UNIVERSIDADE FEDERAL DO PARANÁ

ANDRÉ LUIZ GOLLO

DEVELOPMENT OF A PLANT CULTURE MEDIUM COMPOSED WITH VINASSE ORIGINATED FROM *Haematococcus pluvialis* CULTURE

CURITIBA

2015

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DEVELOPMENT OF A PLANT CULTURE MEDIUM COMPOSED WITH VINASSE ORIGINATED FROM *Haematococcus pluvialis* CULTURE

Dissertação apresentada como requisito parcial à obtenção do grau de Mestre em Engenharia de Bioprocessos e Biotecnologia, no Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Carlos Ricardo Soccol

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UNIVERSIDADE FEDERAL DO PARANÁ
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia
Departamento de Engenharia de Bioprocessos e Biotecnologia
UFPR Setor de Tecnologia

RELATÓRIO DE DEFESA DE DISSERTAÇÃO DE MESTRADO

Aos trinta dias do mês de janeiro de 2015, na Sala de Aula do Prédio do CENBAPAR do Centro Politécnico da Universidade Federal do Paraná, Jardim das Américas, foi instalada pelo Profº Drº Júlio César de Carvalho, Coordenador do Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, a banca examinadora para a Nonagésima Quarta Defesa de Dissertação de Mestrado, área de concentração: Agroindústria e Biocombustíveis. Estiveram presentes no Ato, além da Coordenadora do Curso de Pós–Graduação, professores, alunos e visitantes.

A Banca Examinadora, atendendo determinação do Colegiado do Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia ficou constituída pelos membros: Profº Dr. Eduardo Andrea Lemus Erasmo (UFT), Profº Dr. André Luís Lopes da Silva (UFPR), e Profº Dr. Carlos Ricardo Soccol (UFPR – orientador da dissertação).

Às 14h00, a banca iniciou os trabalhos, convidando o candidato **André Luiz Gollo** a fazer a apresentação da dissertação intitulada: "**DEVELOPMENT OF A PLANT CULTURE MEDIUM COMPOSED WITH VINASSE ORIGINATED FROM *Haematococus pluvialis* CULTURE**". Encerrada a apresentação, iniciou-se a fase de arguição pelos membros participantes.

Tendo em vista a dissertação e a arguição, a banca composta pelos membros Profº Dr. Eduardo Andrea Lemus Erasmo, Profº Dr. André Luís Lopes da Silva e Profº Dr. Carlos Ricardo Soccol, declarou o candidato Aprovado (de acordo com a determinação dos Artigos 59 a 68 da Resolução 65/09 de 30.10.09).

Curitiba, 30 de janeiro de 2015.

Profº Dr. Eduardo Andrea Lemus Erasmo

Profº Dr. Carlos Ricardo Soccol

Profº Dr. André Luís Lopes da Silva

DEDICATÓRIA

Aos meus pais, Itamar e Vera que sempre acreditaram em mim e pelo incondicional amor e apoio, principalmente a partir do dia 21/04/2014. Aos amigos Eliezer Tasca, William Busatto e João Gabriel Camargo Rocco, que passaram rapidamente pela minha vida, mas ensinaram os maiores valores que podemos ter: Amor, Aceitação e Gratidão.

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“Agradeço todas as dificuldades que enfrentei, não fosse por elas, eu não teria saído do lugar. As facilidades nos impedem de caminhar. Mesmo as críticas nos auxiliam muito”.

Franciso Cândido Xavier

(Médium e Filantropo)

1910-2002.

“O que somos é consequência do que pensamos”

Buda

“Não ganhe o mundo e perca sua alma, sabedoria é melhor que prata e ouro”

Bob Marley

(Cantor e Compositor)

1945-1981

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RESUMO

Os nutrientes minerais presentes na vinhaça são utilizados por algas e plantas para o seu crescimento. A cultura de alga produz alguns compostos orgânicos e inorgânicos que ficam solúveis no meio da cultura líquida. Estas substâncias podem ser úteis para a formulação de um meio de cultura para plantas, ja que os meios de cultura convencionais são constituídos por componentes dessa natureza. Portanto, o objetivo deste estudo foi desenvolver um meio de cultura para plantas utilizando a vinhaça usada para o cultivo de *Haematococcus pluvialis*; investigar possíveis efeitos benéficos desses biocompostos presentes no filtrado algal sobre a micropropagação de *Nidularium procerum* (Bromeliaceae) no acúmulo de quercetina e avaliar a citotoxicidade desta vinhaça por meio do bioensaio com *Artemia salina*. A vinhaça proveniente da cultura do *H. pluvialis* pode ser utilizada para formular meios de cultura de tecidos de plantas. Usando uma concentração de 3%, seus nutrientes minerais podem suportar o crescimento *in vitro* das plantas, mas alguns nutrientes precisam ser suplementados para otimização. Um protocolo eficiente para a micropropagação de *N. procerum* foi desenvolvido. As plantas regeneradas foram transferidas satisfatoriamente para casa-de-vegetação (aclimatizadas). O desenvolvimento deste meio de cultura representa a possibilidade de reutilização da vinhaça, além de ser uma alternativa racional para o descarte desse resíduo industrial, agregando valor ao que é atualmente considerado um remanescente indesejado. Além disso, este processo pode reduzir os custos de produção de mudas clonais e/ou compostos bioativos em biofábricas. Não foi observado nenhum efeito bioestimulante do filtrado de algas na morfogênese *in vitro*, por outro lado, este filtrado algal aumenta a produção de quercetina. A cultura do *H. pluvialis* em vinhaça diminuiu a citotoxicidade e os compostos fenólicos, evitando a necrose nos tecidos dos explantes.

Palavras-chave: toxicidade, *Artemia salina*, resíduo industrial, Bromeliaceae, quercetina, *Nidularium procerum*.

ABSTRACT

The mineral nutrients presents in vinasse support algae and plant growth. The algal culture produces some organic and inorganic compounds to the liquid culture medium. These substances can be useful to formulate a plant tissue culture medium, whereas tissue culture medium is constituted of organic and inorganic components. Therefore, the aim of this study was to develop a plant culture medium using the vinasse used for *Haematococcus pluvialis* culture, to investigate possible beneficial effects of biocompounds presents in algal filtrate on micropropagation of *Nidularium procerum* (Bromeliaceae), quercetin accumulation and to evaluate the citotoxicity of this vinasse by bioassay with *Artemia salina*. The vinasse originated from *H. pluvialis* culture can be used to formulate plant tissue culture using 3% concentration and Its mineral nutrients can support *in vitro* growth of the plants, but some nutrients must be supplemented. An efficient protocol for micropropagation was developed for *N. procerum*. The micropropagated plants were suitable transferred to the greenhouse (acclimatized). This culture medium represents a reuse of this waste water and a rational alternative to vinasse disposal, adding value to what is currently considered an undesired residue. Moreover, this process can reduce the production costs of clonal seedlings and/or bioactive compounds in biofactories. There was not a biostimulant effect of the algal filtrate on the morphogenesis, on the other hand, this algal filtrate increase the quercetin production. The culture of *H. pluvialis* in the vinasse decreases the citotoxicity and phenolic compounds content, avoiding the explant tissue necrosis.

Keywords: toxicity, *Artemia salina*, industrial residue, Bromeliaceae, quercetin, *Nidularium procerum*

SUMÁRIO

INTRODUCTION

Plant tissue culture represents a powerful tool to promote massive clonal plant propagation in a short time. This technique is use commercially by propagating elite clones to most varied applications, such as potato microtubers (potato seed), ornamental, medicinal, forestry and horticultural plants. Moreover, to produce interspecific hybrids and genetically modified plants.

Although, this technique is very expansive and new technologies to reduce the production costs must be developed. Some progress was already performed, like new models of bioreactors (to automate the process), biotechnological approaches to produce plant growth regulators and others bioactive compounds, like vitamins and amino acids, the use of natural light in growth chambers and so on.

Vinasse is an effluent originated from ethanol industry and the reason for a strong environmental concern, but it is rich in mineral nutrients. This nutrients were already used to establish successively nutritive solutions to support plant growth in hydroponics, thus this residue could be an excellent source of mineral nutrients to formulate plant culture media too.

The *Haematococcus pluvialis* is a microalgae used to produce astaxanthin, a biopigment and antioxidant molecule. The vinasse is used to formulate algae culture medium, however, it was recognized that the algal vinasse (i.e. vinasse used as culture medium to grow algae), could be used to formulate a plant culture medium more efficient due to possible biostimulants produced by this algae. Moreover, the compounds released by these algae could promote some influence on the secondary metabolites produced by plants cultivated *in vitro*.

GENERAL OBJECTIVE

In order to develop new technologies for plant tissue culture, the aim of this study was to develop a plant culture medium using the vinasse (nutrient source) used for *Haematococcus pluvialis* culture.

SPECIFIC OBJECTIVES

1. In order to establish an alternative micropropagation protocol for *Nidularium procerum*, bromeliad with a promising candidate for antioxidant production;

2. In order to investigate possible beneficial effects of biocompounds presents in algal filtrate on micropropagation of *Nidularium procerum* (Bromeliaceae);

3. In order to investigate quercetin accumulation in the plants;

4. In order to evaluate the cytotoxicity of this vinasse by bioassay with *Artemia salina*.

Chapter I - PLANT TISSUE CULTURE – A REVIEW

1. INTRODUCTION

Plant tissue culture is a technology or collection of techniques which draws in the culture of small pieces of tissues (explants), plant cells an organs on synthetic media with controlled conditions (i.e. light, temperature, humidity) and axenic environment (EVANS *et al*, 2003). The first experiment involving isolated plant cells were reported by Gottlieb Haberlandt (1902) (KRIKORIAN and BERQUAM 1969).

Nowadays there is a greater knowledge about this issue and the cell and plant tissue culture is widely used to produce clones. Because of *in vitro* conditions, the ability of morphogenesis is controlled by cell totipotency, a special characteristic of plant cells and meristems in which they retain a latent capacity to produce a whole plant (REINERT and BACKS, 1968, VERDEIL *et al*., 2007). Single cells, plant cells without cell walls (protoplasts), pieces of leaves, stem or roots can often be used to generate a new plant (embryogenesis) on culture media given the required nutrients and plant hormones by inducing somatic embryos (EMBRAPA, 2006). It is also possible to direct the totipotency to organogenesis, inducing buds, shoots or roots (DA SILVA *et al*., 2012).

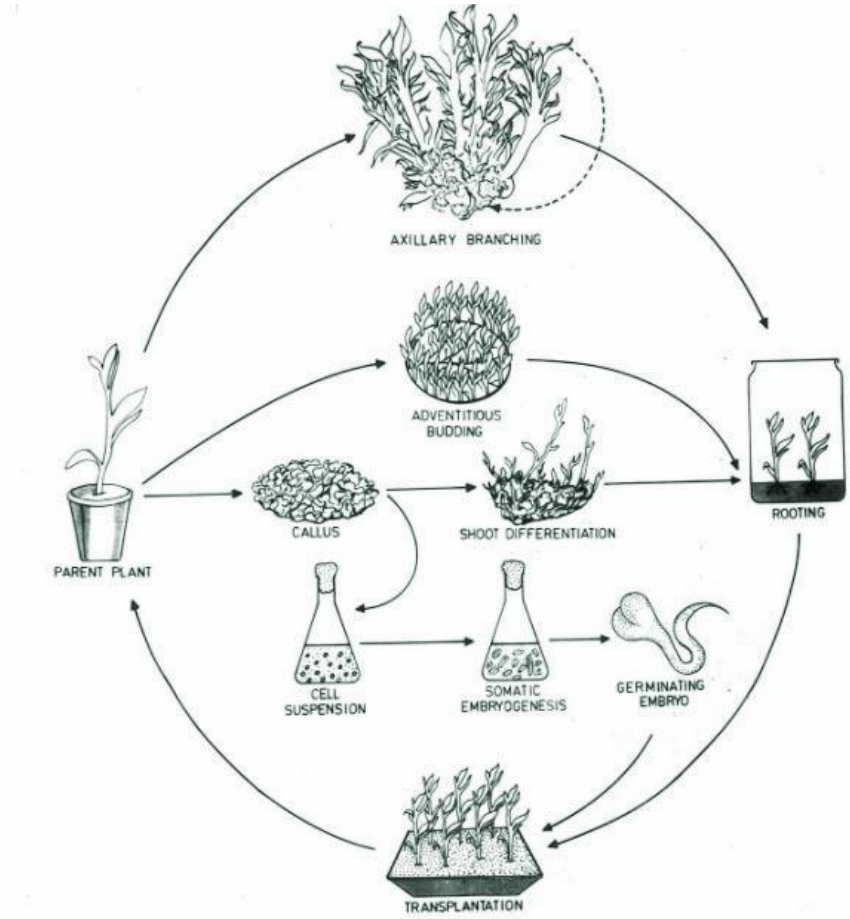

FIGURE 1.1. Diagrammatic representation of the steps involved in *in vitro* multiplication of plants. Source: www.dbtmicropropagation.nic.in

2. CALLUS, CELLS, SINGLE CELL AND PROTOPLAST CULTURE

The culture of undifferentiated mass of cell on agar media produced from an explant of a seedling or other plant part is called callus culture. For callus formation, auxin and cytokines, both are required. The main use of callus culture is focused on maintaining cell lines and morphogenesis (CHAWLA, 2009).

Other technique used by lots of botanist is cell suspension cultures. With correct media composition, explants proliferate into a callus (compact mass of cells) of dedifferentiated cells, which can be screened for the objective of interest and then isolated and transferred to liquid medium to create suspension of single cells and cell clumps. In this technique, the cells grow much faster than in callus culture, hence need to be subcultured more frequently.

There is even a third type of culture, single cell culture. Free cells are isolated from plant organs or cell suspensions and grown as single cells under *in vitro* conditions, thus producing a clone of identical cells. It can be isolated by mechanical or enzymatic methods (CHAWLA, 2009). Other kind of single cell culture is the protoplast culture. Protoplast is the “naked” cell, just the living cytoplasm of each cell, bounded by the plasma membrane. This kind of plant cell is very useful to make genetic transformation, since the cell wall would otherwise block the passage of DNA into the cell (DAVEY *et al*., 2005). It may be regenerated into whole plants, first by growing into a group of plant cells that develops into a callus and then by regeneration of shoots, callogenesis (THORPE, 2007).

These techniques of cell cultures can be applied in lots of situations like mutant screening and selection, once that induces mutagens, producing more frequency of mutants than spontaneous ones (MACHADO et al., 2005). Cell cultures are even effectively for production of chemicals, like heterologous proteins on a commercial scale for enhanced yield and better production control, once that plant systems allow for proper protein assembly, folding and glycosylation, without the threat of contaminants such as endotoxins and pathogens (WILSON and ROBERTS 2012, MIRALPEIX *et a*l., 2013). It is even used to make protoplast culture (WANG, 2011), *in vitro* mutagenesis (KIM *et al*., 2011) and the produce of pure lines (WANG *et al*., 2013).

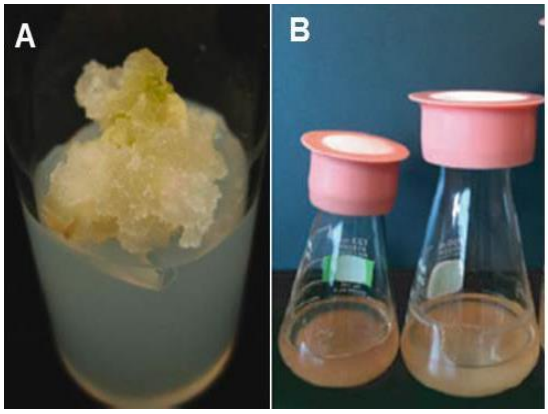

FIGURE 1.2. A) Grow of dedifferentiated cells showing callus formation in a culture media with correct nutrients and hormones combination. B) Callus cells are transplanted into liquid media, creating a suspension culture.

Source: a) DAGLA (2012). b) WILSON and ROBERTS (2012).

3. SOMATIC EMBRIOGENESIS

Somatic embryogenesis is a technique used to induce genetic modifications on vegetal cells. It is a very important technique utilized on tissue culture, because allows the change of natural cells function. In example, somatic cells – ordinary plant tissue – are “reprogrammed” and can originate cells with embryogenic potential (ZENG *et al*., 2006). Somatic embryos can be generated directly from the explant or from for callus culture. They are mainly produced on *in vitro* conditions and requires plant growth regulators (PGR`s – auxin and cytokinin) to be developed (QUIROZ-FIGUEROA *et al*., 2006).

Applications of this process include: high rate of multiplication when compared with any propagation process, production scheduling by the culture maintenance in liquid medium, clonal propagation of genetically uniform plant material and elimination of viruses (CARVALHO *et al*. 2006).

The main problem in the somatic embryogenesis process is the efficiency maturation of the embryos. In general, somatic embryo germination is altered by culture conditions - embryo induction and maturation; therefore, it generally results in different degrees of germination and plantlet development (VAHDATI *et al,* 2008). Accordingly, it is necessary to apply maturation treatments in order to maximize the development of embryos in later stages (CORREDOIRA *et al.,* 2003; MIGUEL *et al.,* 2004).

4. MICROPROPAGATION

Micropropagation or *in vitro* propagation is a technique which utilizes plant tissue culture on *in vitro* conditions. It is widely used in the plant science, forestry, industry and horticulture (GEORGE and DEBERG, 2008; HARNEY, 1982; PENNEL, 1987). This technique produces a large number of progeny plants which are genetically identical to the stock plant. Besides the advantage in produces perfect clones, it produces a massive number of individuals in a relatively short period of time compared to traditional methods of propagation (DOBRÁNSKI *et al*. 2010).

These individuals can be important to the pharmaceutical, medicinal and agricultural industries, because with this technique, they can also produce a role of secondary metabolites, i.e. antioxidants, which can be improved by growing on aseptic and specifics conditions (DA SILVA, 2013). Beside it, micropropagation can even shows a large number of applications and advantages:

- It can help on restoration of degraded regions, reintroducing plants into their native environment, like a strategy in plant conservation and protected areas management, selecting the best clones, micropropagating them and recovery of species at extinguishment (i.e. endangered orchid reestablishment - DECRUSE *et al*., 2013).
- Application on phytoremediation researches (LEDUR, 2009).
- The commercial production (domestic and international markets) of plants used as potting, landscape and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals (ANDRADE *et al*., 2011).
- The use of cell culture (i.e. suspension) to research plant cells with advantageous characters, like herbicide resistance/tolerance (CAO *et al*., 1992), production of secondary metabolites, as anticancer molecules (MALIK *et al*., 2011), antioxidants and research their activity on stressed situation (PALACIO *et al*,. 2011, GOMES-JUNIOR *et al*., 2006), recombinant protein used as biopharmaceuticals (GEORGIEV *et al*., 2009), lipid profile characterization (CORREA and ATEHOURTUA, 2012), inductors of plant regenerators (PACHECO *et al*, 2012).
- The possibility in maintenance a large-scale production of theses valuables compounds, whereas plant cells can growth in liquid cultures in bioreactors (GEORGIEV *et al*., 2009).
- Production of a lot of seedlings with medicinal interest, free from contamination and external agents (SONG *et al*., 2014).
- To rapidly study the molecular basis for physiological, biochemical, and reproductive mechanisms in plants, for example *in vitro* selection for stress tolerant plants (MANOJ *et al*., 2011) and *in vitro* flowering studies (AINA *et al*., 2012a).
- For chromosome doubling and induction of polyploidy. Like doubled haploids, tetraploids and other forms of polyploids. This is usually achieved by application of antimitotic agents such as colchicine or oryzalin (AINA *et al.,* 2012b).

- *In vitro* propagation through meristem culture is the best possible means of virus elimination and then transferred to the environment (ROUT *et al*., 2006).
- Produce particularly good flowers, fruits, or have other desirable traits and to quickly produce mature plants (SATHYANARAYANA, 2007).
- The production of multiples plants in the absence of seeds or necessary pollinators to produce seeds (SHUG *et al*, 2009).
- The production of plants from seeds that otherwise have very low chances of germinating and growing, such as Orchids and Nepenthes *(*CHYUAM *et al.*, 2011; FAY, 19992).

Micropropagation generally involves four distinct stages: initiation of cultures, shoot multiplication, *in vitro* elongation and rooting of shoots, and acclimatization (FIGURE 1.1.). The first stage: culture initiation or *in vitro* establishment, depends on explant type or the physiological stage of the donor plant at the time of excision. Explants from actively growing shoots are generally used for mass scale multiplication. The second stage: shoot multiplication is crucial and achieved by using Plant Growth Regulators like auxin and cytokinin. The third stage: the elongated shoots, derived from the multiplication stage, are subsequently rooted either *ex vitro* or *in vitro*. In some cases, the highest root induction occurs from excised shoots from liquid medium when compared with semi-solid medium (BIDARIGH and AZARPOUR 2013). The fourth stage: acclimatization of *in vitro* grown plants that is an important and the last step in micropropagation (ROUT *et al.,* 2006). The micropropagated plants have a big vulnerability in loose humidity once they were shifted from high humidity/low irradiance conditions to low humidity/high irradiance conditions. Their stomata do not show a perfect function - closing and opening when necessary (JIN *et al.*, 2013). In this case most individuals cannot survive if transferred direct to the field, and a pre-acclimatization step is required to enabling than to survive under ‘adverse’ environments (SILVA *et al.,* 2011).

Propagation *in vitro* can be a very efficient alternative to solve a lot of problems. But is a complex technique that requires the choice of two critical factors: choose of an explant and culture medium (MURASHIG and SKOOG, 1962). The first one depends on the goals of the project and the characteristic of the tissue in question. And the second one is what promotes the plant´s growing and the standard development on *in vitro* conditions. It is very difficult to stabilize, due its sterilized

process, maintenance of regulators and growing promoters, like hormones in a very low concentration (CALDAS *et al.,* 1998).

5. KNUDSON AND MS CULTURE MEDIUM

There are two most useful culture mediums well known to orchids and bromeliads. The first one was proposed by Knudson (KNUDSON, 1946). It is a simple and a cheaper one, but some important nutrients are lacking. Its composition is based on the small amount of reagents (TABLE 1.1.). Knudson medium besides be incomplete, can be useful because of its price. It is cheaper than the others and for some plants can works very well.

These nutrients are usually effective to orchid tissue culture, like multiplication of *Cattleya ioddigesti* and elongation when supplemented with 2,5mg/L^{-1}GA3 (SOARES, *et al.* 2009). It is recommended in the propagation of some other genus: *Laelia*, *Laeliocattleya* and *Brassocattleya* (ARDITTI and ERNEST 1993, Schneiders *et al.,* 2012). In most cases Knudson medium shows positive results in multiplication of shoots and leafs and elongation (SOARES *et al*., 2009, VILLA *et al.,* 2014). Promote rooting with this medium is more difficult, but Lee *et al*., (2011) shows that is even possible when added some growth regulators and some quantity of active charcoal on *Cymbidium sinense* micropropagation culture. Besides the most efficiency of Knudson medium is on Orchids microcultivation, it can be uses in the others plant tissue culture like *Blenchum spp*. and *Pelaea roduntifolia* (Forst.) (JANSSENSAND SEPELIE, 1989).

In the other way, the most widely used medium for micropropagation culture was MS, proposed by Murashige and Skoog (MURASHIG and SKOOG, 1962). This inorganic salt formulation represents one of the major achievements in the history of cell and tissue culture (GOULD and SMITH 1989). It is used for almost all the species used in micropropagation and could be find in different strengths and variations. Most researches utilizes the primordial medium - MS complete - (TABLE 1.1.). Others find effectivity in a half concentration of salts (SCHNEIDERS *et al*., 2012), in addition to many other variation.

TABLE 1.1. Nutrients composition of the two most widely used medium cultures for orchid, KNUDSON C formulation (1946) and Murashige and Skoog medium (MS) (1962).

KNUDSON	**MS**		
Salts	**Salts**	**Vitamins**	**Grow Factors**
$Ca(NO_3)_2.4H_2O$	NH_4NO_3	Nicotinic acid	Cytokinin (BAP)
$FeSO_4.7H_2O$	KNO_3	Pyridoxine,	Auxin (NAA, AIB)
KH_2PO_4	H_3BO_3	Thiamin	
$MgSO_4.7H_2O$	KH_2PO_4	Glycine	
$(NH_4)_2SO_4$	$NaMoO_4.2H_2O$	Mio-Inositol	
$MnSO_4.4H_2O$	KI		
	CaCl2.2H2O		
	CoCl2.6H2O		
	$MgSO_4.7H_2O$		
	$MnSO_4.4H_2O$		
	$ZnSO_4.7H_2O$		
	$CuSO_4.5H_2O$		
	$FeSO_4.7H_20$		
	Na_2.EDTA		

Knudson (1946), Murashig and Skoog (1962).

Murashige and Skoog (1962) culture medium is used in the propagation of a large number of specie, like some examples: production of *Vitis labrusca, Vitis vinifera* plants for beverage industry (CARVALHO *et al.*, 2013); research on solving hyperhydricity in micropropagation of *Lavandula angustifolia (*MACHADO *et al.,* 2014) - plant which produces an important oil presented in multiples hygienic products and medicinal and in *Jatropha curcas* organogenesis (COSTA *et al*., 2014), a specie with a large potential for biofuel production; *Nidularium procerum and Nidularium innocentii,* species with ornamental and medicinal interest (SILVA *et al.,* 2012) and several others species and cultivars.

Knudson medium can be used for orchid micropropagation, because its cost is lower than MS medium and it shows significant results. But when compared with MS medium have, in most cases, production disadvantage. Studies demonstrated that for Dendrobium ”Sonia” orquid, MS medium was the most efficient in inducing protocorm-like bodies (PLBs) (POOSHOA, 2004). For *Anoectochilus formosanus,* MS

medium is found more suitable than Knudson for shoot tip culture (KET *et al.,* 2004). In the comparison of these two mediums for the micropropagation of *Geodorum densiflorum* orchid, Sheelavantmath *et al*., (2000) demonstrated that the explants cultured on KC medium did not show any response and turned brown in 6-8 weeks. However, the explants cultured on MS medium responded well. In fact, the regenerated shoots rooted on MS only.

6. ALTERNATIVE CULTURE MEDIUMS

In literature there are a few others culture media developed for *in vitro* tissue culture, Hyponex (H3) (KANO, 1965), Vacin and Went (VW) (VACIN *&* WENT, 1949), B5 (GAMBORG *et al.,* 1968), Woody plant medium - WPM (LLOYD *and* MCCOWN 1981) and KC medium with MS vitamins (VILLA *et al.,* 2014), but they are specific for some species and goals.

As already shown, micropropagation is a tool widely used and with a tendency to increase its use commercially in the next years. There is a big industry interest, the forests are in extinction and reforestation is the topic on environmentalists' conferences and the large demand of food in the world is increasing in astronomic rates, making the fields being depleted. MS medium is a very efficient culture medium, but it is still expansive. Therefore is necessary to reduce the micropropagated plants production costs, increasing its competition in the market.

One alternative is utilize industrial residues, like vinasse, to promote plant growth. Vinasse is the main effluent of sugar industry, in the ethanol production. Sugarcane or sugar beet is processed to produce crystalline sugar, pulp and molasses. The last one is further processed by fermentation to ethanol, ascorbic acid or other products. The industrial production of ethanol by fermentation results in the discharge of large quantities of high-strength liquid wastes (distillation stage) generally called stillages, distillery slops or vinasse (SILES *et al*., 2011). It has a 93% water and 7% solid compound, with a low to high dark color, bad odor, acid pH, high temperature and high levels of salt (among 24,000 to 80,000 mg/l) and organic material (4,000 a 64,000 mg/l) and for every liter of ethanol produced between 12-20 liters of vinasse are produced (HIDALGO, 2009).

This residue is rich in several mineral nutrients therefore it is used as soil fertilizing. This is a polemic question, studies conducted by Camargo *et al*. (1983), Glória and Orlando Filho (1983), Laime *et al.* (2011) and Jiang *et al.* (2012) in the disposal of sugarcane vinasse in the soil have reported beneficial effects on crops and physic-chemical properties of the soils, because it increases moisture retention, potassium levels, electric conductivity and porosity, in addition to biological activity. However, few studies have assessed the real polluting potential of vinasse in the soil and water (LYRA *et al*., 2003; TENÓRIO *et al*., 2000). The effects of the application of this residue on the soil depend on various factors, such as the quantity applied in the soil, soil type and chemical composition (CHRISTOFOLETTI *et al.,* 2013).

When in large concentration, vinasse can be approximately one hundred times more pollutant than household sewage for aquatic environmental (FREIRE and CORTEZ, 2000; KANNAN and UPRETI, 2008) and in terrestrial environment can modify the lifecycle of lot species (Pedrosa et al., 2005, Yesilada 1999) and cause soil salinity mainly due to the high potassium level (Hassuda, 1989). However, these mineral nutrients are essential for plant growth, which can also increase the crop yield (Silva and Orlando Filho 1981). Then, these mineral nutrients can be used to formulate plant culture medium (Lopes da Silva *et al*., 2014), hydroponics solution (Santos et al 2013) and this process can be a useful alternative to aid to vinasse disposal.

In addition, some microrganisms like the microalgae *Scenedesmus sp., Chlamydomonas reinhardii* can grow on vinasse medium (Ramirez et al., 2014, Kadloglu and Algur 1992). Another specie capable in grow at vinasse medium is *Haematococcus pluvialis,* used in this work.

7. *Haematococcus pluvialis*

Haematococcus pluvialis is a freshwater specie of Chlorophyta from the family Haematococcaceae and can be naturally found in temperate regions, from African lakes to the cold waters of Scandinavia. It is unicellular and mobile. This species is well known for its high content of the strong antioxidant Astaxanthin ($C_{40}H_{52}O_4$. 3,30-dihydroxy-b,b-carotene-4,40-dione), which is important in aquaculture and cosmetics (Lorentz and Cyewski 2000).

The high amount of astaxanthin is present in the resting cells, which are produced and rapidly accumulated when the environmental conditions – bright light, high salinity and low availability of nutrients - become unfavorable for normal cell growth (Boussiba and Vonshak 1991, Grunewald et al 2001, Santos and Mesquita 1984). Their resting cysts are often responsible for the blood-red color seen in the bottom of dried out rock pools and bird baths. This color is caused by astaxanthin, which is believed to protect the resting cysts from the detrimental effect of UV-radiation, when exposed to direct sunlight (Dore and Cysewski 2003).). Lorenz and Cysewski (2000) showed that *H. pluvialis* accumulates up to 5% dry weight of astaxanthin.

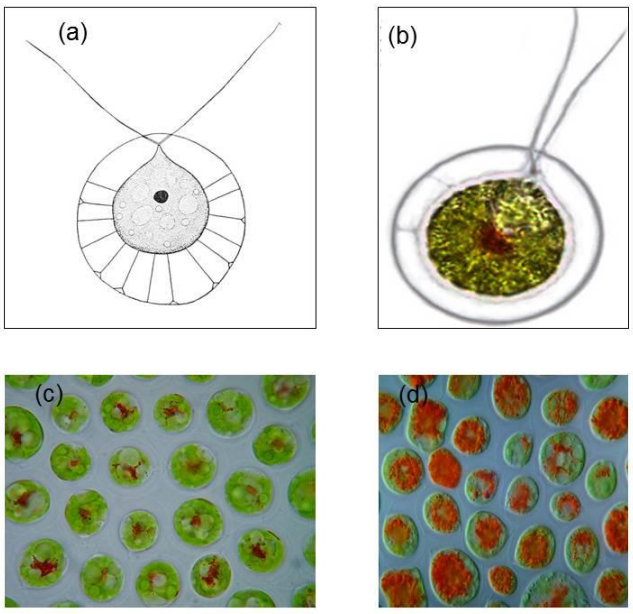

FIGURE 1.3. a) Schematic figure of *Haematococcus pluvialis.* b) Single cell of *H. pluvialis.* c) *H. pluvialis* culture growing in normal conditions of environment. d) H. pluvialis culture in stressful situation to promote astaxanthin production.
Source: a) Collins 1909 b) www.gsbioe.com 41-3329-3675c) e d): www.photomacrography.net.

8. SECUNDARY METABOLITES PRODUCTION

A hole of secondary metabolites can be produced natural by organisms like the study of Coca et al (2014), which the production was up to 168 ± 18mg.L^{-1}.d^{-1} of protein with *Spirulina platensis* algae.

The same way as microalgas, plants can even produce secondary metabolites. Plant secondary metabolites are low-molecular weight compounds that aid in the adaptation of plants to their environment. Because of their significant biological activity, plant secondary metabolites have been used in traditional medicine for centuries (Wilson and Roberts, 2012). Currently, over 60% of anticancer drugs and 75% of drugs for infectious disease are either natural products or analogues of natural products (Newman et al., 2003; Cragg and Newman, 2009). Secondary metabolites are also commonly used as insecticides, dyes, flavors and fragrances (Wilson and Roberts, 2012). (TABLE 1.2.).

TABLE 1.2. Examples of secondary metabolites commercialized trough natural harvest (Wilson and Roberts, 2012).

Use	Compound/product	Plant source	Yield (% dry weight)	References
Anticancer agent	Camptothecin	*Camptotheca acuminata*	0.4–0.5	Lopezmeyer et al. (1994)
	Vinblastine	*Catharanthus roseus*	0.01	Ishikawa et al. (2009)
	Vincristine	*C. roseus*	0.0003	Ishikawa et al. (2009)
Analgesic	Morphine	*Papaver somniferum*	0.05–0.1	Odell et al. (2008)
	Codeine	*P. somniferum*	0.01–0.1	Odell et al. (2008)
Insecticide	Pyrethrins	*Tanacetum cinerariifolium*	1.8–2.5	Morris et al. (2006)
	Neem (Azadirachtin)	*Azadirachta indica*	0.1–0.9	Morgan (2009)

9. *Nidularium procerum*

In this study was used *Nidularium procerum* Lem*.,* a plant which belongs to the family Bromeliaceae. Bromeliaceae is one of the largest botanical families of the world. According to Givnish (20005) is the fifth-largest family in number of species among the monocotyledons and the second-largest among tropical epiphytes (Schulte *et al*. 2005). It is distributed extensively in the coastal Atlantic Forest in tropical America, with exception of *Pitcairnia feliciana* from West Africa (Chedier and Kaplan, 1996). According to APG III (2009) belongs to Poales order.

Bromeliads show a great variety of terrestrial, rupicolous or epiphytic life forms (FIGURE1.4.). The family is a prime example for colonization of extreme habitats like coastal plains, humid mountains forests and high Andean savannahs, and many species are particularly well adapted to xeric conditions (Givnish *et al*. 1997, Benzing 2000, Crayn *et al*. 2004). About half of all Bromeliaceae are epiphytes and apart from the Orchidaceae, they make up the largest portion of epiphytic vascular plants within the Neotropics (Benzing 1990).

Bromeliads are perennial, rosette herbs with a short axis or rarely an elongated stem. The roots are absorbing in terrestrial species but in epiphytes their vascular system is reduced and water uptake is accomplished by absorptive trichomes (Peters 2009). Inflorescences are terminal, sessile or pedunculate, simple to paniculate and often bear conspicuously colored bracts (Peters 2009).

The last study published in taxonomy of Bromeliaceae by Luther (2008) shows that this family presently contains 3.172 species distributed in about 58 genera, and subdivided in 3 subfamilies: Pitcairnioideae, Tillandsoideae and Bromelioideae, majoring present in Brazil. As a matter of fact, recent studies by Givnish *et al*. 2007 based on molecular data established a new concept of eight bromeliad subfamilies: Tillandsioideae, Bromelioideae, Brocchinioideae, Lindmanioideae, Hechtioideae, Puyoideae, Navioideae and Pitcairnioideae (FIGURE 1.5.). Despite of these works, online data on The Plant List (2014), shows that Bromeliaceae family currently include 52 plant genera, with 6.815 scientific plant names of species rank. Of these, 3.320 are accepted species names.

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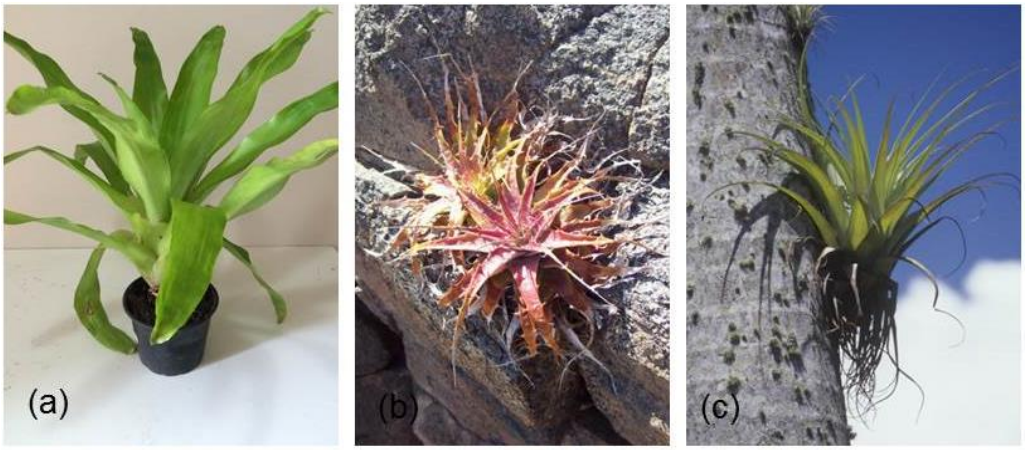

FIGURE 1.4. Different habits of bromeliads. (a) Terrestrial. (b) Rupicolous. (c) Epiphytic. Source: (a) Prived picture. (b) www.skyflowershop.com. (c)www.art.com.

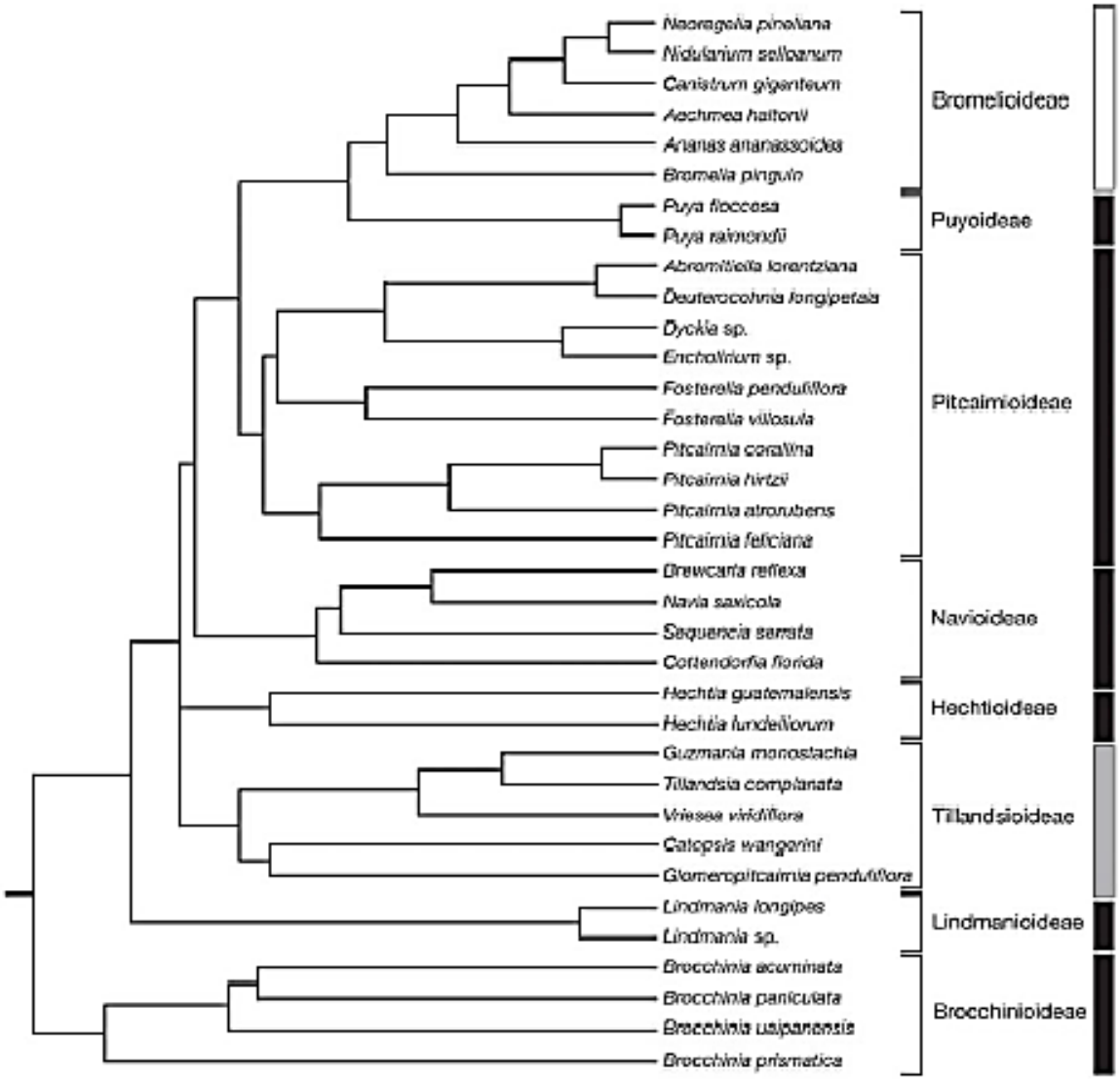

FIGURE 1.5. Molecular phylogeny of Bromeliaceae based on *ndh*F data. Assignment of the new subfamilies to the traditional subfamilies is indicated by shaded bars. Hollow bar: Bromelioideae, grey bar: Tillandsioideae, solid bars: Pitcairnioideae. Source: Givnish et al. (2007).

In Brazil they are in all national territory - 44 genera with approximately 1.290 species which 1.145 are endemic, but the coastal rain forest is the habitat preferred by Bromeliaceae (81.8%), where there is the greatest genetic diversity and the highest degree of endemism (Fontoura *et al*., 1991). Species of Nidularium are well spread in the coastal rain forest from Bahia State, in the northeast region of Brazil, to Rio Grande do Sul State, in the south.

This family has ornamental and medicinal characteristics. Profile of bromeliads includes antioxidant activity and this 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. Cinnamic acid derivatives (Takata and Scheuer, 1976), flavonoids, diterpenoids (Bringmann et al., 2000; Harbone, 1988; Raffauf et al., 1981), triterpenoids and steroids (Atallah and Nicholas, 1971; Borin and Gottlieb, 1993; Marker et al., 1943).

The most common representatives are *Nidularium procerum and N. innocentii* (Leme 2000). Leaves of *N. procerum* have potent analgesic (Amendoeira et al. 2005a) and anti-inflamatory activity (Amendoeira et al 2005b). These species presents also potent anti-eosinophil activity (i.e. Anti-allergic properties) (Vieirade-Abreu *et al*., 2005). They have attracted substantial interest in recent years as it is the candidates to develop new medicaments and as source of several secondary metabolites. Some studies had already shown that *N. procerum* has an antioxidant potential, including polyphenols. Among polyphenolic compounds are the flavonoids, and among these are the flavonoids, like quercetin (Lopes da Silva 2013).

10. QUERCETIN

Quercetin (also named as 3,3',4',5,7-pentahydroxy flavone) is one of most widely distributed flavonoids in plants, its chemical structure is showing in FIGURE 1.6. 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 understood yet (Wei *et al*., 1994). Bardy *et al* (2013) showed that quercetin may also induce insulin secretion by activation of L-type calcium channels in the pancreatic β-cells. However, due to the various preventive effects in human healthy, quercetin is already used as a food supplement available in the commerce.

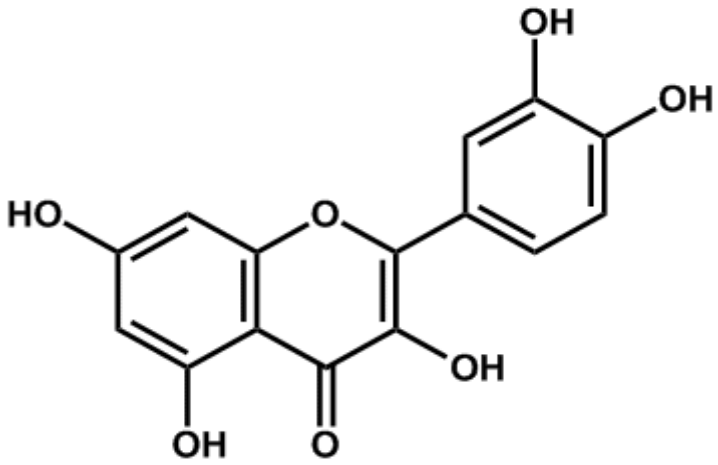

FIGURE 1.6.Quercetin molecule.

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), cranberry (15 mg/100g), sweet potatoe (10 mg/100g) and tomatoes (0.8-65 mg/100g) (Bhagwat et al., 2013) (TABLE 1.3.). 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 the another study carried out by Lopes da Silva *et al* (2013), the *N. procerum* (around 34mg/100g) and *N. innocentii* (around 70mg/100g) are excellent source of quercetin and may be explored commercially being produced *in vitro* (Lopes da silva 2013).

TABLE 1.3. Quercetin concentration (mg/100mg for edible portion) contained in different kinds of foods.

FOOD CONTAINING QUERCETIN	QUERCETIN (mg/100g)
Onion	28.4-48.6
Kale	11
Broccoli	3
Tomatoes	0.8-65
Cranberry	15
Bilberry	3
Tea, black or green	2
Apple	2.1-7.2
Crowberry	5
Sweet potato	10

Bhagwat et al., (2013); Hertog et al., (1992).

11. *Nidularium procerum* MICROPROPAGATION

To understand the enhance in production of antioxidants on *in vitro* conditions, is important knows that the algae can produce a large role of biomolecules in its lifecycle. And these molecules can stay intracelullarly or be expelled to the extracellular middle, adding some molecular value to the culture medium.

Bromeliads are threatened and facing extinction due to predatory collect of its habitats. *Cryptanthus fosterianus*, *Neoregelia binotiie* and *Nidularium utriculosum* are already considered extinguished, and in the Atlantic Forest species registered, about 40% were threatened and facing extinction (WorkshopBiodiversitas 2005). 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 (Silva *et al*., 2012). *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).

Despite the lack of the studies in the Nidularium genera, it is well known the nutrients that culture medium, for micropropagation, needs carry on. Dorris M. (2010) shows the importance of some nutrients for Bromeliads in general. In example of nutrients that are in high quantity (macronutrients), Nitrogen and Sulfur are needed to make proteins. Phosphorous is a critical element in plant structure, energy and part of DNA, Its lack makes pl ants purple. Potassium is involved in enzymes function, Magnesium is part of chlorophyll constitution and Calcium is involved in a large number of processes, helping keeps plants green and blooming. There are still those that need to be find in low quantity (micronutrients) in the medium, in example: Iron, Manganese, Zinc, Boron, Copper, Iodine and Molybdenum.

The plant tissue culture can be a good alternative to be employed in a large arrangement of goals, like industries production, medicine and laboratories. It is possible because there is a lot kind of plant tissue culture techniques that was developed. Callus, cell, single cell, protoplast culture, somatic embryogenesis and micropropagation are some of them which are more utilized currently, because are advantageous, spending less time and in a not distant future, less money.

They are not new techniques, but steel needs improvement to get cheaper and be competitive in the market. The main problem is the medium culture cost. It is therefore evident that there is a need for further studies to develop alternative culture mediums that can improve both secondary metabolites and plant production.

REFERENCES

AINA, K. O.; QUESENBERRY, K.; GALLO, M**.** Photoperiod Affects in Vitro Flowering in Wild Peanut *Arachis paraguariensis*. **American Journal of Plant Sciences**, v. 3, n. 5, p. 567-571. 2012.

AINA, K. O.; QUESENBERRY, K.; GALLO, M. *In vitro* induction of tetraploids in *Arachis paraguariensis***. Plant cell tissue and organ culture**, v. 111, n. 2, p. 231-238, 2012.

ANDRADE, R. A.; MARQUES, T. F.; JASPER, S. P.; DAMATTO JUNIOR, E. R.; FUZITANI, E. J.; NOMURA, E.S. Micropropagação de mudas de bananeira em meio líquido*.* **Comunicata Scientiae,** v. 2, n. 3, p: 156-159, 2011.

ARDITTI, J.; ERNST, R. Micropropagation of Orchids**. New York: John Wiley**. 682p., 1993.

ATALLAH, A. M.; NICHOLAS, H. J. Triterpenoid and steroid constituents of Florida Spanish moss. **Phytochemistry**, v. 10, p. 3139–3145, 1971.

BARDY, G.; VIRSOLVY, A.;, QUIGNARD, J. F.; RAVIER, M .A.; BERTRAND, G.; DALLE, S.; CROS, G. MAGOUS, R.; RICHARD, S.; OIRY, C. " Quercetin induces insulin secretion by direct activation of L-type calcium channels in pancreatic beta cells". **Br. J. Pharmacol***.* v. 169, n. 5, p. 1102–13, 2013.

BENZING, D. H. Vascular epiphytes. **Cambridge University Press**, New York, 1990.

BENZING, D. H. Bromeliaceae – Profile of an adaptive radiation. **University Press Cambridge,** p. 98–105, 2000.

BHAGWAT, S.; HAYTOWITZ, D .B.; HOLDEN, J. M. Beltsville Human Nutrition, Research Center Nutrient Data Laboratory, **U.S. Department of Agriculture**, 2013.

BIDARIGH, S.; AZARPOUR, E. Study Effect of MS Medium and Shoot Tip Length Levels on Rooting in Micro Cuttings of Poinsettia. **International Journal of Agriculture and Crop Sciences,** v.5, n. 8, p. 857-860, 2013**.**

BORIN, M.R.D.M.B.; GOTTLIEB, O.R. Plant chemosystematics and phylogeny: steroids, taxonomic markers? **Plant Systematics and Evolution**. v. 184, n. 41–76. 1993.

BOUSSIBA, S.; VONSHAK, A**.** Astaxanthin Accumulation in the Green Alga *Haematococcus pluvialis*. **Plant and Cell Physiology.**v.32, n. 7, p. 1077-1082, 1991.

BRINGMANN, G.; OCHSE, M.; ZOTZ, G.; PETERS, K.; PETERS, E.M.; BRUN, R.; SCHLAUER, J. 6-Hydroxyluteolin-7-O-(1''-alpha-rhamnoside) from Vriesea sanguinolenta Cogn.And Marchal (Bromeliaceae). **Phytochemistry** 53, 965–969, 2000.

CALDAS, L. S. Cultura de tecidos e transformação genética de plantas. **Brasília: EMBRAPA/CNPH**. p.87-132, 1998.

CAMARGO, O. A.; VALADARES, J. M. A. S.; GERALDI, R. N. Características químicas e físicas de solo que recebeu vinhaça por longo tempo. **Instituto Agronômico**, Campinas, 1983.

CARVALHO, D. C.; SILVA, A. L. L. DA.; SCHUCK, M. R.; PURCINO, M.; TANNO, G. N.; BIASI, L. A. Fox grape cv. Bordô (Vitis labrusca L.) and grapevine cv.

Chardonnay (Vitis vinifera L.) cultivated in vitro under different carbohydrates, amino acids and 6-Benzylaminopurine levels**. Brazilian Archives of Biology and Technology**, v. 56, p. 191-201, 2013.

CARVALHO, J. M. F. C.; LIMA, M. M. DE A.; AIRES, P. S. R.; VIDAL, M. S.; PIMENTEL, N. W. Embriogênese Somática. **EMBRAPA 152**, p. 36, 2006.

CASTELLANOS, H.; SÁNCHEZ-OLATE, M.; RÍOS D. Embriogénesis somática como alternativa potencial para la regeneración *in vitro* de género *Nothofagus.* p. 59-74. *In* Gutiérrez, B., O. Ortiz, y M.P. Molina (eds.) Clonación de raulí. Estado actual y perspectivas**. Informatic Cefor,** n. 16p. 54-59 Concepción, Chile. 2005.

CELESTINO, C., HERNÁNDEZ, I*.,* CARNEROS, E., LÓPEZ-VELA, D.,TORIBIO, M. La embriogénesis somática como elemento central de la biotecnología forestal. Investigación Agraria. **Sistemas y Recursos Forestales,** n.14, p. 345-357, 2005.

CHAWLA, H. S. Introduction to Plant Biotechnology. **Science Publishers**, 698p., 2009.

CHEDIER, L. M., KAPLAN, M. A. C. Chemical ecology of three species of Bromeliaceae. **National Museum Univ. Rio de Janeiro Brazil,** v. 3, p. 25–31, 1996.

CHRISTOFOLETTI, C. A.; ESCHER, J. P.; CORREIA, J. E.; MARINHO, J. F. U.; FONTANETTI, C. S. Sugarcane vinasse: Environmental implications of its use. **Waste Management,** v. 33, n. 12, p. 2752–2761, 2013.

CHUGH, S.; GUHA, S.; RAO, I. U. Micropropagation of orchids: A review on the potential of different explants. **Scientia Horticulturae,** v. 122, n. 507–520, 2009.

CHYUAM-YIH, N.; SALEH, N. M**.** *In vitro* propagation of Paphiopedilum orchid through formationof protocorm-like bodies. **Plant Cell Tissue Organ Culture**, v. 105, n. 2, p. 193–202, 2011.

Coca M.; Barrocal V.M.; Lucas S.; González-Benito G.; García-Cubero, M. T. Protein production in *Spirulina platensis* biomass using beet vinasse-supplemented culture media.food and bioproducts processing. **Food and Bioproducts Processing**, Short communication**.**p. 1-7,2014.

COLLINS F.S. The Green Algae of North America. **Tufts College**, v. 2, p 79-480, 1909.

Correa, S. M.; Lucia Atehortua, L. Lipid profile of *in vitro* oil produced through cell culture of *Jatropha curcas*. **Journal of AOAC International,** v. 95, n. 4, p1161, 2012.

CORREDOIRA, E.; BALLESTER, A.; VIEITEZ, A. M. Proliferation, maturation and germination of *Castanea sativa* Mill.somatic embryos originated from leaf explants. **Annals of Botany,** v. 92 p.129-136, 2003.

Costa, J. L.; Silva, A. L. L. Da; Bordignon-Junior, S. E.; Chagas Júnior, A. F.; Scheidt, G. N.; Veras Júnior, R. V. Organogenesis in zygotic embryos and hypocotyls of

physic nut (Jatropha curcas L.) under 6-benzylaminopurine levels**. Advances in Forestry Science**, v. 1, p. 71-73, 2014.

CRAGG, G. M. AND NEWMAN, D. J. Nature: a vital source of leads for anticancer drug development. **Phytochemical Review**. v. 8, p. 313–331, 2009.

CRAYN, D M.; WINTER K; SMITH, J. A. Multiple origins of crassulacean acid metabolism and the epiphytic habit in the Neotropical family Bromeliaceae. **Proceedings of the National Academy of Sciences of the United States of America. v.** 101, n. 10, p. 3703–3708, 2004.

DAGLA H. R. Plant Tissue Culture Historical Developments and Applied Aspects. **RESONANCE**, v.17, n. 8, p. 759-768, 2012.

DAVEY, M. R.; ANTHONY, P. P.; POWER, B. J.; LOWE, K. C. Plant protoplasts: status and biotechnological perspectives**. Biotechnology Advances,** v. 23, p. 131–171, 2005.

DECRUSE, S. W.; RENY, N.; SHYLAJAKUMARI, S.; KRISHNA, P. N.N *In vitro* propagation and field establishment of Eulophiacullenii (Wight) Bl., a critically endangered orchid of Western Ghats, India through culture of seeds and axenicseedling-derived rhizomes. **In Vitro Cellular and Developmental Biology – Plant,** v. 49, n. 5, p.520–528, (2013).

DOBRÁNSKI, J.; TEIXEIRA DA SILVA, J. A. Micropropagation of apple – a review. **Biotechnology Advances,** v. 28, n. 4, p. 462-488, 2010.

DORE J. R.; CYSEWSKI G. R. Haematococcus algae meal as a source of natural astaxanthin for aquaculture feeds. **Cyanotech Corporation**. Hawaii, 2003.

DORRIS, M. What is Plant Tissue Culture? (Cultivation). **Journal of the Bromeliad Society.** v. 60, n. 4, p. 172, 2010.

EMBRAPA, 2006. Embriogenese somática. **Embrapa Algodão**, v. 152, Campina Grande, São Paulo, Setembro 2006.

EVANS, D. E.; COLEMAN, J. O. D.; KEARNS A. Plant Cell Culture.Bios.Scientific Publishers**, Taylor & Francis Group**, London, p.1, 2003.

FONTOURA, T.; COSTA, A.; WENDT, T. Preliminary checklist of the Bromeliaceae of Rio de Janeiro state, Brazil. **Selbyana**, v. 12, p. 5–45, 1991.

FREIRE, W. J.; CORTEZ, L.A. Vinhaça de cana-de-açúcar**. Agropecuária Guaíba**, 2000.

GAMBORG O. L.; MILLER R.A.; OJIMA K. Nutrient requirements of suspension cultures of soybean root cells. **Experimental Cell Research**. v. 50, p. 151-158, 1968.

GEORGE, E. F.; DEBERGH, P. C. Micropropagation: uses and methods. In: George EF, Hall MA, De Klerk GJ, editors. Plant propagation by tissue culture. 3rd ed. Dordrecht, Netherlands**: Springer;** p. 29–64, 2008.

GEORGIEV, M. I. G.; WEBER, J.; MACIUK, A. Bioprocessing of plant cell cultures for mass production of targeted compounds. **Applied Microbiology and Biotechnology**, v. 83, n. 5, p. 809–823, 2009.

GIVNISH, T. J.; SYTSMA, K. J.; SMITH, J. F.; HAHN, W. J.; BENZING D. H.;. BURKHARDT. E. M. Molecular evolution and adaptive radiation in *Brocchinia* (Bromeliaceae: Pitcairnioideae) atop tepuis of the Guayana Shield. In: GIVNISH, T.J. & K.J. SYTSMA (eds.) **Molecular Evolution and Adaptive Radiation:** p. 259–311. Cambridge University Press, Cambridge, UK., 1997.

GIVNISH, T. J. Hallmarks of the New World .**Systematic Biology**. 54, p. 340 – 344, 2005.

GLÓRIA, N.A., ORLANDO FILHO, J. Aplicação de vinhaça como fertilizante. **Boletim Técnico do Planalsucar,** v. 5, p. 5–38, 1983.

GOMES-JUNIOR, R. A..;MOLDES, C. A.; DELITE, F. S.; POMPEU, G. B.; GRATÃO, P. L.; MAZZAFERA, P.; PETER J. L.; AZEVEDO, R. A. Antioxidant metabolism of coffee cell suspension cultures in response to cadmium. **Chemosphere,** v. 65, n. 8, p. 1330–1337, 2006.

GOULD, J. H.; SMITH, R. H.A Non-Destructive Assay for GUS in the Media of Plant Tissue Cultures. **Plant Molecular Biology Reporter**, v. 7, n. 3, 1989.

GRUNEWALD, K.; HIRSCHBERG, J.; HAGEN, C. Ketocarotenoid biosynthesis outside of plastids in the unicellular green alga *Haematococcus pluvialis*. **Journal of Biological Chemistry,** v. 276, p. 6023–6029, 2001.

GUERRA, M. P.; TORRES, A. C.; TEIXEIRA, J. B. Estabelecimento de um protocolo regenerativo para a micropropagação do abacaxizeiro. **Pesquisa Agropecuária Brasileira**, v.34 n.9, p.1557-1563, 1999.

HARBONE, J. B. The Flavonoids: Advances in Research Since 1980**. Chapman & Hall**, London, 1988.

HARNEY, P. M. Tissue culture propagation of some herbaceous horticultural plants. pp. 187-208 in D.T, Ellis B. E., Harney P. M., Kasha K. J.; Peterson R. L. Application of Plant Cell and Tissue Culture to Agriculture and Industry. **Plant Cell Culture Centre**, Univ. of Guelph, Ontario, Canada, 1982.

Hassuda, S. Impactos da infiltração da vinhaça de cana no Aqüífero Bauru. **Masther´s thesis,** 92p., IG/USP, São Paulo-SP, 1989.

HIDALGO, K. Vinasse in feed: Good for animal and environment. **Feed Technology**, v. 13, n. 5, p. 18-20, 2009.

JANSSENS, J.; SEPELIE, M.In vitro multiplication of *Blechnum* spp. and *Pelaea rotundifolia*(Forst.) Hook by homogenization. **Scientia Horticulturae**, v. 38, n. 1–2, p. 161–164, 1989.

JIANG, Z. P.; LI, Y. R.; WEI, G. P.; LIAO, Q.; SU, T.M.; MENG, Y.C.; ZHANG, H.Y.; LU, C.Y. Effect of long-term vinasse application on physico-chemical properties of sugarcane field soils. **Sugar Technology,** v. 14, p. 412–417, 2012.

JIN, M. Y.; PIAO, X. C.; XIU, J. R.; PARK, S .Y.; LIAN, M. L. Micropropagation using a bioreactor system and subsequent acclimatization of grape rootstock '5BB'**. Scientia Horticulturae,**v. 164, p. 35–4017, 2013.

CAO, J.; DUAN, X.; MCELROY, D.; WU, R. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. **Plant Cell Reports**, v. 11, p. 586 - 591, 1992.

KADLO A. Tests of Media with Vinasse for *Chlamydomonas reinhardii* for Possible Reduction in Vinasse Pollution. **Bioresource Technology,** v. 42, p. 1-5, 1992.

KANNAN, A.; UPRETI, R. K**.** Influence of distillery effluent on germination and growth of mung bean (Vigna radiata) seeds. **Journal of Hazardous Materials**, v. 153, p. 609–615, 2008.

KANO, K. Studies on the media for orchid seed germination. **Memoirs of the Factories Agriculture,** Kagawa University v. 20, p. 1-68, 1965.

KET, N. V.; HAHN, E. J.; PARK, S. Y.; CHAKRABARTY D.; PAEK, K. Y. Micropropagation of an endangered orchid *Anoectochilus formosanus.* **Biologia Plantarum,** v.48, n. 3. p: 339-344, 2004.

KIM, J.; KIM, J. H.; CHOI, K. H.; KIM, J. H.; SONG, Y. S.; CHA, J**.** Enhancement of the catalytic activity of a 27 kDa subtilisin-like enzyme from bacillus amyloliquefaciens CH51 by *in vitro* mutagenesis. **Journal of Agricultural and Food Chemistry.** v. 59, n. 16, p. 8675-8682, 2011.

KNUDSON, L. A new nutrient solution for the germination of orchid seeds**. American Orchids Society Bulletin**, v. 15, p. 214-217, 1946.

KRIKORIAN, A. D.; BERQUAM, D.L. Plant cell and tissue cultures: The role of Haberlandt. **Botanical Review**. v. 35, p. 59-88, 1969.

LAIME, E. M. O., FERNANDES, P. D., OLIVEIRA, D. C. S., FREIRE, E. A. Possibilidades tecnológicas para a destinação da vinhaça: uma revisão. **Revista Trópica Ciências Agrárias e Biológicas**. v. 5, p.16–29, 2011.

LEME, E. M. C. Nidularium – bromélias da Mata Atlântica. **Sextante**, Rio de Janeiro, 2000.

LLOYD, G.; MCCOWN, B. Commercially feasible micropropagation of montain laurel, Kalmia latifolia, by use of shoot tip culture. **International Plant Propagation Society**, v.30, p.421-327, 1981.

LOPES DA SILVA, A. L. Atividade antioxidante, teor de quercetina e desenvolvimento de meio de cultura a base de vinhaça para micropropagação de plantas. Thesis doctoral degree, disponible in **Federal University of Parana**, 76p., Curitiba , Brazil, 2013.

LORENZ, R. V. T.; CYSEWSKI, G. R. Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. **Trends of Biotechnology,** v. 18, p. 160–167, 2000.

LUTHER, H. E. An alphabetic list of Bromeliad binomials. 10 th ed . **Sarasota : Bromeliad Society International**, 2008.

LYRA, M. R. C. C.; ROLIM, M. M.; SILVA, J. A. A. Topossequência de solos fertirrigados com vinhaça: contribuição para a qualidade das águas do lençol freático. **Revista Brasileira de Engenharia Agrícola Ambiental**, v. 7, p. 523–532, 2003.

MACHADO, M. P. ; SILVA, A. L. L. DA ; BIASI, LUIZ ANTONIO ; DESCHAMPS, C. ; BESPALHOK FILHO, J. C. ; ZANETTE, F. Influence of calcium content of tissue on hyperhydricity and shoot-tip necrosis of in vitro regenerated shoots of Lavandula angustifolia Mill. **Brazilian Archives of Biology and Technology**, v. 57, p. 636-643, 2014.

MACHADO, M. P.; FILHO, E. R.; TEREZAN, A. P.; RIBEIRO, L. R.; MANTOVANI, M. S. Cytotoxicity, genotoxicity and anti-mutagenicity of hexane extracts of *Agaricus blaze*i determined *in vitro* by the comet assay and CHO/HGPRT gene mutation assay. **Toxicology in Vitro**, v.19, n. 4, p. 533-539, 2005.

MALIK, S.; CUSIDÓ, R. M.; MIRJALILI, M. H.; MOYANO, E.; PALAZÓN, J.; BONFILL, M**.** Production of the anticancer drug taxol in *Taxus baccata* suspension cultures: A review. **Process Biochemistry,** v. 46, n 1, p. 23–34, January 2011.

MARKER, R. E.; WAGNER, R. B.; ULSHAFER, P. R.; WITTBECKER, E .L.; GOLDSMITH, D. P. J.; ROUF, C. H. Isolation and structures of thirteen new steroidal sapogenins. New sources for known sapogenins. **Journal of American Chemical Society.** v. 65, p. 1199–1208, 1943.

MERKLE, S. A.; DEAN, J. F. D.. Forest Tree Biotechnology. **Current Opinion in Biotechnology,** v.11, p. 298-302, 2000.

MIGUEL, C., GONZALVES, S., TERESO, S., MARUM, L. AND OLIVEIRA, M. M. Somatic embryogenesis from 20 open-pollinated seed families of Portuguese plus trees of maritime pine. **Plant Cell Tissue and Organ Culture,** v.76, p.121-130, 2004.

MICHAEL, F. F. Conservation of rare and endangered plants using in vitro methods. **In Vitro Cellular and Developmental Biology.** v. 28, p. 1-4, 1992.

MIDDLETON, J. R. 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,** p. 619-652, 1994

MIRALPEIX, B.; RISCHER, H.; HAKKINEN, T.; RITALA, A.; SEPPANEN-LAAKSO, T.; OKSMAN-CALDENTEY, K. M.; CAPELL, T.; CHRISTOU, P. Metabolic Engineering of Plant Secondary Products: Which Way Forward? **Current Pharmaceutical Design**, v. 19, n. 31, p. 5622-5639, 2013.

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

NEWMAN, D.J.; CRAGG, G. M.; SNADER, K. M**.** Natural products as sources of new drugs over the period 1981–2002**. Journal of Natural Products**, v. 66, p. 1022–1037, 2003.

PACHECO, G.; GARCIA, R.; LUGATO, D.; VIANNA, M.;MANSUR, E. Plant regeneration, callus induction and establishment of cell suspension cultures of *Passiflora alata* Curtis. **Scientia Horticulturae,** v. 144, p. 42–47, 2012.

PALACIO, L.; CANTERO, J. J.; CUSIDÓ, R.; GOLENIOWSKI, M. Phenolic compound production by *Larrea divaricata* Cav. plant cell cultures and effect of precursor feeding. **Process Biochemistry,**v. 46, n. 1, p. 418–422, 2011.

PENNELL D. Micropropagation in Horticulture. **Grower Books, London**, 1987.

PEDROSA, E. M. R.; ROLIM, M. M.; ALBUQUERQUE, P. H. S.; CUNHA, A. C. Supressividade de nematóides em cana-de-açúcar por adição de vinhaça ao solo. **Revista Brasileira de Engenharia Agrícola Ambiental**, v. 9, p. 197–201, 2005.

PETERS, J. Revision of the genus *Fosterella* (Bromeliaceae). Dissertation in Natural Sciences, present in the **Faculty of Natural Sciences, University of Kassel**, 2009.

PUCHOOA, D. Comparison of Different Culture Media for the *In Vitro* Culture of *Dendrobium* (Orchidaceae). **International Journal of Agriculture & Biology,** v. 6, n. 5 p. 884–888, 2004.

QUIROZ-FIGUEROA, F. R., ROJAS-HERRERA, R., GALAZ-AVALOS, R. M., AND LOYOLA- VARGAS, V. M. Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. **Plant Cell Tissue and Organ Culture**. v.86, p.285–301, 2006.

RAI, M. K.; RAJWANT, K. K.; SINGH, R.; GANGOLA, M. P.; DHAWAN, A. K. Developing stress tolerant plants through in vitro selection—An overview of the recent progress, **Environmental and Experimental Botany**, v. 71, n. 1, p. 89–98, 2011.

RAFFAUF, R. F.; MENACHERY, M. D.; LE QUESNE, P. W. Diterpenoid and Flavonoid constituents of *Bromelia pinguin*. **The Journal of Organical Chemical.** v. 46, p. 1094–1098. 1981.

RAN, L. O.; DEOK-CHUN, Y.; HAE-JOON, C.; BYUNG-HOON, M. Efficient *in Vitro* Plant Regeneration from Hybrid Rhizomes of *Cymbidium sinense* Seeds. **Horticulture Environmental Biotechnology,** v. 52, n. 3, p.303-308. 2011.

REINERT J, BACKS D. Control of totipotency in plant cells growing *in vitro*. **Nature,** v. 1.p. 220:1340, 1968.

ROUT, G.R.; MOHAPATRA, A.; MOHAN J. Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects**. Biotechnology Advances**, v. 26, n. 6, p. 531–560, 2006.

SANTOS, J. D. DOS ; SILVA, A. L. L. DA ; 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.

SANTOS F. M.; MESQUITA J. Ultrastructural study of *Haematococcus lacustris* (Girod.)**.** Rostafinski (Volvocales): I. some aspects of carotenogenesis. **Cytologia,** v.49, p. 215–228, 1984.

SATHYANARAYANA B. N. Plant Tissue Culture: Practices and New Experimental Protocols. I. K. **International Publishing House,** p. 106, 2007.

SCHNEIDERS, D.; PESCADOR, D.; ROSETE, B.; RAITZ, M.; SUZUKI, R. M. Germinação, crescimento e desenvolvimento *in vitro* de orquídeas (*Cattleya* spp., Orchidaceae). **Revista Ceres**, Viçosa, v. 59, n. 2, p. 185- 191, 2012.

SCHULTE, K.; HORRES, R.; ZIZKA, G. Molecular phylogeny of Bromelioideae and its implications on biogeography and the evolution of CAM in the family (Poales, Bromeliaceae).**Senckenbergiana Biologica,** v. 85, p. 113 – 125, 2005.

SHEELAVANTMATH, S. S.; MURTHY, H.N.; PYATI, A.N.; ASHOK-KUMAR, H.G.; RAVISHANKAR, B.V. *In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam.) Schltr. through rhizome section culture. **Plant Cell, Tissue and Organ Culture,** v. 60, p. 151–154, 2000.

SILVA, A. L. L. da.; 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.

SILVA, A. L. L. Da.; OLIVEIRA, Y. de.; COSTA, J. L.; SCHEIDT, G. N.; CARVALHO, D. C.; SANTOS, J. D. dos; GUERRA, E. P. Pré-aclimatização e aclimatização em cultivo hidropônico de plantas micropropagadas de Eucalyptus saligna Sm**. Revista Acadêmica: Ciências Agrárias e Ambientais – PUCPR,** v. 9, p. 179-184, 2011.

SILVA, G. N., ORLANDO FILHO, J. Concentração da Composição Quimica dos diferentes Tipos de Vinhaça do Brasil. **Boletim Tecnico PLANALSUCAR**, 1981.

SOARES, J. D. R.; ARAUJO, A. G.; PASQUAL, M.; RODRIGUES F. A.; ASSIS, F. A Salts concentrations of medium Knudson C and gibberellic acid in vitro growth orchid plantlets/Concentracoes de sais do meio Knudson C e de acido giberelico no crescimento in vitro de plantulas de orquidea**. Ciência Rural,** v. 39, n. 3, p772, 2009.

SONG, J. Y.; NAYLOR-ADELBERG, J.; WHITE, S. A.; MANN, D. A.; ADELBERG, J. Establishing clones of *Veratrum californicum*, a native medicinal species, for micropropagation. **In Vitro Cellular Development Biology — Plant,** v. 50, p. 337–344, 2014.

TAKATA, R. H., SCHEUER, P. J. Isolation of caffeic and p-coumaric acids from pineapple steam. **Lloydia,** v.39, 409–411,1976.

TENÓRIO, Z.; CARVALHO, O. S.; SILVA, O. R. R .F.; MONTES, J. M. G. LÓPEZ, F. G**.** Estúdio de la actividad biológica de los suelos de los tabuleros costeros del NE de Brasil enmendados com residuos agrícolas: vinaza y torta de caña de azúcar**. Revista Brasileira de Engenharia Agrícola Ambiental,** v. 4, p. 70–74, 2000.

THE PLANT LIST. Online link: **http://www.theplantlist.org,** accessed in 14/12/2014.

THORPE, T. A. "History of plant tissue culture". **Molecular Biotechnoogyl.** v. 37, n. 2, p. 169–80., 2007.

VACIN, E.; WENT, F. Some pH changes in nutrient solution. **Botanical Gazette,** v. 13, p. 110 - 605, 1949.

VAHDATI, K.; BAYAT, S.; EBRAHIMZADEH, H.; JARITEH, M. AND MIRMASOUMI, M**.** Effect of exogenous ABA on somatic embryo maturation and germination in Persian walnut *(Juglans regia* L.). **Plant Cell Tissue and Organ Culture.** v. 93, p. 163-171, 2008.

VERDEIL, J-L.; ALEMANNO, L.; NIEMENAK, N.; TRANBARGER, T.J. Pluripotent versus totipotent plant stem cells: dependence versus autonomy? **Trends in Plant Science**; v. 52, p. 12-245, 2007.

VILLA, F.; PASQUAL, M.; SILVA, E. F. Micropropagação de híbridos de orquídea em meio knudson com adição de vitaminas do meio ms, benzilaminopurina e carvão ativado. **Semina: Ciências Agrárias**, Londrina, v. 35, n. 2, p. 683-694, 2014.

ZENG, F.; , ZHANG, X. ; ZHU, L.; TU, L.; GUO, X.; NIE, Y. Isolation and characterization of genes associated to cotton somatic embryogenesis by suppression subtractive hybridization and macro-array. **Plant Molecular Biology**, v.60, p.167–183, 2006.

WANG, X.; WU, R.; LIN, X.; BAI, Y.; SONG, C.; YU, X.; XU, C.;ZHAO, N.; DONG, Y.; LIU, B. Tissue culture-induced genetic and epigenetic alterations in rice pure-lines, F1 hybrids and polyploids. **BMC Plant Biology,** p.13-77, 2013.

WANG, Y. H. An efficient protocol for stimulating cell development in protoplast culture of Scaevola. **Plant Growth Regulators**, v. 64, p. 293–299, 2011.

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.

WILSON, S. A.; ROBERTS, S. C. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. **Plant Biotechnology Journal**, v. 10, n. 3, p. 249–268, 2012.

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.

WORKSHOP BIODIVERSITAS**.** Revisão da Lista da Flora Brasileira Ameaçada de Extinção. **http://www.biodiversitas.org.br.** 2005, accessed November, 2014.

YESILADA, E. Genotoxic activity of vinasse and its effect on fecundity and longevity of Drosophila melanogaster. **Bulletin of Environmental Contamination and Toxicology**, v. 63, p. 560–566, 1999.

CHAPTER II - DEVELOPMENT OF A PLANT CULTURE MEDIUM COMPOSED WITH VINASSE ORIGINATED FROM *HAEMATOCOCCUS PLUVIALIS* CULTURE

1. INTRODUCTION

The plant culture media are constituted of organic and inorganic substances that are carbohydrates, vitamins and macro and micronutrients; and can be supplemented with amino acids, plant growth regulators, complex mixtures and others substances depending of the culture objective. Nowadays, the plant tissue culture is used in several parts of the world in biofactories to produce clonal seedlings and bioactive compounds. However, it is necessary to develop alternatives to reduce the production costs. Industrial wastes are a cheap source of nutrients and organic compounds and can be used to formulate plant culture medium.

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

The photosynthetic freshwater unicellular green microalga *Haematococcus pluvialis* (Chlorophyceae) is one of the best sources of astaxanthin, because of its ability to accumulate a superior amount of astaxanthin (up to 4% of its dry weigh) compared to other sources as *Phaffia rhodozyma* (about 0.04% of its dry weight) (Boussiba *et al.,* 1999). The ketocarotenoid astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione), is a pigment commonly found in the marine environments, where it is produced by phytoplankton and it is conserved through the food chain in some marine animals, leading to the prominent color of the flesh and shell (Lorenz and Cysewski, 2000). This pigment is used as feed additive in aquaculture for the

production of salmon in confinement, whereas it is the pigment responsible by the coloration of the salmon. In the nature, the salmon acquire its coloration by its natural diet (i.e., the pigment is present in diet originated from food chain) and in confinement this pigment must be inserted in its diet as feed additive.

Algal filtrate and algal extracts are used as biostimulants for enhance the resistance and yield of plants. The vinasse originated from *Haematococcus pluvialis* culture (algal filtrate) is rich of mineral nutrients and it liberates several organic compounds into the culture medium, nevertheless, it was suggested that some these substances can be beneficial and improve plant growth and morphogenesis. Therefore, the aim of this study was to develop a plant culture medium using the vinasse used for *Haematococcus pluvialis* culture, to investigate possible beneficial effects of biocompounds presents in algal filtrate on micropropagation of *Nidularium procerum* (Bromeliaceae), quercetin accumulation and to evaluate the citotoxicity of this vinasse by bioassay with *Artemia salina*.

2. MATERIAL AND METHODS

2.1. *Haematococcus pluvialis* culture. Mixed must vinasse from sugarcane was used as 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. It was used 0.4 $g.L^{-1}$ inoculum for the initial culture (cells in vegetative growth). This inoculum was prepared using the same vinasse medium described above. The culture was performed with 0.5 vvm air, at 25o C and after 15 days of culture (cells in vegetative growth, no formation of cysts occurs) the algal biomass was separated and the vinasse resultant (algal filtrate) was used to compose the plant culture media.

2.2. Plant culture media composed with vinasse from *H. pluvialis* culture. In order to develop the vinasse culture media, the vinasse originated from *H. pluvialis* culture (15 days of culture) was analyzed for the sulfate, chloride, phosphate, nitrate,

ammonium, calcium, magnesium, sodium, potassium, manganese, zinc, copper 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, copper and manganese, were quantified by atomic absorption spectroscopy (Clesceri, 1998). We developed our vinasse media based on the quantity of ions contained in the KC medium (Knudson, 1946) (Table 1) and MS medium (Murashige and Skoog, 1962) (Table 2). These vinasse media formulations were performed with addition of reagents with analytical degree. In order to prepare media using vinasse above 3%, the vinasse was concentrated using a roto evaporator at 55°C.

2.3. *In vitro* establishment of *N. procerum.* The seed disinfection of *Nidularium procerum* 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 (Type I, Himedia). 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 7 $g.L^{-1}$ agar.

2.4. *In vitro* multiplication on media composed with vinasse from *H. pluvialis* culture. *N. procerum* isolated shoots (2 cm height) were removed from clusters propagated *in vitro* were used as explants. The KC-based vinasse media were: (VH1) It was composed with 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)

composed with 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: (MSVH 2.7%) It was composed with 2.7% vinasse supplemented with salts and vitamins of MS medium and modified its Ca and Mg sources for 390 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 330 mg.L-1 $MgSO_4.7H_2O$, (MSVH 5.4%) it was constituted with 5.4% vinasse supplemented with salts and vitamins of MS medium and modified its Ca and Mg sources for 342 $mg.L^{-}1$ $CaCl_2.2H_2O$ and 295 $mg.L^{-1}$ $MgSO_4.7H_2O$ and (MSVH 10.8%) It was composed with 10.8% vinasse supplemented with salts and vitamins of MS medium and modified its Ca and Mg sources for 245 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 225 $mg.L^{-1}$ $MgSO_4.7H_2O$. As control were tested the KC and MS media. All media were supplemented with 30 $g.L^{-1}$ sucrose, 2 µM NAA, 4 µM BAP and solidified with 7 g.L-1agar. All vinasse used for media formulation were originated from *H. pluvialis* culture at 15 days. The shoot height (cm), shoot number, shoot percentage, root number, root percentage, leaf number, fresh mass (mg) and chlorosis percentage were evaluated after 60 days of *in vitro* culture.

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

2.6. Acclimatization. Plantlets from all the rooting media were removed from flasks and their roots were washed with tap water (faucet). The acclimatization consisted on the culture of the plantlets in substrate PlantmaxTM HT in the greenhouse with intermittent nebulization during 15 days. At the end of 15 days of acclimatization, these plantlets were removed from intermittent nebulization, but they were kept

inside the greenhouse with manual irrigation. Survival percentage was evaluated after 30 days of culture *ex vitro*.

2.7. Methanol extract. Shoots cultured in all multiplication media (after 60 days of *in vitro* culture) had their fresh mass (1 g) macerated and extracted in 10 mL methanol (1:10 w/v) during 24h at room temperature under 80 rpm agitation (in the dark), after they were filtered with Whatman No*.* 1filterpaper. The extracts were stored at -20°C.

2.8. 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. All assays were performed in triplicate.

2.9. Bioassay with *Artemia salina.* The modified method of Solis et al., (1992) was employed in the toxicity analysis of vinasse. Brine Shrimps (*Artemia salina*) were hatched using 0.025 g cysts in a plastic bottle (250 mL), filled with artificial sea water (prepared using Aquasalt (41.4 $g.L^{-1}$), from Aqua OneTM and adjusted to pH 8.5 under constant aeration for 48h (at the light). After hatching, active nauplii free from cysts were harvested from 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. The vinasse dilutions were added in each well and the volume completed to 1 mL, totalizing 2 mL per well. It was tested three vinasse types: (1) crude vinasse, (2) treated vinasse (filtered and decanted) according the procedure of Santos et al. (2013) and (3)

vinasse originated from *H. pluvialis* culture (after 15 days of culture), both at the levels of 0, 0.5, 1.0, 2.0, 3.0, 6.0 and 9.0%. After 24h of treatment exposition, the number of dead and alive naupplii was counted and these data were used to estimate the LC50. All assays were performed in triplicate.

2.10. Total phenolic content of vinasses. They were tested three vinasse types: (1) crude vinasse, (2) treated vinasse and (3) vinasse originated from *H. pluvialis* culture. These vinasses were diluted to 3% and 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) were added, and after five minutes, 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 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 blank. Results were expressed in $\mu g.mL^{-1}$ of gallic acid equivalent.

2.11. Culture conditions and statistical analysis. All media had their pH adjusted to 5.8 and were autoclaved at 1.5kgf/cm^2, 121ºC for 2min. The cultures were kept at 25 ± 2ºC under white fluorescent light (28 µM m^{-2} s^{-1}) with a 16h photoperiod. In all experiments were used culture flasks with 5cm diameter and 8.5cm height and with 30mL culture medium. The experimental design was completely randomized with six replicates (per treatment) of five explants. The data were 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). The LC50 (lethal concentration, 50%) and the confidence intervals (95%) were calculated by the Trimmed Spearman-Karber method, using the software TSK, version 1.5 (USEPA, 1990).

3. RESULTS AND DISCUSSION

3.1. Vinasse composition originated from *H. pluvialis* culture. Chemical analysis of the vinasse originated from *H. pluvialis* culture demonstrated that the largest levels of mineral nutrients were SO_4 and Na, followed by Cl and Ca (Table 3 and 4). The high level of SO_4 is commonly found in mixed must vinasse – utilized in the present study; on the contrary the juice must vinasse has a low content SO_4. In a study to determine the composition of the vinasse originated from different types of most (considering the amount for 3% vinasse), Marques (2006) obtained 111.9, 192.0 and 22.8 $mg.L^{-1}$ SO_4 for mixed must vinasse, molasse must vinasse and juice most vinasse, respectively. In this study was obtained 172.1$mg.L^{-1}$ SO_4. This variation in SO_4 content in mixed must vinasse is due to its origin; whereas this vinasse is produced by the mixture of juice most vinasse with the molasse must vinasse. The juice most vinasse is the more suitable to development of plant culture medium due to low content SO_4, which can be used more concentrated than others types (i.e., molasse must vinasse and mixed must vinasse), allowing larger mineral nutrient availability and it aid to avoid the salt stress occurrence caused by the high level of SO_4.

The high content of Cl and Na in the treatments in comparison with the control was due to the supplementation of vinasse with 0.7% NaCl (Table 3 and 4), whereas *H. pluvialis* is a sea alga, it is necessary to simulate the medium similar to its environment. Another ion content that can be highlighted is the PO_4, Fe and NH_4, which were drastically low compared to other vinasses, suggesting a larger consume by alga. The first type vinasse used to formulate a plant culture medium was the juice most; therefore, it was used to compare with the results of this present study. However, in order to formulate a plant culture medium using vinasse is important to determine its mineral nutrient composition to perform the necessary ionic adjusts (i.e., supplementation with reagents).

Table 3. Comparative mineral salt content of different vinasse sources and different KC-based media used for *in vitro* culture of *Nidularium procerum.* All vinasse used for media formulation was originated from *H. pluvialis* culture at 15 days.

Ion (mg.L^{-1})	PV[1]	TV[2]	VH[3]	KC[4]	VH1[5]	VH5[6]
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]PV - 3% Crude vinasse, adapted from Santos *et al*., (2013).

[2]TV - 3% Treated vinasse (filtered and decanted).

[3] VH - 3% Vinasse used for culture of *Haematococcus pluvialis* after 15 days.

[4] KC - Knudson C medium.

[5] VH1 - 3% vinasse, 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} $MgSO_4.7H_2O$.

[6] VH5 - 3% vinasse, 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} $NaH_2PO_4 \cdot H_2O$.

Table 4. Comparative mineral salt content of different vinasse sources and different MS-based media used for *in vitro* culture of *Nidularium procerum.*

Ion ($mg.L^{-1}$)	CV[1]	TV[2]	VH[3]	MS[4]	MSVH[5] 2.7%	MSVH[6] 5.4%	MSVH[7] 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] CV - 2.7% Crude vinasse, Adapted from Santos et al., (2013).
[2] TV - 2.7% Treated vinasse (filtered and decanted), no available (this has the levels of MS salts, nevertheless the levels presents in vinasse are unknown.
[3] VH - 2.7% vinasse used for culture. of *Haematococcus pluvialis* after 15 days.
[4] MS - Murashige and Skoog (1962) culture medium (MS).
[5] MSVH 2.7% - 2.7% vinasse supplemented with salts and vitamins of MS medium and modified their Ca and Mg sources for 390 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 330 $mg.L^{-1}$ $MgSO_4.7H_2O$.
[6] MSVH 5.4% - 5.4% vinasse supplemented with salts and vitamins of MS medium and modified their Ca and Mg sources for 342 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 295 $mg.L^{-1}$ $MgSO_4.7H_2O$.
[7] MSVH 10.8% - 10.8% vinasse supplemented with salts and vitamins of MS medium and modified their Ca and Mg sources for 245 $mg.L^{-1}$ $CaCl_2.2H_2O$ and 225 $mg.L^{-1}$ $MgSO_4.7H_2O$.

3.2. *In vitro* multiplication. There were no statistical differences for all variables evaluated for the KC-based vinasse media on multiplication (Table 5). These media performed using the nutrients present in vinasse for KC-based medium were suitable, however, the shoot number varied from 5.6 to 7.4 shoots per explant, while for this same species cultivated on MS medium supplemented with the same

plant growth regulators, it was reached 14.9 shoots per explant, but in this another report the explants were evaluated after 120 days - the double-time (Lopes da Silva *et al*., 2012). Comparing the KC medium with MS medium, we can observe a great difference in amount and availability of ions, the KC medium does not have several ions presents in MS medium, that are: Na, Cl, Zn, Cu, Co, MoO_4 and B. The MS medium presents a highest salt concentration than KC medium for the NO_3, NH_4, Mn, K, Fe and Mg. The KC medium had the larger levels of Ca, SO_4 and PO_4 than MS medium (Table 3 and 4).

The VH1 could be used commercially and its main advantages will be the cost reduction (even compared to KC and VH5, due to use less reagents than these media) and it is possible that the $MgSO_4.7H_2O$ could be removed without influence the multiplication efficacy, this consideration was based in 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 1000mg L^{-1} $Ca(NO_3)_2 \cdot 4H_2O$ and 65mg.L^{-1} $MnSO_4 \cdot 4H_2O$ and it was obtained suitable results for micropropagation of *Oncidium leucochilum* (Orchidaceae) (Lopes da Silva, 2013). Moreover, the amount of Mg presents in vinasse from *H. pluvialis* culture is higher (0.89mg.L^{-1} more) than the treated vinasse (decanted and filtered) (Table 3). Other advantage of the commercial use of VH1 is the bureaucracy decrease, whereas this medium uses the $Ca(NO_3)_2 \cdot 4H_2O$ as nitrogen source which is a reagent not controlled by the Armed Forces, on the contrary of the NH_4NO_3 and KNO_3 which are controlled by varies safety agencies in several countries due to possibility of the explosive manufacture.

The *in vitro* multiplication on MS-based vinasse media presented statistical differences for the shoot number, leaf number and fresh mass. The best result for multiplication was found in MS (control) which reached 9.4 shoots per explant (Table 6), while the KC medium produced 7.4 shoots per explant (Table 5). There were no statistical differences for shoot height, shoot percentage, root number, root percentage and survival percentage evaluated for the MS-based vinasse media (Table 6).

Increasing vinasse concentration in the culture medium formulations increased sodium and sulfate content (Table 6) and decreased the shoot number, leaf number and fresh weight (Table 6). The results found in MS-based vinasse media suggests the occurrence of salt stress, whereas the MS presents already an elevated salinity

compared to KC medium and mixed with the vinasse concentrated, the levels of Na and SO_4 reached enough levels to decrease the multiplication efficacy (Table 5 and 6). The inhibition of the growth and yield is due the reduction in the osmotic potential caused by the excess of salts and/or to their toxicant effect. The Na and SO_4 can contribute significantly to salt stress occurrence, in spite of, high levels of Na inhibit the K uptake (Taiz and Zeiger, 2004), and it also reduces the efficiency of use of others nutrients (Rego *et al*., 2011), mainly by competition during the uptake process. Other fact that sustained the salt stress occurrence is that the salinity often reduces shoot growth more than root growth (Läuchli and Epstein, 1990), as it was observed in multiplication (Table 5 and 6).

The methodology employed for the development of MS-based vinasse media was different for the KC-based vinasse medium, which the media were supplemented with reagents and the dilution vinasse used at 3%. For the MS-based vinasse media formulations, there was also the supplementation with reagents, but the vinasse was concentrated in different levels (2.7, 5.4 and 10.8%), the aim was to identify effects of putative bioactive compounds presents in algal filtrate that be able to promote the growth of explants, and it was cogitated the possibility these biocompounds showed this putative effect only in high concentrations. However, the high levels of Na and SO_4 impeded the observation of possible effects due to salt stress occurrence (Table 6). However, other studies must be carried out eliminating these excessive ions to avoid the negative interference in the plant growth.

3.3. *In vitro* rooting. There were no statistical differences for all variables evaluated for the KC-based vinasse media on rooting (Table 5). The root number and root percentage varied from 1.4 to 1.5 roots per explant and 60 to 86.6% rooting (Table 5). However, none plant growth was tested and the shoot height used as explant was small (1 cm height), whereas the shoot length is often correlated to rooting percentage (Silva *et al*., 2006; Bisognin *et a*l., 2008). In another study with *N. procerum*, it was used explants with 2 cm height and the largest rooting rate and root number obtained was 96% and 4.1 root per explant, respectively, after 50 days of culture on MS medium (free of plant growth regulators) in 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, other condition that can

favor the rooting is the supplementation of vitamins in the culture medium. The KC medium supplemented with the MS vitamins favored the root development of an orchid hybrid named *Brassocattleya* Pastoral x *Laeliocattleya* Amber Glow (Fernandes da Silva *et al.,* 2009). The presence of activate charcoal was beneficial for rooting of *Orthophytum mucugense*, even so in culture medium with 1.11 µM IBA or free of plant growth regulators, it raised approximately 20% more rooting than its absence (Cerqueira Lima *et al.,* 2012).

The root formation in isolated shoots of *N. procerum* occurred in culture medium free of plant growth regulators, this observation is similar to the results found in *Dyckia macedoi* (Mercier and Kerbauy, 1993), *Dyckia agudensis* (Silva *et al*., 2007), *Dyckia maritima* (Silva et al., 2008), *Vriesea scalaris* (Silva *et al*., 2009) and *Orthophytum mucugense* (Cerqueira Lima *et al*., 2012).The IBA did not favor the rooting in *Vriesea scalaris* (Silva *et a*l., 2009) and *Orthophytum mucugense* (Cerqueira Lima et al., 2012); on the other hand, the rooting percentage can be increased with the addition lower auxins concentration, especially NAA, in the concentrations 1.1 µM and 0.1 $mg.L^{-1}$, respectively (Mercier and Kerbauy, 1992; 1993). However, if the rooting obtained without the use of plant growth regulators does not interfere in plant survival during acclimatization, it represents a cost reduction for micropropagation process and suggests that these species are good candidate to establish suitable protocols for *ex vitro* rooting due to easiness of rooting.

The survival rate varied from 80 to 86.6% (Table 5), this result probably is associated to explant length, whereas explants much small can dehydrate depending of osmotic potential of culture medium, if the culture medium was more hypertonic than the explant content, the water moves from explant to culture medium promoting the explant dehydration. Other fact that sustained this consideration is the explant length (2 cm height) used in multiplication in this study. In order to overcome this problem a prior phase for shoot elongation can aid to elevate the survival rate of explants obtaining larger explants.

During the rooting phase also occurred the lateral shoot formation in the explant base, the shoot rate and shoot number varied from 73.3 to 86.6% and 1.8 and 2.4 shoot per explant, respectively (Table 5). This morphogenetic process occurs due to presence of the endogenous levels of cytokinins supplemented in culture medium for multiplication phase (i.e., to promote the multiple shoot formation). These

explants originated from multiplication medium containing cytokinins, even so, transferred to another culture medium free of plant growth regulators, they continue to proliferate and this effect is named habituation. A prior phase of shoot elongation aids to consume these endogenous cytokinins and this favored the rooting, guided the energy used to produce shoots to produce roots. However, this process can be accelerated as observed in *Vriesea fosteriana*, which the addition of 0.54 µM NAA was necessary to stop these lateral shoots proliferation as well as to restabilize the apical growth of the shoots, in this way; the rooting was easily induced (Mercier and Kerbauy, 1992).

The *in vitro* rooting on MS-based vinasse media presented statistical differences for the rooting percentage, root number, shoot percentage, shoot number, shoot height, leaf number and fresh mass (Table 6). There were no statistical differences for survival rate among the treatments, and none explant has died. The MSVH 10.8% medium decreased the shoot percentage (80%) and the others media did not influence it. The media formulated with different vinasse dilutions, 2.7, 5.4 and 10.8% obtained a significant decrease for rooting percentage, root number, shoot number, shoot height, leaf number and fresh mass (Table 6). These results is involved with the salt stress occurrence, likely attributed to high levels of SO_4 and Na as discussed previously in this present study.

Different vinasse dilutions (decanted and filtered), 2.5, 5 and 10% were used for the *in vitro* culture of the *Oncidium leucochilum* orchid, and it was concluded that the 2.5% vinasse dilution was ideal for plant tissue culture and did not affect explant survival and favored the rooting rate, in spite of smaller vinasse dilutions (5 and 10%) possessed a significant phytotoxic effect on explants (Silva, *et al.,* 2013). The vinasse dilution is an important variable in formulating culture medium due its direct influence on the explant survival.

Table 5. *In vitro* multiplication and rooting of *Nidularium procerum* cultivated in KC-based vinasse media after 60 days. All vinasse used for media formulation was originated from *H. pluvialis* culture at 15 days.

***In vitro* multiplication**								
Medium	**H[1]**	**SN[2]**	**SP[3]**	**RN[4]**	**R[5]**	**LN[6]**	**FM[7]**	**S[8]**
KC[*]	2.5 a	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
***In vitro* rooting**								
Medium	**H[1]**	**RN[4]**	**SP[3]**	**SN[2]**	**R[5]**	**LN[6]**	**FM[7]**	**S[8]**
KC	3.2 a	1.4 a	86.6 a	2.4 a	60.0 a	16.2 a	55.1 a	86.6 a
VH1	3.1 a	1.5 a	73.3 a	1.8 a	80.0 a	13.2 a	36.3 a	86.6 a
VH5	2.8 a	1.5 a	80.0 a	1.9 a	86.6 a	12.0 a	34.3 a	80.0 a
CV (%)	18.3	14.6	15.7	16.4	24.7	12.1	28.2	16.8

[1]Shoot height (H cm).
[2]Shoot Number (SN).
[3]Shoot Percentage (SP %).
[4] Root Number (RN).
[5]Root Percentage (R %).
[6]Leaf Number (LN).
[7]Fresh Mass (FM mg).
[8]Survival Percentage (S %).
KC (Knudson C medium).
VH1 (3% vinasse, 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} $MgSO_4.7H_2O$).
VH5 (3% vinasse, 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} $NaH_2PO_4 \cdot H_2O$).
[*]Means within a column followed by the same letter do not differ from each other by the Duncan´s test at $P<0.05$.

Table 6. *In vitro* multiplication and rooting of *Nidularium procerum* cultivated in MS-based vinasse media after 60 days. All vinasse used for media formulation was originated from *H. pluvialis* culture at 15 days.

***In vitro* multiplication**								
Medium	**H[1]**	**SN[2]**	**SP[3]**	**RN[4]**	**R[5]**	**LN[6]**	**FM[7]**	**S[8]**
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
***In vitro* rooting**								
Medium	**H[1]**	**RN[4]**	**SP[3]**	**SN[2]**	**R[5]**	**LN[6]**	**FM[7]**	**S[8]**
MS	3.4 a*	4.0 a	100 a	4.5 a	92 a	26.5 a	109.4 a	96.0 a
MSVH 2.7%	2.3 b	1.8 b	100 a	2.6 b	64 b	16.5 b	54.0 b	83.2 a
MSVH 5.4%	2.6 b	0.9 b	100 a	2.3 b	60 b	15.7 b	39.9 b	96.0 a
MSVH 10.8%	2.3 b	1.4 b	80 b	2.1 b	50 b	13.8 b	36.6 b	83.2 a
CV(%)	16.6	16.6	11.7	16.2	31.8	14.1	45.2	19.7

[1]Shoot height.
[2]Shoot Number.
[3]Shoot Percentage.
[4] Root Number.
[5]Root Percentage.
[6]Leaf Number.
[7]Fresh Mass.
[8]Survival Percentage.
MS - Murashige and Skoog (1962) culture medium.
MSVH 2.7% - 2.7% vinasse supplemented with salts and vitamins of MS medium and modified their Ca and Mg sources for 390 mg.L^{-1} $CaCl_2.2H_2O$ and 330 mg.L^{-1} $MgSO_4.7H_2O$.
MSVH 5.4% - 5.4% vinasse supplemented with salts and vitamins of MS medium and modified their Ca and Mg sources for 342 mg.L^{-1} $CaCl_2.2H_2O$ and 295 mg.L^{-1} $MgSO_4.7H_2O$.
MSVH 10.8% - 10.8% vinasse supplemented with salts and vitamins of MS medium and modified their Ca and Mg sources for 245 mg.L^{-1} $CaCl_2.2H_2O$ and 225 mg.L^{-1} $MgSO_4.7H_2O$.
*Means within a column followed by the same letter do not differ from each other by the Duncan´s test at $P<0.05$**.**

3.4. Acclimatization. The micropropagated plants survival rate obtained in this study varied from 78.3 to 100% (Fig. 1A-B). These results were suitable, whereas above 80% is already a convenient survival rate, considering the difficult to transfer plants from *in vitro* conditions to *ex vitro* conditions, what implies in the

necessity of gradual physiological changes, mainly to avoid the excessive water loss from tissues to environment by transpiration. These plant gradual changes must promote the epicuticular wax and functional stomata formation (Sutter and Langhans, 1982), both is associated to avoid plant loss water and consequently increase the plant survival rate.

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

Micropropagated plants originated from MS-based vinasse media reached from 78.3 to 95.7% survival rate (Fig. 1B). The best results were found in MS (control) and MSVH 2.7%, obtaining a survival rate of 95.8 and 94.7%, respectively. Different salt composition of culture medium and supplementation with different levels of microorganisms filtrates 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) and in microshoots of *Lavandula angustifolia* cultivated on LS medium supplemented with different levels of fermented extract of *Fusarium moniliforme*, which presented 66.6 to 91.6% survival rate (Lopes da Silva *et al*., 2013). These results can be associated to the different nutritional status of the plants cultivated in different culture media, whereas different culture media have different nutrient sources and amounts, this can influence also in the available and uptake of the nutrients. Moreover, in this study, these results can also be associated to the salt stress promoted in the media containing largest vinasse concentrations, increasing vinasse concentration in the culture medium formulations increased sodium, sulfate and potassium content and decreased survival rate (Fig. 1B).

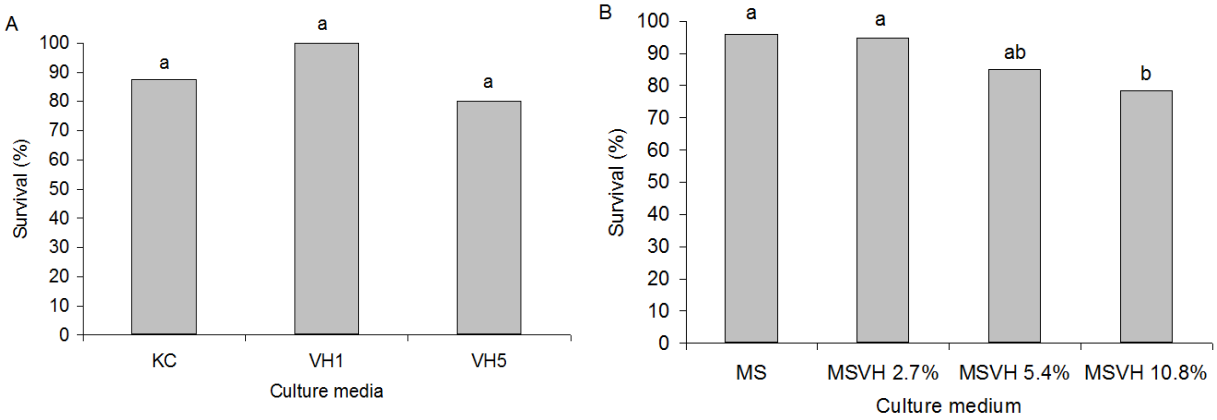

Fig. 1. Acclimatization of micropropagated plants of *Nidularium procerum* originated from different vinasse (used to culture *Haematococcus pluvialis*) media after 30 days of *ex vitro* culture, (A) KC-based vinasse media and (B) MS-based vinasse media. The formulation of vinasse media are detailed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

3.5. Quercetin content. Plantlets cultured on KC medium presented higher quercetin content than MS medium, being 54.5mg/100g^{-1} fresh weight compared to 47.3 mg/100gg^{-1} fresh weight, respectively. The larger amount of SO_4 and PO_4 in KC medium can be associated with the highest quercetin production, when it is compared with the MS composition (Table 1 and 2). The highest amount of SO_4 and PO_4 can influence a larger production of the compound 4-coumaroyl-CoA ($C_{30}H_{42}N_7O_{18}P_3S$), whereas it has P and S and this compound is an intermediate in phenylpropanoids pathway, where it occurs the quercetin biosynthesis (Taiz and Zeiger 2004).

The presence of algal filtrate had increased the quercetin content. The VH1 and VH5 media, presented 57.2 and 57.1 mg/100 g^{-1} fresh weight, respectively. The results observed in VH1 and VH5 media compared to KC medium (54.5 mg/100 g^{-1} fresh weight) represents an increment of approximately 4.7% of 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 weight, respectively. These results obtained in plantlets cultivated in MSVH media represent an increment of approximately 59.6% of quercetin production when it is compared to MS medium (Fig. 2B). The low quercetin production obtained by VH media, when compared to MSVH media can be associated with a low nutrients level and absence of some nutrients, such as Co, MoO_4 and B.

Calli obtained from young leaf explants of *Pluchea lanceolata* (Asteraceae) were exogenous supplemented with cinnamic acid (5 mg/100ml) or phenylalanine (50 mg/100ml), these calli had an increase in its yield for total quercetin about 7 to 8 fold and only 1 to 2 fold in comparison to control, respectively. In this study, they concluded that cinnamic acid is more effective precursor than phenylalanine (Arya and Patni, 2013). However, it is possible that the algal filtrate have in its composition, phenylalanine and/or cinnamic acid, this could explain the highest quercetin production promoted by algal filtrate, due to the fact that these compounds are precursors of quercetin biosynthesis.

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

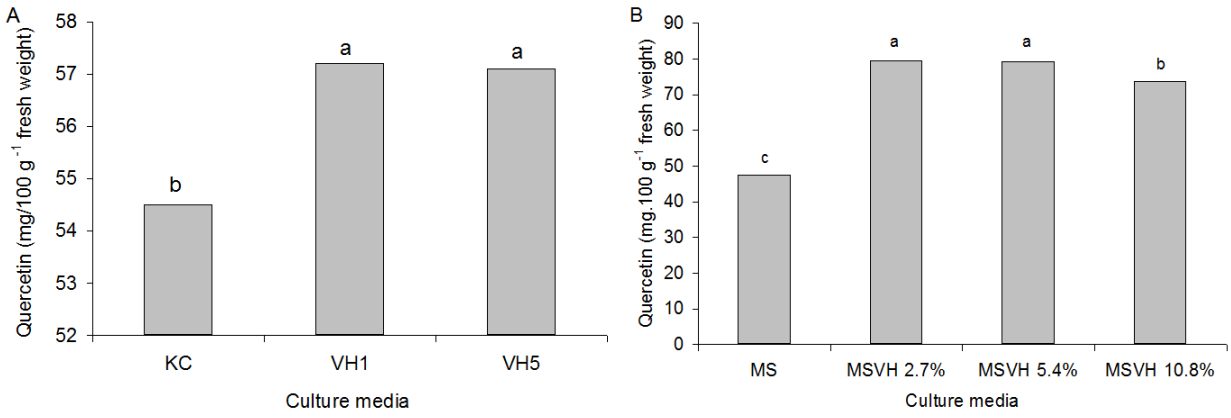

Fig. 2. Quercetin content of methanolic extracts of fresh plants of *Nidularium procerum* multiplicated on different vinasse (used to culture *Haematococcus pluvialis*) media after 60 days of *in vitro* culture, (A) KC-based vinasse media and (B) MS-based vinasse media. The formulation of vinasse media are detailed in material and methods. Means followed by the same letter do not differ from each other by the Duncan´s test at P<0.05.

3.6. Citotoxicity and total phenolic content of vinasses. The crude vinasse presented the highest toxicity with a LC50 of 0.68 (0.42-1.11%), followed by treated vinasse (decanted and filtered) with a LC50 of 2.06 (1.68-2.54%). This process used to treat the vinasse, using decantation and filtration according Santos

et al. (2013), allowed a toxicity reduction, which resulted in a difference of 1.38% compared to crude vinasse. This treated vinasse was also evaluated, because it was used to formulate the first plant culture medium performed with vinasse, however, this plant culture medium promoted some region of necrosis in the explants (37.5%) (Lopes da Silva, 2013). This phytotoxicity is not desired in a plant culture tissue and can increase the browning rate in explants. It is possible that this toxicity can be caused mainly by the presence of phenolic compounds in vinasse. However, in vinasse these compounds are produced by catabolism of lignin originated from sugarcane biomass (Parnaudeau *et al*. 2008). Therefore, an early culture of an alga could decrease the vinasse toxicity, whereas algae need a great amount of carbon and phenolic compounds are a carbon source that the algae can also use. However, the vinasse originated from *H. pluvialis* did not show toxicity until 9%, the largest level tested in this study. Moreover, the vinasse level used to perform the best plant culture medium was 3%.

The results of total phenolic content found for crude vinasse, treated vinasse and vinasse used to *H. pluvialis* culture presented 11.3, 7.8 and 0.32 $\mu g.mL^{-1}$, respectively. This represent a decrease of 31 and 97.2% total phenolic in treated vinasse and vinasse used to *H. pluvialis*, respectively when compared to crude vinasse. These results had supported the suggestion that phenolic compounds are the main substances involved in vinasse toxicity. The early culture of alga before formulate plant culture medium is an excellent form to remove the vinasse toxicity.

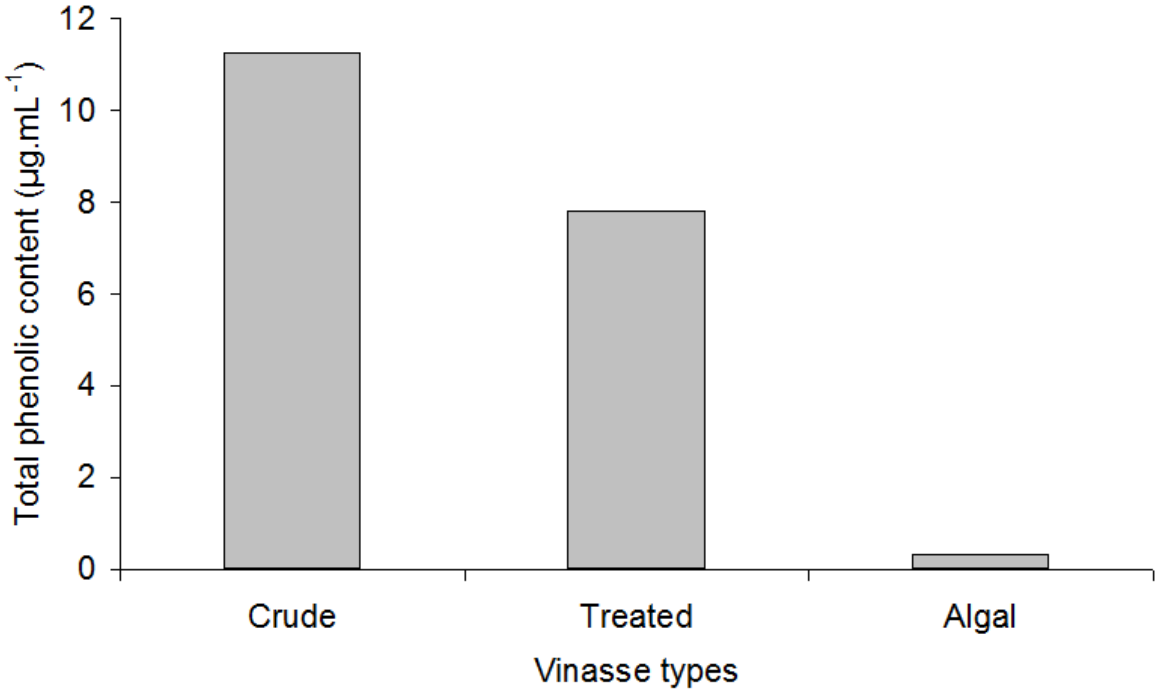

Fig. 3. Total phenolic content in crude vinasse, treated vinasse (decanted and filtered) and vinasse originated from *H. pluvialis* culture.

4. COST ANALYSIS OF KC FORMULATION

The KC formulations using vinasse presented a cost reduction of 20.3% and 6.43% compared with KC medium (Knudson, 1946) for VH1 and VH5, respectively (Table 7). The component more expansive of these formulations was the nitrogen source - Ca(NO3)2.4H2O, representing 66% of KC medium value. For the KC formulations using vinasse this nitrogen source represents 98.1% and 81.4% of cost for VH1 and VH5, respectively. The use of an alternative source of nitrogen could reduce drastically the cost of these vinasse culture media.

Table 7. Costs involved in the production of culture media for bromeliads plants: KC (1946), VH1 and VH5.

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 VH1 medium (R$)	Cost of VH5 medium (R$)
$Ca(NO_3)_2.4H_2O$	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	11,00	11,00
$(NH_4)_2SO_4$	0.5000	1000	20,00	10,00	-	-
$MnSO_4.4H_2O$	0.0075	500	37,00	0,55	0,42	0,42
$NaH_2PO_4.H_2O$	0.2400	500	24,00	-	-	11,52
3% Vinasse[1]	-	-	-	-	-	-
Total cost of culture medium (R$)				83,30	66,42	77.94
Cost reduction in percentage				-	20.3%	6.43%

[1]The cost of the vinasse transport was inconsiderate due to differences among the distances of the ethanol industries.

5. CONCLUSIONS

The vinasse originated from *H. pluvialis* culture can be used to formulate plant tissue culture medium using 3% dilution, its mineral nutrients can support *in vitro* growth of the plants, but some nutrients must be supplemented. An efficient protocol for micropropagation was developed for *N. procerum*. The micropropagated plants were suitable transferred to the field (acclimatized). This culture medium represents a reuse of this waste water and a rational alternative to vinasse disposal and adds value to what is currently considered an undesired residue. Moreover, this process can reduce the production´s cost of clonal seedlings and/or bioactive compounds in biofactories. It was not observed a biostimulant effect of the algal filtrate on the morphogenesis; on the other hand, this algal filtrate represented an increment of approximately 59.6% of quercetin production when compared with the control. Furthermore, the culture of *H. pluvialis* in the vinasse decreases the cytotoxicity and phenolic compounds content, avoiding the explant tissue necrosis.

6. SUGGESTIONS FOR FUTURE RESEARCH

- Another algae species could be explored;
- This vinasse algal can be used as an elicitor to enhance the accumulation of secondary metabolites, but its influence in other compounds classes must evaluated;
- Algal biomass extraction using different technologies must be performed and these extracts could be tested in different *in vitro* morphogenesis processes.

REFERENCES

AHMAD, F.; KHAN, A. U.; YASAR, A. The potential of *Chlorella vulgaris* for wastewater treatment and biodiesel production. **Pakistan Journal of Botany**.,v. 45, n. S1, p. 461-465, 2013.

ARYA, D., PATNI, V. Comparative analysis of total flavonoids and quercetin content *in vivo* and *in vitro* and enhancement of quercetin via precursor feeding in *Pluchea lanceolata* Oliver & Hiern. **International Journal of Pharmacy and Pharmaceutical Sciences**, v. 5, n. 3, p. 617-62, 2013.

BOUSSIBA, S.; W. BING, J. P.; YUAN.; CHEN, F. Changes in pigment profiles of *Haematococcus pluvialis* during exposure to environmental stresses. **Biotechnology Letters**. v. 21, p. 601-604, 1999.

CLESCERI, L. S.; GREENBERG, A. E.; EATOM, A. D. Standard Methods for the Examination of Water and Wastewater. **American Journal of Public Health,** Washington, 1998.

EMBRAPA. Núcleo Tecnológico para Informática. **EMBRAPA SOC–Software Científico**. Campinas, 1990.

FERNANDES DA SILVA, E.; VILLA, F.; PASQUAL, M. Meio de cultura Knudson modificado utilizado no cultivo in vitro de um híbrido de orquídea. **Scientia Agraria**, v.10, n.4, p.267-274, 2009.

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

LÄUCHLI, A.; EPSTEIN, E. Plant responses to saline and sodic conditions. In K.K. Tanji (ed). **Agricultural salinity assessment and management**.**manuals and reports on engineering practice** v. 71. p 113-137, 1990.

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, 2007.

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.

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.

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**.v. 45, n. 6, p: 2057-2064, 2013.

LOPES DA SILVA, A. L. Atividade antioxidante, teor de quercetina e desenvolvimento de meio de cultura a base de vinhaça para micropropagação de plantas. 75f. Tese (Doutorado em Engenharia de Bioprocessos e Biotecnologia) – Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, **Universidade Federal do Paraná**, Curitiba, 2013.

LORENZ, R. T.; CYSEWSKI, G. R. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. Trends in Biotechnology. v. 18, p. 160–167, 2000.

MARQUES, M. O. Aspectos técnicos e legais da produção, transporte e aplicação de vinhaça. In: SEGATO, S. V.; PINTO, A. S.; JENDIROBA, E.; NÓBREGA, J. C. M. (Org.). **Atualização em produção de cana-de-açúcar**. Piracicaba: Livro Ceres, p. 369-375, 2006.

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.; F. SKOOG. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum**, 15: 473-497, 1962.

MUKLESTAD, S.; HAUG, A. Production of carbohydrates by the marine diatom *Chaetoceros affinis*var. *willei* (Gran) Hustedt. I. Effect of the concentration of nutrients in the culture medium. **Journal of Experimental Marine Biology and Ecology**. v. 9, n. 2, p. 125-136, 1972.

OLIVEIRA, Y.; PINTO, F.; SILVA, A. L. L.; GUEDES, I.; BIASI, L. A.; QUOIRIN, M. An efficient protocol for micropropagation of *Melaleuca alternifolia* Cheel. ***In Vitro* Cellular and 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.

PARNAUDEAU, V.; CONDOM, N.; OLIVER, R.; CAZEVIEILLE, P.; RECOUS, S. Vinasse organic matter quality and mineralization potential, as influenced by raw material, fermentation and concentration processes. **Bioresource technology**, v. 99, n. 6, p. 1553-1562, 2008.

REGO, S. S.; FERREIRA, M. M.; NOGUEIRA, A. C.; GROSSI, F.; SOUSA, R. K.; BRONDANI, G. E.; ARAÚJO, M. A.; SILVA, A. L. L. Water and Salt Stress in the Germination of *Anadenanthera colubrina* (Veloso) Brenan Seeds. **Journal of Biotechnology and Biodiversity**, v. 2, n. 4, p. 37-42, 2011.

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 Environmental Manage**. v. 114, p. 8-12, 2013.

SAYWELL, L. G.; CUNNINGHAM, B. B. Determination of Iron: Colorimetric o-Phenanthroline Method. **Industrial and Engineering Chemistry Research.** *Anal. Ed.*, 9: 67-69, 1937.

SILVA, A. L. L. da; COSTA, J. L.; GOLLO, A. L.; SANTOS, J. D. dos ; FORNECK, H. R.; BIASI, L. A.; Soccol, V. T.; CARVALHO, J. C.; SOCCOL, C. R. Development of a vinasse culture medium for plant tissue culture. **Pakistan Journal of Botany.** v. 46, p. 2195-2202, 2014.

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

Solis, P. N.; Wright, C. W.; Anderson, M. M.; Gupta, M. P.; Phillipson J. D. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). **Planta Medica.** v. 59, p. 250-252, 1993.

SUTTER, E.; LANGHANS, R. W. Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot-tip culture. **Canadian Journal of Botany**, v. 60, p. 2896-2902, 1982.

TAIZ, L.; ZEIGER, E. Fisiologia Vegetal. Porto Alegre, Artmed; USEPA, 1990.Trimmed spearman-karber (TSK) program version 1.5 ecological monitoring research division. **Environmental Monitoring Systems Laboratory**, **United States Environmental Protection Ageny**. Cincinnati, O.H., 2004.