


Embryos and Lateral Buds Culture of *Tapeinochilos Ananassae* (Hassk). K. Schum.

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Abstract: Zygotic embryos and lateral buds of *Tapeinochilos ananassae* were inoculated into full or half-strength MS medium ($\frac{1}{2}$ MS) containing three antioxidants: ascorbic acid, activated charcoal, and PVP at concentrations of 0.25; 3.0 and 0.5 g.L⁻¹ respectively. The $\frac{1}{2}$ MS medium supplemented with 3.0 g.L⁻¹ activated charcoal resulted in the best embryo establishment and plant development. The lateral buds showed no significant development, an intense phenolic oxidation and high microbial contamination (mainly bacterial). Enzymatic analysis of oxidized lateral buds showed a decline in peroxidase activity and an increase in polyphenoloxidase activity.

Key-words tropical flowers, embryo culture, peroxidase, polyphenoloxidase

Cultura de embriões e gemas laterais de *Tapeinochilos ananassae* (Hassk). K. Schum.

Resumo: Embriões zigóticos e gemas laterais de *Tapeinochilos ananassae* foram inoculados em meio MS ou $\frac{1}{2}$ MS suplementado com três tipos de antioxidantes: ácido ascórbico, carvão ativado e PVP, nas concentrações 0,25; 3,0 e 0,5 g.L⁻¹, respectivamente. O meio $\frac{1}{2}$ MS suplementado com 3,0 g.L⁻¹ de carvão ativado propiciou o melhor estabelecimento dos embriões e o melhor desenvolvimento das plantas. As gemas laterais não apresentaram desenvolvimento significativo e sim uma intensa oxidação fenólica e elevada contaminação microbiana (sobretudo bacteriana). Análises enzimáticas das gemas laterais oxidadas mostraram um decréscimo da atividade da peroxidase e aumento na atividade da polifenoloxidase.

Palavras chave Flores tropicais, cultura de embriões, peroxidase, polifenoloxidase

Introduction

Tapeinochilos ananassae Hassk. K. Schum. (Costaceae) is a tropical flower with high commercial acceptance on the international market due to the brilliant red inflorescence and the spiral form of the stem. The species is a rhizomatous herbaceous perennial, with vegetative stems and leaves arranged in spiral. The inflorescences – located beneath the foliage, emerging directly from the rizome – are formed by rigid bracts rounded texture (Ferrero, 2001).

The low viability of the seeds makes vegetative

propagation its principal form of commercial reproduction, but can facilitate the spread of pests and illnesses among the planting stock (Paiva & Loges, 2005).

The tissue culture techniques can offer important tools to solve problems that limit the growth of this important ornamental culture. In vitro propagation (micropropagation) has been widely applied to produce, in a short time and at any time of the year, a large scale of high quality plantlets, ensuring varietal authenticity (Oliveira et al., 2011). Zygotic embryos and buds have been widely used as explant sources to



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initiate in vitro cultures because their juvenile state and totipotency (Grattapaglia & Machado, 1998; Bona et al., 2012). Explant establishment is a crucial step for in vitro culture, but there are serious problems that can occur during this process, including microbial contamination and phenolic oxidation (García-González et al., 2010).

As there is a notable absence of published information about the in vitro culture of *T. ananassae*, the present work evaluated the establishment of different explants types as well as biochemical markers involved in oxidative processes.

Material and Methods

In vitro culture

Lateral buds and zygotic embryos were isolated and cultivated in complete MS (Murashige & Skoog, 1962) or half-strength medium ($\frac{1}{2}$ MS) supplemented with three types of antioxidants: citric acid, activated charcoal or polyvinylpyrrolidone (PVP). Eight different treatments were elaborated ($\frac{1}{2}$ MS; $\frac{1}{2}$ MS + 0.25 g.L⁻¹ acid citric; $\frac{1}{2}$ MS + 3.0 g.L⁻¹ activated charcoal; $\frac{1}{2}$ MS + 0.5 g.L⁻¹ PVP; MS; MS + 0.25 g.L⁻¹ acid citric; MS + 3.0 g.L⁻¹ activated charcoal; MS + 0.5 g.L⁻¹ PVP). 6.5 g.L⁻¹ of agar was added to nutritive media, the pH adjusted to 5.8, and autoclaving at 121 °C (1 atm. pressure) for 20 min. The plants were grown in 20 x 150 mm test tubes containing 10 mL of nutritive media. The cultures were maintained in a growth room at 28±1 °C.

During the first 8 days, the cultures remained in the dark followed by a photoperiodic regime of 16 h (50 $\mu\text{mol}\cdot\text{m}^2\cdot\text{s}^{-1}$). The explants were observed during 30 days to analyze plant development (shoot and root), undeveloped explants, and oxidation. The experimental design was completely randomized with 15 replicates for zygotic embryos and 20 for lateral buds per treatment.

The results obtained were submitted to the variance analysis (ANOVA) and statistical analysis was completed with Assistat-Statistical Assistance Software, version 7.5 beta. The z test was used to analyze proportions of plant, undeveloped explants,

and oxidation, with significance at $\alpha=5\%$. The homogeneity of the microbial contamination was evaluated using the Qui-square test (χ^2), with significant corrections at $\alpha=5\%$ (Vieira, 2003).

Histological analyses

Histological analyses were undertaken of the lateral buds when these failed to show regenerative structures. In order to verify the integrity of the bud tissue, samples were fixed in FAA 50 (Johansen, 1940), cut manually in transversal sections, cleared with 30% sodium hypochlorite, washed in distilled water, and then stained with Safrablau (Kraus & Arduin, 1997). The slides were examined and photographed under an optical microscope (Coleman).

Biochemical analyses

Peroxidase (POD) and polyphenoloxidase (PPO) enzyme activities of lateral buds inoculated into MS medium were evaluated after 7, 14, and 21 days of inoculation. Fresh tissue (0.05 g) was macerated in liquid nitrogen, and 2.5 mL of 0.1 M sodium phosphate buffer pH=6.5 at 0 to 4 °C was added. The extracts were centrifuged for 15 min at 8,000 rpm and the supernatant maintained at -20 °C until the analyses were performed. All analyses were carried out with three replicates: total soluble proteins (Bradford, 1976), peroxidase activity (Vieira & Fatibello-Filho, 1998) and polyphenoloxidase activity (Kar & Mishra, 1976). The data was submitted to polynomial regression analysis and expressed in U.min⁻¹.mg⁻¹ protein.g⁻¹ fresh weight.

Results and Discussion

Establishment of zygotic embryos

After 30 days, the zygotic embryos cultivated in $\frac{1}{2}$ MS supplemented with 3.0 g.L⁻¹ of activated charcoal demonstrated higher leaves and roots number than the others treatments (Table 1). The proportion of plant development in this treatment was 80% (p=80), and presented the lower levels of oxidation, 20% (p=20), and all embryos developed plants (non developed embryos p=0) (Table 2).

Table 1. Leaves and roots numbers and fresh weight of *Tapeinochilos ananassae* on the 30th day of zygotic embryos culture, in ½ MS and MS media with distinct concentration of acid citric, activates charcoal and PVP.

Tabela 1. Número de folhas, raízes e peso fresco de *Tapeinochilos ananassae* no 30º dia de cultivo de embriões zigóticos em meio ½ MS e MS com distintas concentrações de ácido cítrico, carvão ativado e PVP.

Treatments	Nº leaves	Nº roots	Fresh weight
½ MS	0,40 c	0,40 d	0,39 c
½ MS + 0.25 mg. L ⁻¹ acid citric	0,89 b	1,22 b	0,73 b
½ MS + 3.0 mg. L ⁻¹ activated charcoal	1,41 a	1,81 a	0,79 a
½ MS + 0.5 mg. L ⁻¹ PVP	0,91 b	1,05 b	0,74 ab
MS	0,51 c	-	0,43 c
MS + 0.25 mg. L ⁻¹ acid citric	0,98 b	0,80 c	0,78 a
MS + 3.0 mg. L ⁻¹ activated charcoal	0,74 b	0,80 c	0,73 b
MS + 0.5 mg. L ⁻¹ PVP	0,80 b	0,84 bc	0,75 ab

Means followed by the same letter do not differ by Tukey's test at 5% probability

Médias seguidas da mesma letra não apresentam diferença significativa pelo teste de Tukey a 5% de probabilidade

Table 2. Proportions of formation of plants, oxidation and non developed embryos in different culture media during in vitro establishment of zygotic embryos of *Tapeinochilos ananassae* (n= number of individuals; Z= standard reduced; p(%)= overall proporcion)

Tabela 2. Proporções de formação de plantas, oxidação e embriões não desenvolvidos em diferentes meios de cultura durante o estabelecimento in vitro de embriões zigóticos de *Tapeinochilos ananassae* (n= número de indivíduos; Z= padrão reduzido; p(%)= proporção geral)

Treatments	Formation of plants			Oxidation		Non developed embryos	
	N	p(%)	Z	p(%)	Z	p(%)	Z
½ MS	15	46.7	-0.512	40	0.105	6.7	1.433
½ MS + 0.25 acid citric (g.L ⁻¹)	14	57.1	1.702	42.9	0.692	0	3.393*
½ MS + 3.0 activated charcoal	15	80.0	6.695*	20	2.885*	0	3.465*
½ MS + 0.5 PVP	15	46.7	-0.512	46.7	1.599	6.7	1.433
MS	15	26.7	3.395*	20	2.885*	40	6.697*
MS + 0.25 acid citric	12	50	0.029	25	1.577	25	1.870
MS + 3.0 activated charcoal	15	46.7	-0.512	33.3	-0.105	13.3	-0.600
MS + 0.5 PVP	15	13.3	6.277*	60	4.589*	26.7	2.632*

* Significant values, considering Z values greater than 1.96 or less than -1.96 (α= 5%)

* Valores significativos, considerando-se valores de Z superiores a 1,96 ou inferiores a -1,96 (α= 5%)

The positive effects of activated charcoal are associated with its capacity to adsorb toxic substances, such as phenolic compounds and their degradation products that are liberated by plant tissue during in vitro culture (Cid & Teixeira, 2010). Activated charcoal at 2.5 g.L⁻¹ added in the culture medium increased the germination and the plant growth of *Piper hispidinervum* C. DC. (Guedes et al., 2006) and 2 mg.L⁻¹ could effectively induce large protocorms of *Cimbidium giganteum* (Hossain et al., 2010). Embryos of *Lychnophora pinaster* Mart.

(Souza et al., 2003) likewise better development in half strength MS medium. The reduced concentrations of nutrient in the tissue culture media represent considerable decrease in the osmotic potential (Paiva & Otoni, 2003) and, in particular reduced concentrations of metals such as iron, copper and zinc that are important to prevent the formation of the highly toxic hydroxyl radical via the metal-dependent Haber-Weiss or the Fenton reactions (Mittler, 2002) increasing the susceptibility of the explants to oxidations.

The addition of PVP to the culture media did not result in significant gains in terms of establishment of the zygotic embryos of *T. ananassae*. When PVP was associated with MS medium, a greater number of oxidized (p=60) and undeveloped (p=26.7) embryos were observed, as well as reduced numbers of plant formation (p=13.3). PVP (0.4 g.L⁻¹) did not control oxidation in *Syagrus oleraceae* MART. BECC (Melo et al., 2001). Conversely, both activated charcoal and PVP promote a higher establishment of *Lippia sidoides* Cham. zygotic embryos and their transformation into weel-formed plants eventually need different species to oxidant agents (Costa et al., 2007).

Anatomical aspects of lateral buds and their establishment

No development was observed in any of the treatments used to promote establishment of the lateral buds of *T. ananassae*. The explant showed organogenic potential, the stem apices was composed of undifferentiated cells indicating a potentially functional meristem, the internal tissue of the sheaths show less differentiation than the tissue of external sheath that had functional mature xylem and phloem. The epidermis was composed by only one layer of cells with a thin cuticle, as well as unicellular epidermal hairs (Figure 1A and B).

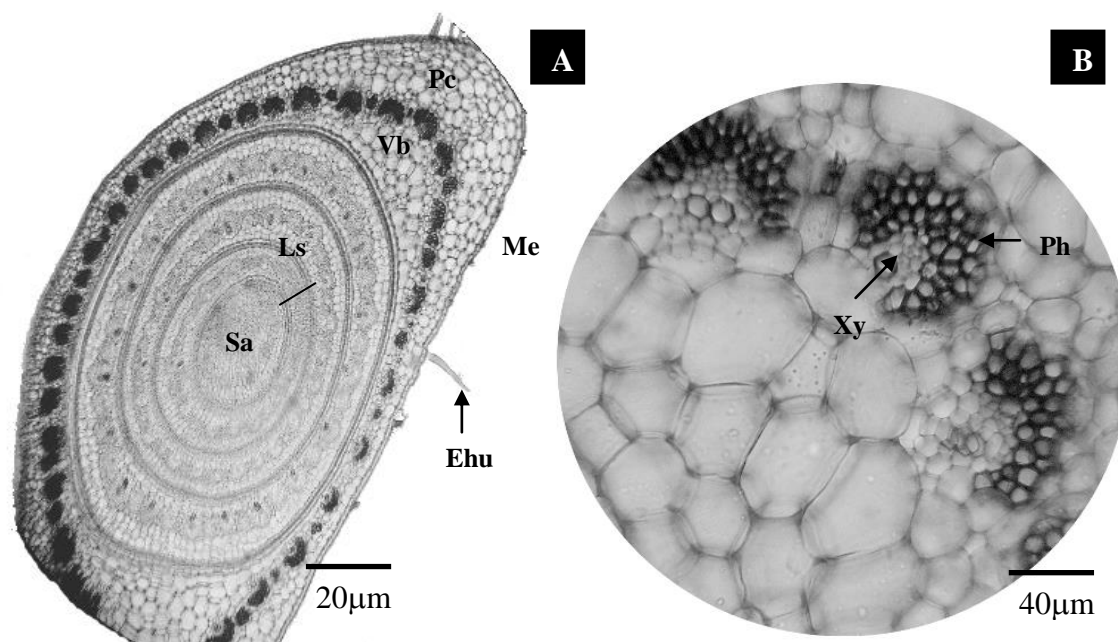


Figure 1. Transversal cut of lateral bud of *Tapeinochilos ananassae*. (A) Overview of the histological section showing stem apex (Sa), leaf sheath (Ls), parenchyma cells (Pc), monostratified epidermis (Me), vascular bundles (Vb) and epidermal hairs unicellular (Ehu); (B) phloem (Pl) and xylem (Xy)

Figura 1. Corte transversal da gema lateral de *Tapeinochilos ananassae*. (A) Seção histológica indicando o ápice caulinar (Ac), bainha foliar (Bf), células do parênquima (Cp), epiderme monoestratificada (Em), feixes vasculares (Fv) e pelos epidérmicos unicelulares (Peu); (B) floema (Fl) e xilema (Xi)

The cultures of lateral buds of *T. ananassae* demonstrated elevated phenolic oxidation levels and microbial contamination, negatively affecting explant development. The lateral buds showed total oxidation of the explants after the period of 20 to 30 days, regardless of the added antioxidant. According to Grattapaglia & Machado (1998) phenolic oxidation may be inherent of the tissues, reducing the effectiveness of any antioxidant used. Lateral buds of *Strelitzia reginae* also demonstrated high levels of oxidation that impeded explant development (North et al., 2010).

Lateral buds from basal branches demonstrated greater frequencies of contamination by fungi (36.2%

of all contaminations) and bacteria (42.5% of all contaminations) with χ^2 values of 18.06 and 29.17 respectively (p<0.05). Lateral buds recently formed presented 2.5% and 12.5% contamination for fungi and bacteria respectively. The lateral buds of basal branches generally have irregular and pilose surfaces associated with their less juvenile origin, which makes disinfection more difficult (Pasqual et al., 2010). During in vitro establishment of *Aniba rosaeodora* Ducke, there were lower levels of bacterial and fungal contamination when more recently formed buds were used (Handa et al., 2005).

The specific activity of peroxidase and polyphenoloxidase in lateral buds

During culture of lateral buds, peroxidase activity decreased 26.5% after 7 days, 51.2% after 14 days, and 58.8% after 21 days (Figure 2A). This decrease in peroxidase activity was coincident with the increase oxidation observed in the explants, these tissues did not also demonstrate any organogenic development during the 21 days of culture. In agreement with the results obtained in the present work, Andersen (1986), reported that the low POD activity may be related to the loss of morphogenic potential of *Rhododendron* cells culture, as well as the callus tissue of *Panax ginseng* (Bonfill et al., 2003) and *Hemerocallis* sp (Debiasi et al., 2007).

POD acts on the hydrogen peroxide and phenol (Lima et al., 1998), which are known to induce membranes, proteins and DNA damage. The loss or

decrease in activity of this enzyme can lead to the elevated production of toxic metabolites and subsequent cell program death (Jaleel et al., 2009). POD also plays a role in growth and differentiation, and their high activity could be correlated to the process of differentiation that occurs during shoot or root induction (Díaz-Vivancos et al., 2011) and somatic embryogenesis (Silva, 2010).

Polyphenoloxidase activity presented a discrete decrease after the seventh day, and then continued to fall until the 7th day. At 21 days the activity levels increased to almost three times than observed in recently excised buds (Figure 2B). The elevated PPO activity is directly correlated with quinone polymerization and the darkening of the explant tissue (Huang et al., 2002).

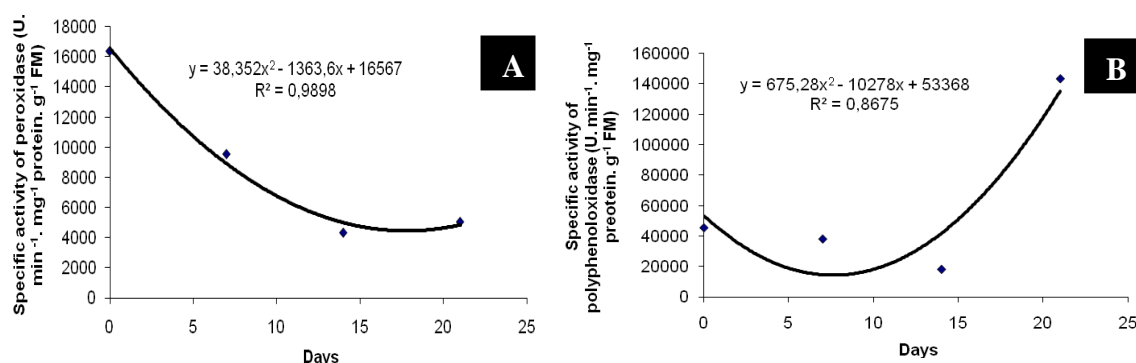


Figure 2. Specific activity of peroxidase (A) and polyphenoloxidase (B) (U.min⁻¹.mg⁻¹ de protein.gm⁻¹ de fresh material) in the lateral buds of *Tapeinochilos ananassae* cultivated in MS medium during 21 days

Figura 2. Atividade específica da peroxidase (A) e polifenoloxidase (B) (U.min⁻¹.mg⁻¹ de proteína.gm⁻¹ de matéria fresca) em gemas laterais de *Tapeinochilos ananassae* cultivadas em meio MS durante 21 dias

In *Tulipa gesneriana* L. cv. Apeldoorn, the elevated polyphenoloxidase activity was accompanied by an increasing degree of oxidation of the tissue (Van Rossum et al., 1997). Likewise, Virginia pine calli showed a lower PPO activity in healthy callus tissue showing low oxidation levels, but its activity was increased in callus under oxidative processes and dark tissue (Tang & Newton, 2004). We observed a gradual darkening of the bud tissue of *T. ananassae* originating in the basal region and extending over time to the entire explant. Darkening of newly formed bud tissue in various bamboo species extended to intact cells, suggesting that the autolysis of injured cells (resulting from the excision of the explants) could provoke autolysis of non-injured neighboring cells (Huang et al., 2002).

Conclusions

The zygotic embryos could be used successfully in the in vitro establishment of *Tapeinochilos ananassae*. The peroxidase and polyphenoloxidase can be used as biochemical markers of organogenesis and phenolic oxidation in lateral buds of *T. ananassae*.

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