ISSN 1983 1501

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

CHARACTERIZATION AND VIABILITY OF *PIPER HISPIDINERVIUM* (PIPERACEAE) CALLUS FOR CELL SUBMERSE CULTURE

Bruna Grosch Schroeder[1], Clayton Debiasi[2], Rita de Cassia Siqueira Curto Valle[3], Rosete Pescador[4] e Lorena Benathar Ballod Tavares[5]

Abstract: *Piper hispidinervium* C.DC. *is a plant from the Amazon region of economic interest due its high safrole content. The submerse cultivation of its cells has shown promising results for the biochemical engineering. However, the inoculum production represented by the cell mass called as callus is still not fully understood. The knowledge on this material before the establishment of the cultivation in reactor is vital for the success of the process. Therefore, callus established in vitro have been physically characterized and analyzed in relation to the cellular viability for 80 days. In this study the experimental design was completely randomized, consisting of ten repetitions (callus/sample) from each collection (10th, 15th, 35th, 45th, 60th, 75th and 80th days of culture).The viability, determined by reaction with 2,3,5-triphenyltetrazolium chloride, decreased during cultivation and the green coloration, characteristic of recent callus, acquired yellowish coloration until the attainment of brown color, which indicate oxidized cells. The friability presented maximum index in 60 days, while the cell mass present in callus and expressed as grams of fresh mass, obtained the highest amount at the 35th days of culture.*

Keywords: Long pepper. Cell mass. Safrole, TTC.

1 Introduction

Safrole, or 4-allyl-1,2-methylenedioxybenzene, is a phenolic ether from the anyl propanoid group with molecular formula $C_{10}H_{10}O_2$, boiling point around 232 °C and solidification point at 11 °C (MARCONDES et al. 2005). It deals about a natural compound of wide distribution within the vegetable kingdom. However, it is only found in commercial amounts in some species from family Piperaceae, such as leaves of the long pepper (*Piper hispidinervium* C.DC.), a native plant from the Amazon region (PESCADOR et al., 2000). Its economic importance is a result of its capacity of being precursory of a number of compounds, especially catecholic drugs such as dopamine and isoproterenol, biodegradable insecticides (natural piretroids) that fix perfume and endolic auxins (ROSA; REBELO; NASCIMENTO, 2003). This plant species presents average yield of 3.5% of essential oils with safrole content ranging from 88% and 95% (Valle, 2003).

The application of biotechnology for the production of biologically-active natural compounds using vegetal, microbial and animal material including marine organisms and insects has been widely discussed, above all in the three last decades (CALIXTO, 2005). The importance of these plants for industry reveals nature dependence, especially in the pharmaceutics area. Another problem found is the forest extrativism that leads to the disappearance of vegetal species. An example is the prohibition for the cut of the Brazilian cinnamom (*Ocotea odorifera* (Vellozo) Rohwer), main safrole producer extracted in Brazil until the decade of 1990 (ARAÚJO; PESCADOR; REBELO, 1997). In order to overcome this dependence, chemical compounds production processes have been exhaustively studied with the objective of obtaining innovative technologies. One of them is the conduction of biotechnological

[1] E-mail: brunagrosch@gmail.com

[2] E-mail: c.debiasi@sbwbrasil.com.br

[3] E-mail: rscurtovalle@gmail.com

[4] E-mail: rosete@cca.ufsc.br

[5] E-mail: lorena@furb.br
FURB - Universidade Regional de Blumenau, Programa de Pós-Graduação em Engenharia Ambiental. CAMPUS II - Complexo Tecnológico. Rua São Paulo, 3250 – Itoupava Seca. CEP 89.030-000 - BLUMENAU - SC, Brasil

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

processes aimed at the production of secondary compounds from cells cultivated in bioreactors. Several works on the culture of *P. hispidinervium* cells in reactors have been conducted by Valle (2003) and Valle et al. (2006), which obtained alternative process with great safrole production potential.

The culture of cells in bioreactors is based on the use of cellular groups called as callus, which are obtained from *in vitro* cultures. This material represents the inoculum of vegetal cells culture processes. Defining it in terms of cellular standards that will later be multiplied in bioreactors is vital to assure the success of the safrole production process. The knowledge on the behavior of *P. hispidinervium* callus cells in relation to its viability along the culture, as well as on the relation between viability and its characterization are not yet fully cleared.

In vitro viability tests are considered as important tools for the characterization and quantification of cells (PORTER; NGUYEN; BURKE, 1994), as those based on the use of cell dyes such as Evans blue (SWAIN; DE, 1994), Tripan blue (HOU; LIN, 1996) or Neutral red (SWAIN; DE, 1994), and those based on enzymatic activity such as 2,3,5- triphenyltetrazolium chloride test (TTC) (RICH et al., 2001; STEPONKUS; LANPHEAR, 1967). In this context, this work evaluated the physical characteristics of *P. hispidinervium* callus and established standards capable of defining the material as inoculum for bioprocesses aimed at the safrole production from *P. hispidinervium*. This experiment is one step towards the use of cells with a view to future production of safrole in vitro.

2 Materials and methods

2.1 Vegetal material

Seeds collected from the Experimental Station of Itajaí/EPAGRI-SC (material voucher) were used for the attainment of microplants germinated *in vitro*, which provided explants. The seeds were disinfected by immersion in alcohol solution at 70% (v/v) for 1.5 min., followed by a commercial dilute solution of sodium hypochlorite in water at 40% (v/v) for 10 min., under agitation at 130 rpm in a shaker. Seeds were then rinsed with sterile water four times and placed in Petri dishes containing MS medium (MURASHIGE; SKOOG, 1962) added of 30 $g.L^{-1}$ of sucrose and 7 $g.L^{-1}$ of agar. The pH was adjusted to 5.8 before sterilization. Microplant production took place on a growth shelf at 25 ± 2 °C with fluorescent bulbs (50 $\mu M.m^2.s$) with periods of 16 hours of light and 8 hours of dark.

2.2 Callus production (inoculum)

For the callus induction, foliar segments (explants) removed of apices and leafstalk before inoculation were used. The callus induction was performed in MS37 medium (MS0 + 22.62 μM of 2,4-dichlorophenoxyacetic acid (2,4 D), 45.25 μM of 6-benzilaminopurine (BAP)) for 80 days. Callus formation took place on a growth shelf at 25 ± 2 °C with fluorescent bulbs (50 $\mu M.m^2.s$) with periods of 16 hours of light and 8 hours of dark.

2.3 Callus physical characterization

Mass, oxidation intensity, callus consistence and coloration were evaluated at the 10th day at the beginning of the callus formation and then at the 15th, 35th, 45th, 60th, 75th and 80th days of culture. The callus mass was determined in analytical scales and expressed as grams of fresh material. For variable oxidation intensity, a three-level scale based on Flores, Nicoloso and Vasconcellos (2006) was used, where 1 = absence of oxidation; 2 = callus partially oxidized and 3 = callus fully oxidized. The callus coloration was visually determined employing the color scale of Pantone® Textile Color Specifier (1999). The friability, which is indicative of foliar dedifferentiation in cellular mass, was manually measured through the consistency and desegregation of callus using a difference test with a five-level structured scale as follows: 1 = not friable; 2 = very low friability; 3 = low friability; 4 = moderately friable and 5 = friable.

2.4 Viability of the callus cells

Collection, preparation and analysis of callus cell samples followed methodology described by Franklin (1994) modified for *P. hispidinervium*. Samples weighting from 75 to 100 mg (fresh mass) collected at the 10th day of culture were disposed in test tubes and submitted to the 2,3,5- triphenyltetrazolium chloride test (TTC) (3.0 mL) and incubated at 28 °C for 12 hours in the absence of light and

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

added of 7.0 mL of 95% ethanolic solution. Following, the colorful complex was extracted in water bath at 100 ^{o}C during a period of four minutes. The samples were homogenized and centrifuged for 10 minutes at 1000 g for solids precipitation. The supernatant was submitted to reading in spectrophotometer at light range equivalent to 556 nm, and the viability curve was constructed with values expressed as absorbance (nm) per gram of fresh mass.

2.5 Statistical analysis

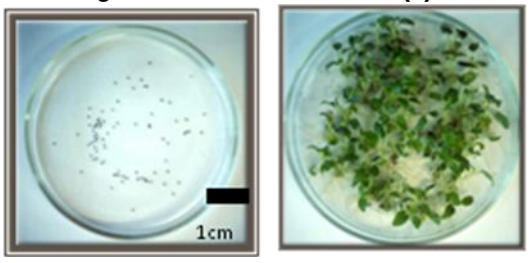
The experimental design was completely randomized, consisting of ten repetitions (callus/sample) from each collection (10th , 15th , 35th , 45th , 60th , 75th and 80th days of culture).

3 Results and discussion

3.1 P.hispidinervium callus induction

Inoculated seeds presented germination rate of 100% at the 15th day of culture (Figure 1). These results corroborate work conducted by Pescador et al. (2000), who studied the germination of *P. hispidinervium* seeds and obtained similar results at the same culture conditions.

Figure 1 – *P. hispidinervium* seeds inoculated in MS medium with 2, 4 D and BAP (a); New plants of *P.hispidinervium* from seeds germinated in MS medium (b)



[a] **[b]**

Authors (2013)

After segmentation, the explants started the dedifferentiation process with the formation of the cellular mass (callus) at the 10th day of culture. This data reflects the earliness of *P.hispidinervium* in relation to the callus production when compared to species such as *Caryocar brasiliense* Camb., which callus formation occurs at the 20th day after inoculation (LANDA et al., 2000). In many *in vitro* systems, modifications in the nutrient contents and growth regulators have resulted in callus induction (PREECE et al., 1995). In some species or even specific organs, it has been observed that for reaching an *in vitro* callus production standard, it is necessary adding synthetic or natural compounds to the culture medium such as coconut water, fruit juices, malt or yeast extract or hydrolyzed protein (TORRES; CALDAS; BUSO, 1998).

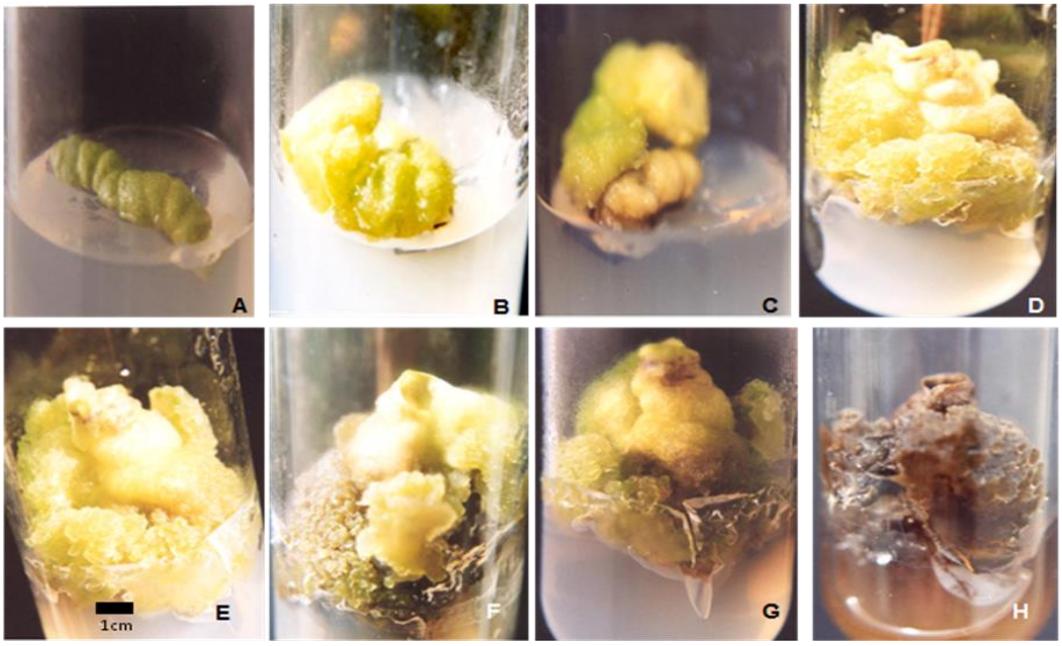
3.2 Characterization of callus for inoculum production

The callus obtained presented color and oxidation intensity alterations (Figure 2 and 3, respectively) along time. At the 10th and 15th days of culture, the predominant color was light green (Figure 2A and 2B - Pantone® 15-6316) and with no oxidation. At the 35th and 45th days of culture, the color remained green, however, with a lighter tonality, being cream-colored from this point on (Figure 2C and 2D - Pantone® 14-7316). In this period, the callus presented a more friable aspect in relation to previous evaluations. However, analyses performed at the 60th day showed the maximum callus production with the following features: fully yellowish (Pantone® 13-6556), friable aspect (Figures 2E and 2F) and easy desegregation. At the last days of evaluation, 75th and 80th days of culture respectively, callus presented brown coloration, very low friability and high oxidation index – necrosis (Figures 2G, 2H and 3).

According to Rodrigues et al. (2009) and Tcacenco (1995), the callus coloration is a very important characteristic for plant regeneration, indicating that callus with lighter coloration and friable aspect are those fit for plants regenerative induction, what also indicates high cellular viability. Low friability rate and high oxidation index of callus cells at the end of the culture period (80th day of culture) may be attributed to the depletion of nutrients and growth regulators available or to the loss of tissues that respond to stimuli from the culture medium. According to Flores, Nicoloso and Vasconcellos (2006), oxidation may be influenced by the type of explants, genotype, medium components and *in vitro* culture conditions.

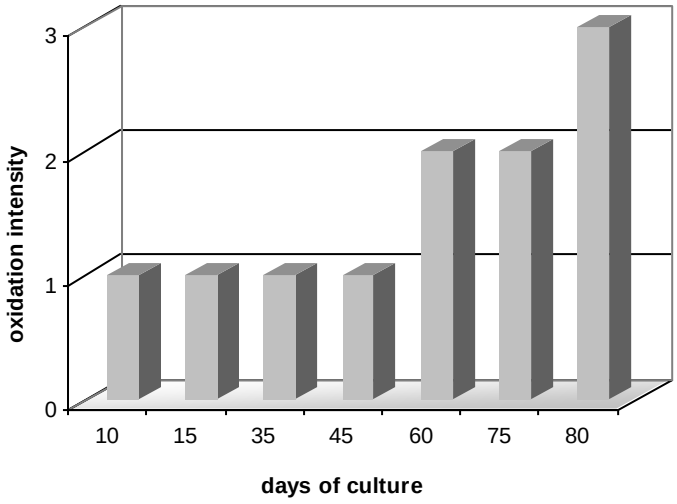
REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

Figure 2 – Color and friability standards of *P. hispidinervium* callus in MS medium added of 5 mg.L^{-1} of 2, 4 D, 10 mg.L^{-1} of BAP,. A: callus at the 10th day of culture; B: callus at the 15th day of culture; C: callus at the 35th day of culture; D: callus at the 45th day of culture; E: callus at the 60th day of culture; F: callus at the 75th day of culture; G: callus at the 80th day of culture.



Authors (2013)

Figure 3 – Average phenolic oxidation intensity in *P.hispidinervium* callus in MS medium with 2, 4 D and BAP along time Y axis:.1- without oxidation; 2- partially oxidized and 3- completely oxidized



Authors (2013)

Debiasi, Fraguas and Lima (2007) report that the oxidation of *in vitro* cultivated tissues may be a result of the nutrient depletion in the culture medium or yet, according to Rodrigues et al. (2009) the occurrence of friable callus may be due the auxin cytokinin ratio. To Castro et al. (2009) the callogenesis in barbatimão (a native species from the brazilian Cerrado) occurred on medium containing 2,4 D in the presence of light. According to França (1999), callus cultivated in the presence of light usually yield more friability than those cultivated in the absence of light. Cells of a given callus may present different morphology, size, color and ploidy, depending on the culture period

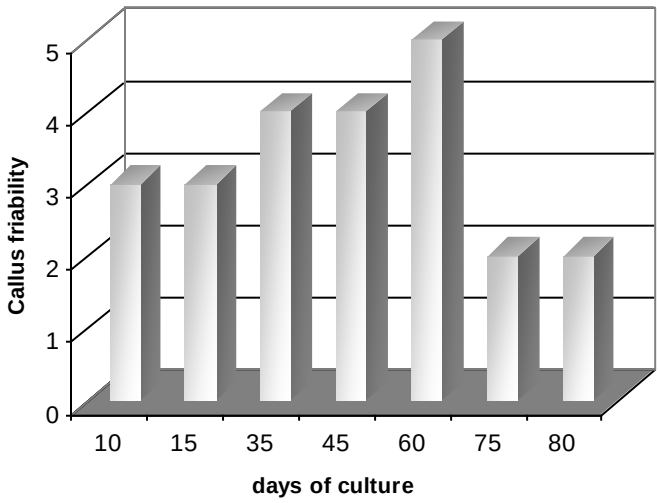
REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

and medium composition. Specifically, in relation to color, callus coloration ranging from yellow to beige may be obtained. According to Pelah et al. (2003) the morphology and texture of callus may be manipulated through variations in the constituents of the nutritious medium. Besides, culture mediums containing relatively high auxin and relatively low cytokinin concentrations produces friable callus of humid aspect and the inverse relation produces a set of compacted cells.

This work revealed a progressive increase on the callus friability up to the 60th day of culture with later reduction as the end of the evaluation period came near (Figure 4). The appearance of friable areas, usually white and translucent, frequently occurs in callus-based *in vitro* plant regeneration. The induction of friable callus depends on the composition of the culture medium and vegetal regulators, on the incidence or absence of light and temperature, and the friability characteristic is usually favored by a high auxin/cytokinin ratio, as well as by the addition of other components to the nutritious medium (TORRES; CALDAS; BUSO, 1998).

Considering the genotype involved, one may suppose that the response capacity of cells to stimuli given is variable, mainly in relation to the growth regulators. Thus, one may affirm that the genotype selected for this work favored the response efficiency to the stimuli given and promoted the callus induction and formation. Hartke and Lorz (1989) studied the callugenesis behavior of some cereal species and considered the existence of variability between genotypes of different species in relation to the callus induction capacity and later plant regeneration based on them.

Figure 4 – Friability level of *P.hispidinervium* callus in MS medium with 2, 4 D and BAP along time. Y axis: 1-not friable;2-very low friable; 3- low friable; 4- moderately friable; 5- friable



Authors (2013)

3.3 Callus variability and mass production

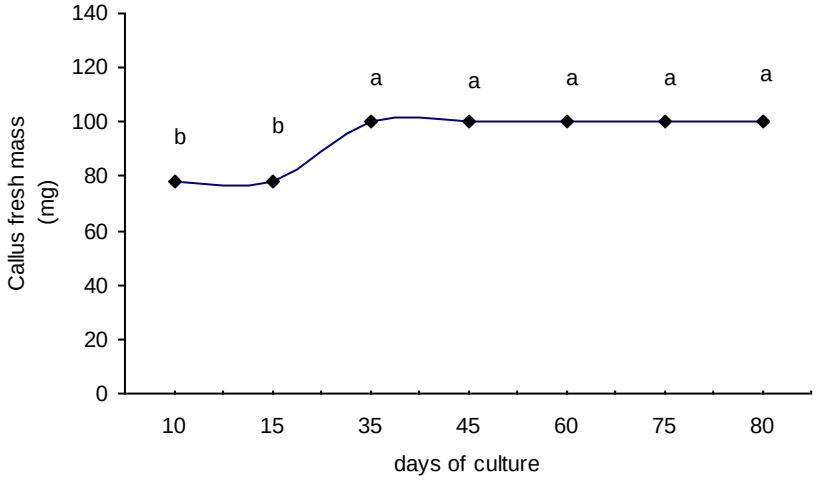
The mass of cells obtained from *P. hispidinervium* callus along the culture presented an increment of 22% from the 35th day on, remaining constant until the end of the experiment, at the 80th day (Figure 5). The viability data, in turn, presented decreasing behavior, represented by a third degree polynomial profile (Figure 6).

The use of triphenyltetrazolium chloride (TTC) enabled verifying that the viability evidences of *P. hispidinervium* callus cells presented variable behavior fully dependent on the permanence time in the culture medium. The efficiency of the TTC biochemical marker in vegetal material for the quantification of the cellular viability, according to Gibon, Sulpice and Larher (2000) and Benson et al. (1996), is recommended for quick tests, since it presents high redox potential when it enters the electron transportation chain into the mitochondria in the respiratory process, which reflects in the activity of this organelle, thus characterizing a viability evidence. Lamarca, Leduc and Barbedo (2009) and Millena et al. (1998) report that in vegetal

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

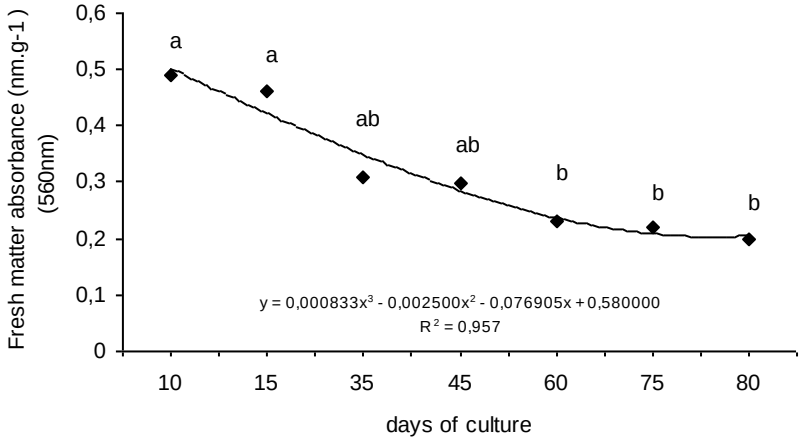
cells, the TTC is partly reduced by the alternative respiratory pathway, which is not given through phosphorylation, but rather through participation when the cytochrome pathway is saturated or inhibited.

Figure 5 –Mass of *P. hispidinervium* callus (g) in MS medium added of 5.0mg.L^{-1} of 2,4 D; 10.0mg.L^{-1} of BAP; at the 10th , 15th , 35th , 45th , 60th , 75th and 80th days of culture



Authors (2013)

Figure 6 – Cell viability given as absorbance per gram of fresh matter of *P. hispidinervium* cells in callus cultivated in MS medium added of 5.0mg.L^{-1} of 2, 4 D; 10.0mg.L^{-1} of BAP; at the 10th , 15th , 35th , 45th , 60th , 75th and 80th days of culture



Authors (2013)

The cell viability remained high at the 15th day of culture with later decrease up to the 60th day. From this point on, levels remained constant at 0.22 nm (figure 6). In the present work, viability results are closely related to the permanence time of *in vitro* callus culture. In other words, long incubation times reduce the mitochondrial rate. According to Lutts, Almansouri and Kinet (2004), studying the viability of whole plants is not an easy task, once organs and differentiated tissues present different sensibilities; however, when one works with tissue cultures, specifically callus cultures, the evaluation reliability increases significantly.

The viability callus cell of *P. hispidinervium* was inversely proportional to the culture time, indicating that the higher is

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

the permanence time, the lower the viability of its cells will be. The existence of three distinct groups in relation to cell viability, statistically different from one another, was verified. The first group was represented by samples from the 10th (0.48144 nm) and 15th (0.4433 nm) days of culture; the second, by samples from the 35th (0.32511 nm) and 45th (0.30700 nm) and the last group by samples from the 60th (0.23944 nm), 75th (0.21511 nm) and 80th (0.20378 nm) days of cultivation.

Working with *in vitro Triticum durum* L. tissues, Lutts, Almansouri and Kinet. (2004) observed significant correlation between growth rate and the TTC reducing potential, once as the growth rate increased, the TTC reducing potential also increased. Pelah et al. (2003) evaluated two cell viability tests (TTC and Neutral red) *in vitro*-cultivated *Hylocereus undatus* (Haw.)*, H. costaricensis* (F. A. C. Weber)*, H. polyrhizus* (F. A. C. Weber) and *Selenicereus megalanthus* (K. Schumann ex Vaupel) tissues under temperature stress. These authors verified variation in the cell viability according to the species under study, presenting reductions for all species at higher temperature (55ºC) when compared to lower temperatures, although it has been the subject of this study.

Observing correlations between coloration and friability physical characteristics and the viability of *P. hispidinervium* callus cells, a progressive variation on the behavior of this correlation was verified. At the first evaluation days (10th and 15th culture days), the cell viability was higher when callus presented light-green coloration with low friability. At the 35th and 45th days, the cell viability decreased when callus presented cream-colored aspect and higher friability. At the 60th day, an increase on the friability of cells was observed just when cell viability decreased and the callus presented yellowish coloration. Finally, at the 75th and 80th days of culture, reductions were observed both for friability and cell viability, and in these periods, callus present brown coloration and intense necrosis characterized by phenolic oxidation.

In relation to necrosis observed at the end of the experimental period, the nutritious depletion of the culture medium or even a direct effect of the cells sensitivity to nutrients or the permanence time could have caused such degenerative process.

4 Conclusion

According to the results obtained, the highest amounts of mean masses and the highest *P. hispidinervium* cell viability occurred at the 35th and 45th days of culture, indicating that this is the maximum permanence time of callus cultivated *in vitro* before transference into bioreactors or even for organogenic/embryogenic cultures.

---

5 Caracterização e viabilidade de calos de *Piper Hispidinervium* (piperaceae) para cultivo submerso de células

Resumo: *Piper hispidinervium C.DC. é uma planta da Amazônia de interesse comercial por sua elevada produção em safrol. O cultivo submerso de suas células tem mostrado resultados promissores para a engenharia bioquímica, no entanto, a produção de inóculo, representada pela massa celular denominada calo, é pouco estudada. O conhecimento deste material antes do estabelecimento do cultivo em reator é condicional para o êxito do processo. Portanto, calos estabelecidos "in vitro" foram caracterizados fisicamente e analisados quanto à viabilidade celular por 80 dias. Nesse estudo o delineamento experimental foi completamente ao acaso, constando de dez repetições (amostra simples) coletadas no 10°, 15°, 35°, 45°, 60°, 75° e 80° dias de cultivo. A viabilidade, determinada por reação com cloreto de 2,3,5 - trifenil tetrazólio foi reduzindo durante o cultivo, e a coloração verde própria de calo recente, foi adquirindo cor amarelada até obtenção de cor marrom, indicativo de células oxidadas. A friabilidade apresentou índice máximo aos 60 dias, enquanto a massa celular presente nos calos e expressos em gramas de massa fresca, obteve maior quantidade aos 35 dias de cultivo.*

Palavras-chave: Pimenta longa. Massa celular. Safrol, TTC.

---

5 References

ARAÚJO, P.S.; PESCADOR, R.; REBELO, R.A. **Obtaining cell lines from plant material of long pepper *Piper hispidinervium***. *Dynamis*, 5 : 19–31 (in Portuguese), 1997.

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

BENSON, E.E.; REED, B.M.; BRENNAN, R.M.; CLACHER, K.A.; ROSS, D. A. **Use of thermal analysis in the evaluation of cryopreservation protocols for *Ribes nigrum* L. germplasm.** *Cryo-Letters*, 17 : 347-362, 1996.

CALIXTO, J.B. **Twenty-five years of research on medicinal plants in Latin America: A personal view. *Journal of Ethnopharmacology***, 100 : 131-143, 2005.

CASTRO, A.H.F.; PAIVA, R.; ALVARENGA, A.A.; MACHADO VITOR, S. M. **Callogenesis and contents of total phenols and tannins in barbatimão [*Stryphnodendron adsrtingens* (Mart.) Coville].** *Ciência e Agrotecnologia*, 33 : 385-390 (in Portuguese, with abstract in English), 2009.

DEBIASI, C.; FRAGUAS, C.B.; LIMA, G.P.P. **Study of polyamines in the morphogenesis *in vitro* of *Hemerocallis* sp.** *Ciência Rural,* 37 : 1014-1020 (in Portuguese, with abstract in English), 2007.

FRANÇA, S.C. **Biotechnological approaches to obtain active substances.** In: Simões, C.M.O.; Schenkel, E.P.; Gosmann, G.; Mello, J.C.P.; Mentz, L.A.; Petrovick, P.R. (Orgs.). Pharmacognosy of the medicinal plant**.** Porto Alegre: UFRGS; Florianópolis: UFSC, 121 pp (in Portuguese), 1999.

FRANKLIN, C.I. **Inhibition and maintenance of callus and cell suspenson culture.** *Plant Cell Cult***.** - *A pratical approach*. Press Oxford. 436 pp, 1994.

FLORES, R.; NICOLOSO, F.T.; VASCONCELLOS, N.J.S. Callus induction and morphogenetic aspects of *Pfaffia tuberosa* (Spreng.) Hicken. *Revista Brasileira de Plantas Medicinai,* 8 : 89-95 (in Portuguese, with abstract in English), 2006.

GIBON, Y.; SULPICE, R.; LARHER, F. **Proline accumulation in canola leaf discs subjected to osmotic stress is related to the loss of chlorophylls and to the decrease of mitochondrial activity.** *Plant Physiology*, 110 : 469–476, 2000.

HARTKE, S.; LORZ H. **Somatic embryogenesis and plant regeneration from varius Indica rice (*O. sativa* L.) genotypes.** *Journal of Genetics and Breeding*, 43 : 205-211, 1989.

HOU, B.H.; LIN, C.G. **Rapid optimization of electroporation conditions for soybean and tomato suspension cultured cells.** *Physiology Plant***,** 111 : 166-223, 1996.

LANDA, F.S.L.; PAIVA R.; PAIVA P.D.O.; BUENO, J.S.S. ***In vitro* callus induction of *Caryocar brasiliense* Camb. leaf.** *Ciência e Agrotecnologia*, 24 : 56-63 (edição especial) (in Portuguese, with abstract in English), 2000.

LAMARCA, E.V.; LEDUC, S.N.M.; BARBEDO, C.J. **Viability and vigor of seeds of *Caesalpinia echinata* Lam. (brazilwood — Leguminosae) by tetrazolium test.** *Revista Brasileira de Botânica,* 32 : 793-803 (in Portuguese, with abstract in English), 2009.

LUTTS, S.; ALMANSOURI, A.; KINET, J.M. **Salinity and water stress have contrasting effects on the relationship between growth and cell viability during and after stress exposure in durum wheat callus.** *Plant Science,* 167 : 9–18, 2004.

MILLENA, A.R.; BENSCHOP, F.F.; WAGNER, J.J.A.M; LAMBERS, H. **The role of the alternative oxidase in stabilizing the in vivo reduction state of the ubiquinone pool and the activation state of the alternative oxidase.** *Plant Physiology,* 118 : 599–607, 1998.

MARCONDES, A.A; BRIGHENTE I.M.C.; AGUIAR, E.M.; REBELO, R.A. **Volatile constituents of Piperaceae from Santa Catarina, Brazil - essential oil composition of *Piper cernuum Vell*. and *Peperomia emarginella* (Sw.) D. DC.** *Journal of Essential Oil Research***,** 17: 286-288, 2005.

MURASHIGE, T; SKOOG, F. **A revised medium for rapid growth and bio-assay with tobacco tissue cultures.** *Physiology Plant*, 15 : 473-497, 1962.

Pantone Textile Color System: Technical Support. Paper edition. Pantone, Inc., ISBN 1-881509-33-8, 1999.

PELAH, D; KAUSHIK, R.A.; NERD, A.; MIZRAHI, Y. **Vialidity of *in vitro* viability tests for predicting response of different vine cacti in the field to high and low temperatures.** *Journal PACD*, 1 : 551-568, 2003.

PESCADOR, R.; ARAÚJO, P.S.; MAAS, C.H.; REBELO, R.A.; GIOTTO, C.R.; WENDHAUSEN JR., R.; LARGURA, G. E; TAVARES, L.B.B. **Biotechnology of *Piper hispidinervium* – Long pepper.** *Biotecnologia Ciência e Desenvolvimento*, 15 : 18-23 (in Portuguese), 2000.

PORTER, D.R; NGUYEN, H.T.; BURKE, J.J. **Quantifying acquired thermal tolerance in winter wheat.** *Crop Science,* 34 : 1686-1689, 1994.

PREECE, J.E.; MCGRANAHAN, G.H.; LONG, L.M.; LESLIE, C.A ***Somatic embryogenesis in walnut* (*Juglans regia*).** In: Jain; S.M.Gupta, P.K.; Newton, R.J. (eds.), Somatic embryogenesis in woody plants Angiosperms. *Dordrecht*, Kluwer Academic Pub. v.2 , 277 pp, 1995.

REA – Revista de *estudos ambientais* (Online)
v.15, n. 2, p. 39-47, jul./dez. 2013

RICH, P.R.; MISCHIS, L.A.; PURTON, S.; WISKICH, J.T. **The sites of interaction of triphenyltetrazolium chloride with mitochondrial respiratory chains.** *FEMS Microbiology Letters*, 202 : 181-187, 2001.

RODRIGUES, M.; PAIVA, R.; NOGUEIRA, R.C.; MARTINOTTO, C.; SILVA JÚNIOR, J.M. ***In vitro* morphogenesis of neem from cotyledonary-derived explants.** *Revista Árvore,* 33 : 21-26 (in Portuguese, with abstract in English), 2009.

ROSA, F.A.F.; REBELO, R,A.; NASCIMENTO, M.G. **Synthesis of new indolecarboxylic acids related to the plant hormone indolacetic acid IAA.** *Journal of the Brazilian Chemical Society*, 14 : 11-15, 2003.

Steponkus, P.L.; Lanphear, F.O. **Refinament of triphenyltetrazolium chloride method of determining cold injury.** *Plant Physiology***,** 42 : 1423-1426, 1967.

SWAIN, D.; DE, D.N. **Vital staining, a technique for rapid screening of plant protoplast viability.** *Indian Journal of Experimental Biology***,** 32 : 501-506, 1994.

TCACENCO, F. **Tissue culture and genetic transformation of brazilian rice germoplasm (*Oryza ativa* L).** Tesis PhD, Louisiana State University Agronomy Departament, 137 pp, 1995.

TORRES, A.C.; CALDAS, L.S.; BUSO, J.A. **Tissue culture and genetic transformation of plants.** EMBRAPA–SPI/ EMBRAPA–CNPH, 120 pp (in Portuguese), 1998.

VALLE, R.C.S.C. Strategies **of cultivation of plant cells of long pepper (*Piper hispidinervium*) and determination of kinetic parameters.** Tesis PhD. Engenharia Química, Universidade Federal de Santa Catarina. 149 pp (in Portuguese), 2003.

VALLE, R.C.S.C.; OCHNER, G.; DEBIASI, C.; TAVARES, L.B.B.; FURIGO JR,A. **Performance of *Piper hispidinervium* cells in submerged system.** *Brazilian Archives of Biology and Technology*, 49 : 43-51, 2006.