transferred to another culture medium free of plant growth regulators, they continued to proliferate. This effect is called habituation. A prior phase of shoot elongation aids in the consumption of these endogenous cytokinins, which favored rooting and guided the energy used to produce shoots to produce roots. However, this process can be accelerated as observed in *Vriesea fosteriana*, in which the addition of 0.54 µM NAA was necessary to stop lateral shoot proliferation and to re-stabilize the apical growth of the shoots; rooting was easily induced in this manner (Mercier & Kerbauy, 1992).

In vitro rooting on MS-based vinasse media presented significant differences in terms of the rooting percentage, root number, shoot percentage, shoot number, shoot height, leaf number and fresh mass (Table 4). There were no significant differences in survival rates among the treatments, and no explants died (100% survival). The MSVH 10.8% medium decreased the shoot percentage (80%) and the other media did not have any influence on this factor. The media formulated with vinasse dilutions of 2.7, 5.4 and 10.8% exhibited a significant decrease in the rooting percentage, root number, shoot number, shoot height, leaf number and fresh mass (Table 4). These results are involved in salt stress occurrence, which could likely be attributed to high levels of SO_4 and Na as previously discussed. The ionic strength the salts has a great influence in the shoot rooting (Silva *et al*., 2015).

Vinasse dilutions (decanted and filtered) at 2.5, 5 and 10% were used for the *In vitro* culture of *Oncidium leucochilum* (orchid), and the 2.5% vinasse dilution was shown to be ideal for plant tissue culture and did not affect explant survival. This dilution favored a high rooting rate, in spite of the finding that smaller vinasse dilutions (5 and 10%) possessed a significant phytotoxic effect on explants (Lopes da Silva *et al*., 2014). The vinasse dilution is an important variable in formulating culture medium because of its direct influence on explant survival.

Acclimatization: The micropropagated plant survival rates obtained in this study varied from 78.3 to 100% (Fig. 1A-B). These results were suitable, whereas above 80% is already a suitable survival rate, considering the difficulty of transferring plants from *In vitro* conditions to *ex vitro* conditions. This difficulty suggests the necessity for gradual physiological changes, primarily to avoid excessive water loss from tissues to the environment by transpiration. These gradual plant changes must promote epicuticular wax and functional stomata formation (Sutter & Langhans, 1982), which are associated to avoid plant loss water, and they consequently increase the plant survival rate.

The plants produced in KC-based vinasse media varied from 80 to 100% in terms of their survival rate; there were no significant differences in plant survival among them (Fig. 1A). However, the VH1 medium exhibited 100% survival in comparison with 80 and 87.5% for VH5 and KC media, respectively (Fig. 1A). A similar result for VH1 medium was found in micropropagated *Nidularium fulgens* plants that were cultivated in a greenhouse irrigated with a nebulization system over 60 days, for which the survival rate was 100% (Paiva *et al*., 2009). In our study, the exposition time to the nebulization system was only 15 days.

Micropropagated plants originating from MS-based vinasse media increased from 78.3 to 95.7% survival rates (Fig. 1B). The best results were found in MS (control) and MSVH 2.7%, which yielded survival rates of 95.8 and 94.7%, respectively. The different salt compositions of the culture medium and supplementation with different levels of microorganism filtrate 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). Microshoots of *Lavandula angustifolia* that was cultivated on LS medium supplemented with different levels of fermented *Fusarium moniliforme* extract yielded 66.6 to 91.6% survival rates (Lopes da Silva *et al*., 2013). These results can be associated with the different nutritional status of the plants cultivated in different culture media, whereas different culture media have different nutrient sources and amounts, which can also influence the availability and uptake of nutrients. Moreover, the results of this study can also be associated with the salt stress promoted in the media containing the highest vinasse concentrations, and increasing the vinasse concentration in the culture medium formulations increased sodium, sulfate and potassium contents and decreased the survival rate (Fig. 1B).

Quercetin content: Plantlets cultured on KC medium presented higher quercetin contents than those grown on MS medium, with 54.5 mg/100 g^{-1} fresh weight relative to 47.3 mg/100 g^{-1} fresh weight, respectively. The larger amounts of SO_4 and PO_4 in KC medium could be associated with the highest quercetin production in comparison with the MS composition (Table 1 and 2). The highest SO_4 and PO_4 amounts could play a part in the greater production of 4-coumaroyl-CoA (C_{30} H_{42} N_7 O_{18} P_3 S); this compound has P and S in its structure and is an intermediate in the phenylpropanoid pathway, which is where quercetin biosynthesis occurs (Taiz & Zeiger, 2004).

The presence of algal filtrate increased the quercetin content. The VH1 and VH5 media yielded 57.2 and 57.1 mg/100 g^{-1} fresh weight, respectively. The results observed in VH1 and VH5 media relative to KC medium (54.5 mg/100 g^{-1} fresh weight) represents an increment of approximately 4.7% of the quercetin production (Fig. 2A). Plantlets cultured on MSVH 2.7%, MSVH 5.4% and MSVH 10.8% presented 79.3, 79.1 and 73.6 mg/100 g^{-1} fresh weights, respectively. The results found for plantlets cultivated on MSVH media represents an increment of approximately 59.6% of the quercetin production in comparison with the MS medium (Fig. 2B). The low quercetin production obtained by VH media relative to that of MSVH media could be associated with a low nutrient level and the absence of some nutrients, such as Co, MoO_4 and B.

Calli obtained from young leaf explants of *Pluchea lanceolata* (Asteraceae) were exogenously supplemented with cinnamic acid (5 mg/100 mL) or phenylalanine (50 mg/100 mL), and these calli had increased yields for total quercetin concentrations of approximately 7 to 8-fold and only 1 to 2-fold in comparison with the control, respectively. The authors concluded that cinnamic acid is a more effective precursor than phenylalanine (Arya & Patni, 2013). However, it is possible that the algal filtrate contains phenylalanine and/or cinnamic acid, which could explain why the highest quercetin production was promoted by algal filtrate because these compounds are precursors of quercetin biosynthesis.

The increased algal filtrate concentration in MSVH media (i.e., 5.4 and 10.8%) did not promote an increase in the quercetin content, and this result can be explained by feedback inhibition in the enzyme phenylalanine ammonia-lyase. This enzyme is an important regulatory step in the formation of many phenolic compounds (Taiz & Zeiger, 2004).

The cytotoxicity and total phenolic contents of vinasses: The positive control yielded an LC_{50} of 45.85 (CI_{95} 27.70-75.89) $\mu g.mL^{-1}$ potassium dichromate. Crude vinasse presented the highest toxicity, with an LC_{50} of 0.68 (CI_{95} 0.42-1.11)%, followed by treated vinasse (decanted and filtered) with a LC_{50} of 2.06 (CI_{95} 1.68-2.54)%. The process used to treat the vinasse, which employed decantation and filtration according Santos *et al*., (2013), reduced the toxicity by 1.38% relative to crude vinasse. This treated vinasse was evaluated because it was used to formulate the first plant culture medium containing vinasse; however, this medium promoted some necrosed regions in the explants (37.5%) (Lopes da Silva *et al*., 2014). This phytotoxicity is not desired in a plant tissue culture and can increase the browning rate of explants. It is possible that this toxicity can be caused primarily by the presence of phenolic compounds in vinasse. However, these compounds are produced in vinasse by the catabolism of lignin originating from sugarcane biomass (Parnaudeau *et al*., 2008). Therefore, an early algal culture could decrease the vinasse toxicity, and algae require a large amount of carbon and phenolic compounds as a carbon source. However, the vinasse originating from *H. pluvialis* did not cause toxicity until reaching 9%, the highest level tested in this study. Moreover, the vinasse level used to perform the best plant culture medium was 3%.

The results of the total phenolic content analysis identified 11.3, 7.8 and 0.32 $\mu g.mL^{-1}$ for crude vinasse, treated vinasse and the vinasse used to culture *H. pluvialis*, respectively. This finding represented a decrease of 31 and 97.2% total phenolics in treated vinasse and the vinasse used for *H. pluvialis*, respectively, in comparison with crude vinasse. These results supported the suggestion that phenolic compounds are the primary substances involved in vinasse toxicity. The prior culture of algae in vinasse must be performed before using the vinasse to formulate plant culture medium, because it is an excellent way to cut vinasse toxicity.

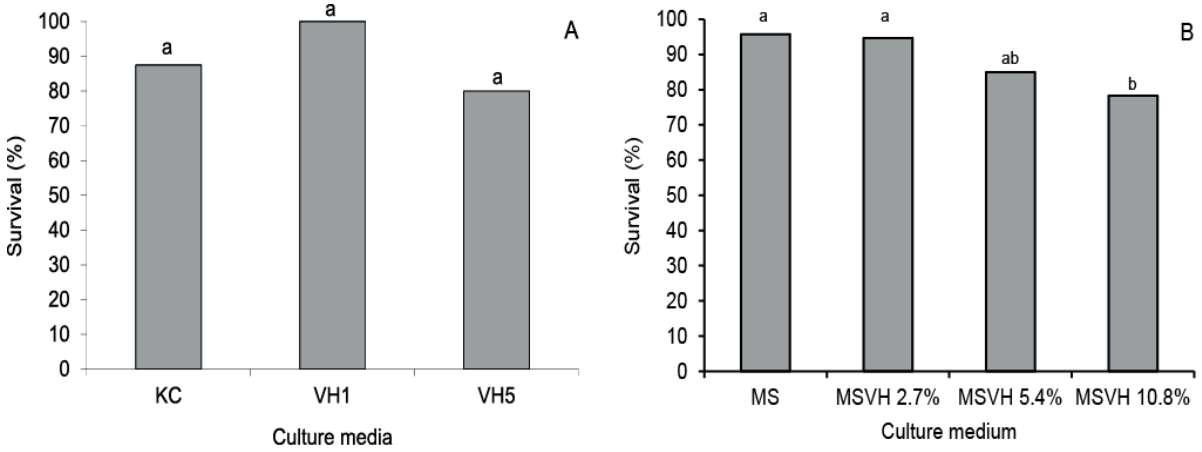

Fig. 1. The acclimatization of micropropagated *Nidularium procerum* plants originating from different vinasse (used to culture *Haematococcus pluvialis*) media after 30 days of *ex vitro* culture, (A) KC-based vinasse media and (B) MS-based vinasse media. The formulations of vinasse media are detailed in the material and methods. Means followed by the same letter do not differ from each other according to Duncan's test at $p<0.05$.

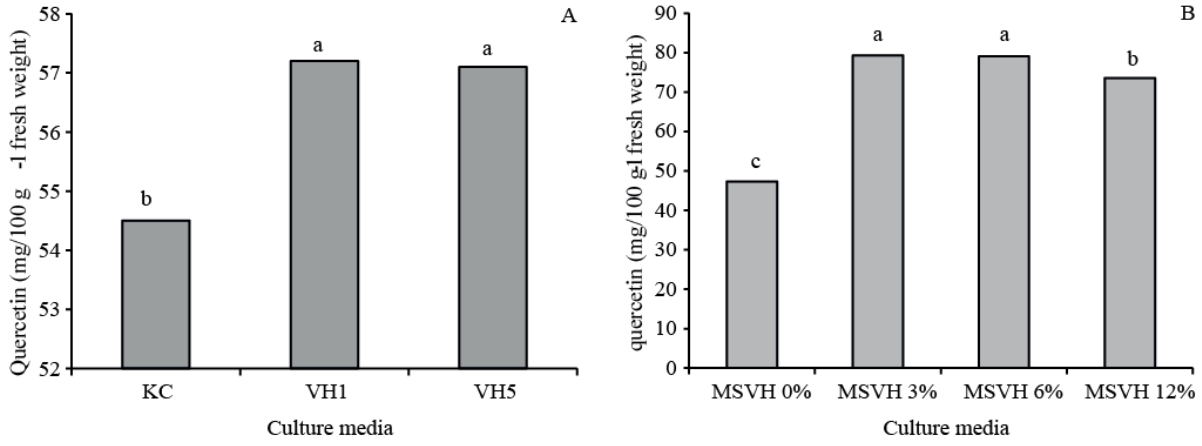

Fig. 2. The quercetin contents for methanolic extracts of fresh *Nidularium procerum* plants that were multiplied on different vinasse (which was used to culture *Haematococcus pluvialis*) media after 60 days of *In vitro* culture, (A) KC-based vinasse media and (B) MS-based vinasse media. The vinasse media formulations are detailed in the material and methods. Means followed by the same letter do not differ from each other according to Duncan's test at $p<0.05$.

Conclusions

Vinasse originating from *H. pluvialis* culture can be used to formulate plant tissue culture at a 3% dilution, and its mineral nutrients can support the *In vitro* growth of plants. However, some nutrients must be supplemented to enhance vinasse efficiency. An efficient protocol for micropropagation was developed for *N. procerum*. The micropropagated plants were suitably transferred to the field (acclimatized). This culture medium represents a reuse method for this wastewater and a rational alternative to vinasse disposal, and it adds value to what is currently considered an undesirable residue. Moreover, this process can reduce the production costs of clonal seedlings and/or bioactive compounds in biofactories. There was no observed biostimulant effect of algal filtrate on morphogenesis, but this filtrate increased quercetin production. *H. pluvialis* culture in vinasse decreases the cytotoxicity and phenolic compounds content, preventing explant tissue necrosis and representing a treatment for this residue for safer disposal in the environment.

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