

FORMATION OF PROTOCORM-LIKE BODIES FROM ROOT APICES OF *CATASETUM PILEATUM* (ORCHIDACEAE) CULTIVATED IN VITRO. II. SOME NON-HORMONAL REQUIREMENTS INVOLVED IN THE REGENERATION⁽¹⁾

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ABSTRACT - (Formation of protocorm-like bodies from root apices of *Catasetum pileatum* (Orchidaceae) cultivated *in vitro*. II. Some non-hormonal requirements involved in the regeneration). The effects of different types of culture media, organic nutrients, activated charcoal, physical state of medium, and the explant size were studied regarding the conversion of *C. pileatum* root tips into protocorm-like bodies (PLB). Modified MS medium was disclosed to be particularly favorable to induction, when compared other more diluted media. Continuous growth of the explanted root tip and PLB formation were considerably dependent on the carbon source. Sucrose, glucose and fructose proved to be the most effective carbon sources. Bacto-peptone improved PLB regeneration. A tendency to multiple PLB regeneration was obtained in liquid medium. Activated charcoal added to the medium showed a conspicuous increase in the conversion of root tips into PLB. An inverse relation was detected between the size of the explanted root tip and the capacity of PLB formation.

RESUMO - (Formação de protocormóides em ápices radiculares de *Catasetum pileatum* (Orchidaceae) cultivados *in vitro*. II. Alguns requisitos não hormonais envolvidos na regeneração). Os efeitos de diferentes tipos de meios de cultura, nutrientes orgânicos, carvão ativado, estado físico do meio e tamanho dos explantes foram estudados em relação a conversão de ápices radiculares de *C. pileatum* em protocormóides (PLB). O meio MS modificado foi o que mais favoreceu a indução de protocormóides quando comparado aos meios mais diluídos. A manutenção do crescimento do explante radicular e a formação de protocormóides foi dependente da presença de uma fonte de carbono no meio: sacarose, glicose e frutose mostraram ser as fontes mais efetivas. Bacto-peptona estimulou a regeneração de protocormóides. Em meio líquido os explantes mostram uma tendência para a regeneração de PLB do tipo múltiplo. O carvão ativado mostrou ser eficaz na transformação dos ápices radiculares em protocormóides. Verificou-se uma relação inversa entre o tamanho do explante e a capacidade de formação de protocormóides.

Key words: *Catasetum pileatum*, orchid, root apex, protocorm-like body.

INTRODUCTION

Techniques of tissue culture at the present time represent an important tool within the areas of both basic and applied research. However, certain specific and local difficulties have so far restricted the application of these techniques to a relatively limited number of plants which deserve attention. The nature of the explants may be mentioned as an important factor in the establishment of any plant tissue culture. As a rule, the organs, tissues, and the cells used as explant sources derive from parts of the shoots of the plants. Consequently, a good deal of knowledge related to the culture of shoot structure has been accumulated in recent years. On the other hand, botanists pay only modest attention to both physiological studies and tissue culture concerned with roots as a whole (Feldman 1984).

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The possibility of orchid propagation *in vitro* was shown by Morel (1964), using shoot tip cultures of *Cymbidium*. Following the method initially described by this author, the micropropagation of several other orchid genera of commercial value was achieved (Morel 1974). However, micropropagation of a great many important genera has so far proved to be impossible (Kerbauy 1985). The process of dedifferentiating mature orchid cells is yet not feasible. Thus, orchid micropropagation depends on the culture of meristematic cells. Beechey (1970) suggested the use of orchid root tips as meristematic cell donors. However, the first publication on this issue caused a certain degree of disappointment. Churchill *et al.* (1972) were unable to regenerate any protocorm-like body using root tips of *Epidendrum "Obrienianum"* (a hybrid orchid). Working with the same material, Stewart and Button (1978) managed to induce the regeneration of only one plantlet *in vitro*. A number of plantlets were obtained from root tips of *Phalaenopsis amabilis* (Tanaka *et al.* 1976). More recently, hybrid of *Catasetum* (Kerbauy 1984a), *Oncidium varicosum* (Kerbauy 1984b, 1988), *Vanilla planifolia* (Philip & Nainar 1986), and *Cattleya* (Kerbauy 1991) have been successfully cloned *in vitro* by means of root tip explants.

There are basically two kinds of behaviour observed in orchid root tips culture *in vitro*. In *Epidendrum* (Stewart & Button 1978), *Oncidium* (Kerbauy 1984b, 1988) and *Cattleya* (Kerbauy 1991), PLB regeneration is preceded by callus formation. In these cases the addition of phytohormone into the medium is essential for its induction. On the other hand, PLB can be directly regenerated from root tips without passing through the callus phase: *Catasetum* (Kerbauy 1984a), as well as allied genera which we have observed in our laboratory, provides such a conspicuous example. With respect to this PLB formation, it is important to point out the fact that the conversion of meristematic root cells into meristematic shoot cells (PLB) seems to occur as a continuous process, with no interruption of cell proliferation (Kraus & Monteiro 1989).

The main goal of the present paper is to study the modulation effect of some non-hormonal requirements on the dramatic conversion of root tips cells into protocorm-like bodies (somatic embryos) *in vitro* culture.

MATERIAL AND METHODS

Root tips obtained from seedlings (8 ± 2 cm tall) of *Catasetum pileatum* Reichb. f., aymbiotically developed, were used for explants.

Three modified culture media were initially compared for regeneration of PLB from root tips: Murashige and Skoog (1962) - medium MMS; formula C Knudson (1946) - medium MK; and Vacin and Went (1949) - medium MVW. Iron was always added in the form of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (27.8 mg.l^{-1}) and Na_2EDTA (37 mg.l^{-1}). The following organic substances were also added to each medium: myoinositol (100 mg.l^{-1}), vitamins (thiamin, 5 mg.l^{-1} ; nicotinic acid, 1 mg.l^{-1} ; pyridoxine, 1 mg.l^{-1}), sucrose (20 g.l^{-1}), and bacto-agar (8 g.l^{-1}). The joint addition of bacto-peptone (1 g.l^{-1}) and activated charcoal (1 g.l^{-1}) was also tested in these media. The pH of the media before autoclaving was adjusted to 5.5. The sterilization was carried out for 15 min at 121°C .

Root tip explants with 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mm in length were inoculated into MMS. From this point on, explants 6 ± 1 mm long were always used.

The following modification of MMS were tested: concentration of salts (halved and doubled), absence of bacto-agar, absence of activated charcoal, and absence of bacto-peptone. The effect of different carbohydrate sources was studied. For this purpose, sucrose was replaced in MMS by: glucose, fructose, ribose, xylose, galactose, mannitol, maltose, and trehalose. Solutions of the carbohydrates were added, one at a time, to MMS at a final concentration of 20 g.l^{-1} . They were milli-

pore-sterilized and added to the autoclaved media after cooling to nearly 40°C. The effect of different sucrose concentrations (0.0, 20.0, 40.0, 60.0, and 120.0 g.l⁻¹) on MMS medium was also studied.

All cultures were kept at 26 ± 2°C under fluorescent light for 16 hours at 9 Wm⁻² exposition. For all experiments, a minimum batch of 25 explants per treatment was used. The experiments were conducted for 30 days.

Photomicrografs were taken of the most important morphological aspects of the PLB formation on the roots cultivated *in vitro* for 30 days.

RESULTS

1 - Morphological aspects of regeneration

Figure 1 shows the sequence of morphological modifications occurring in root tips cultivated *in vitro*, up to the point when the PLB is regenerated and a plant begins to develop.

Root explant may give rise to one single PLB (single regeneration), or to a cluster of PLB (multiple regeneration), as shown in figure 2.

In larger explants (16 and 32 mm long) there also occurred induction of PLB regeneration at certain points along the explant axis (Figure 3). The frequency of occurrence in explants with 6mm long was very low and was not taken into account in the context of this study.

2 - Effects of different chemical and physical factors on root tip conversion into PLB

a. Media types

Figure 4 shows that among the various culture media used, medium MMS was the most favourable for regeneration of PLB when compared to media MVW and MK. The frequency of PLB regeneration was further increased by the addition of bacto-peptone and activated charcoal together. Medium MVW was more effective than medium MK. Such results suggest the use of MMS as basal medium.

b. Explant size

The size of the explants presented a considerable effect on PLB formation (Figure 5). The regenerative expression increased progressively in explants with lengths larger than 1 mm. Furthermore, it was observed that smaller explants (0.25 and 0.5 mm long) showed a conspicuous tendency towards multiple regeneration. A decrease in the formation of multiple PLB was observed by increasing the size of the explants.

c. Salt levels, bacto-agar, bacto-peptone, and activated charcoal

As shown in table 1, the reduction of salt concentration by half induced a decrease in the percentage of PLB formation when compared to MMS. On the other hand, when the concentration of salts was doubled there was no PLB regeneration at all. It is also interesting to note that the frequency of dead explants increased by doubling the salt levels of the media.

Gelled medium (MMS with agar) was more favourable for PLB regeneration in comparison to the equivalent liquid state medium (MMS without agar). However, the latter favoured multiple PLB regeneration. Materials cultured in liquid medium were yellowish in colour when compared to those cultivated in gelled media.

Table 1 - Effects of salt concentration, bacto-agar, bacto-peptone and activated charcoal of MMS medium on PLB formation (%) on root tips of *Catasetum pileatum* Reichb. f. after 30 days of culture.

Medium variant	PLB	R	D
Control	68	32	0
Half salt strength	48	44	8
Double salt strenght	0	52	48
Liquid state	56	40	4
Without peptone	40	56	4
Without charcoal	52	48	0

PLB - root tip with protocorm-like body

R - root tip without protocorm-like body

D - dead root

The addition of a source of organic nitrogen, such as bacto-peptone, proved to be important for the process of root conversion, since in MMS without peptone the percentage of regeneration was lower. The absence of activated charcoal in the medium also led to reduced frequency of regeneration in comparison to complete MMS.

d. Carbohydrates

Table 2 shows the effects of different sugars used as carbon sources. Among the monosaccharides used, fructose and glucose showed results similar to sucrose of the control medium (MMS). Ribose, galactose, and mannitol caused a high explant death rate; in the presence of the former two no regeneration occurred. However, xylose allowed a reasonable degree of PLB regeneration. In relation to disaccharides, sucrose proved to be an efficient carbon source for the process of regeneration, while maltose was reasonably efficient. Trehalose proved to be of little efficiency, and almost half the explants showed no alteration.

Figure 6 shows the effect of varying concentration of sucrose. Regeneration did not occur in the absence of this sugar. Increase of the sucrose content up to a concentration of 20 g.l⁻¹ (MMS) was accompanied by a gradual increase in PLB regeneration. Concentrations above 40 g.l⁻¹ led to an inhibition of PLB regeneration. In a medium with a concentration of 120 g.l⁻¹ no regeneration was observed, and the root explant death rate was 100%.

DISCUSSION

Formation of buds from root explants *in vitro* is a subject which has received relatively little attention (Thomas & Street 1972, Peterson 1975, Chaturvedi & Sinha 1979, Lazzeri & Dunwell 1984a, 1984b). With regard to the roots of orchids, there is even less available information.

Figs. 1-3 - Root explants of *Catasetum pileatum* Reichb. f. after 30 days of culture. 1. Successive stages of conversion of root explant (R) into protocorm-like body (PLB). 2. Single (left) and multiple (right) PLB formation. 3. Lateral PLB formation.

Figs. 1-3 - Explantes radiculares de *Catasetum pileatum* Reichb. f. mantidos em cultura por 30 dias. 1. Estágios sucessivos da conversão do explante radicular (R) em protocormóide (PLB). 2. Regeneração de protocormóides: simples (esquerda) e múltipla (direita). 3. Protocormóides regenerados lateralmente.

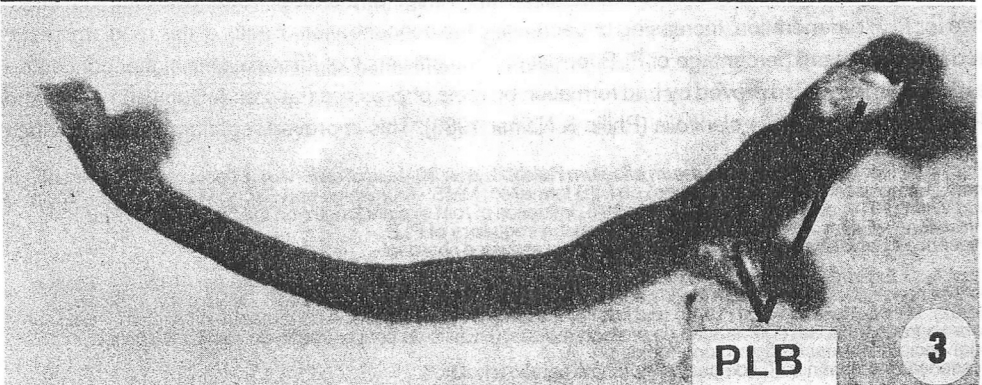
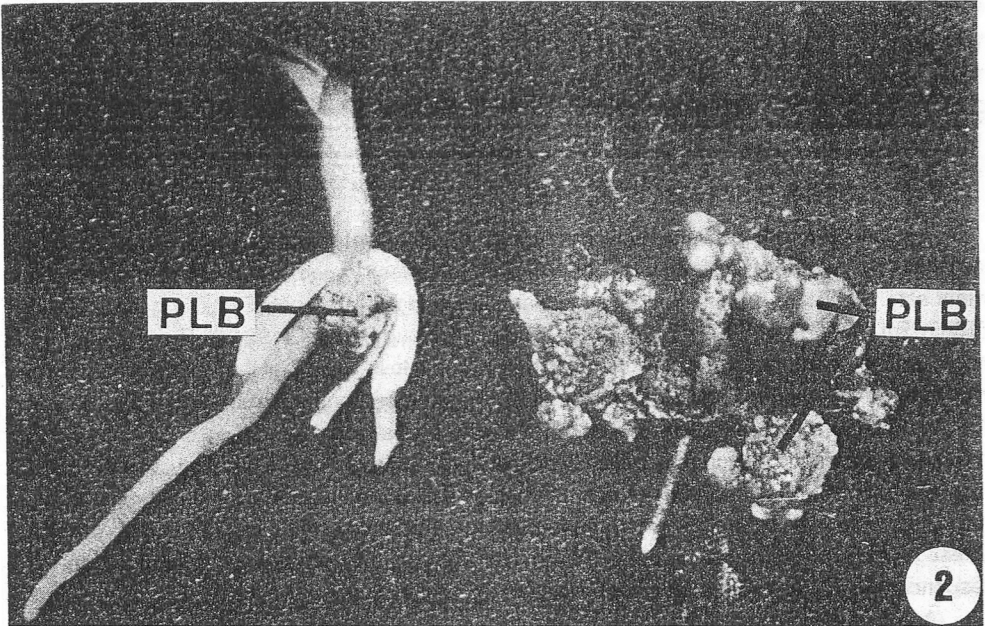
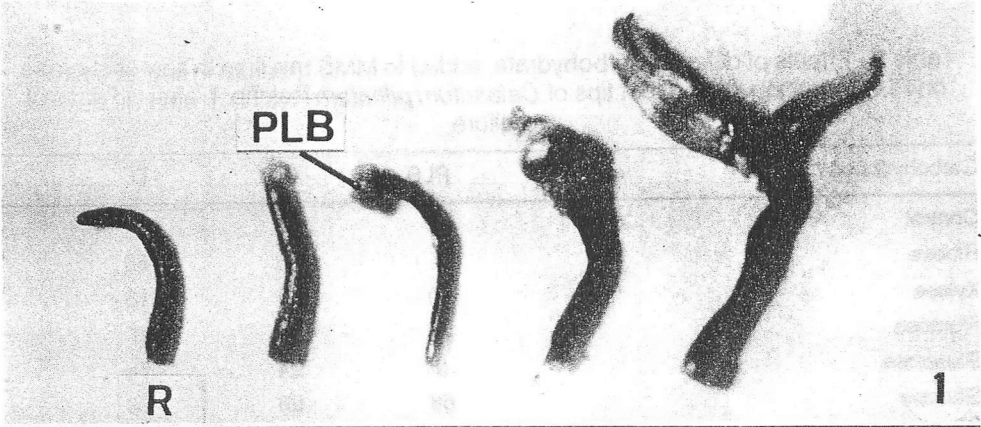


Table 2 - Effects of different carbohydrate, added to MMS medium in lieu of sucrose, on PLB formation (%) on root tips of *Catasetum pileatum* Reichb. f. after 30 days of culture.

Carbohydrates	PLB	R	D
Control	68	32	0
Ribose	0	40	60
Xylose	40	44	16
Fructose	64	36	0
Galactose	0	24	76
Glucose	68	20	12
Mannitol	16	16	68
Maltose	52	32	28
Trehalose	24	48	28

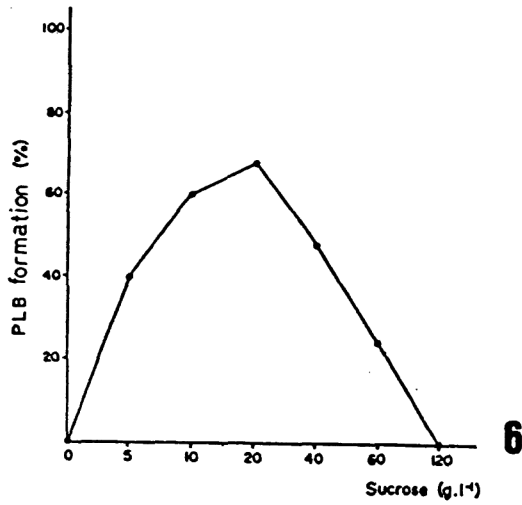
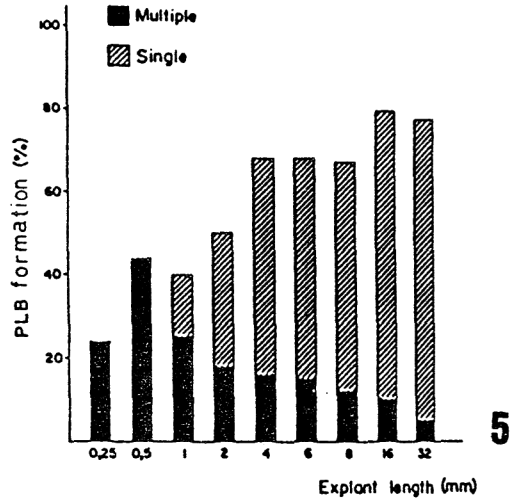
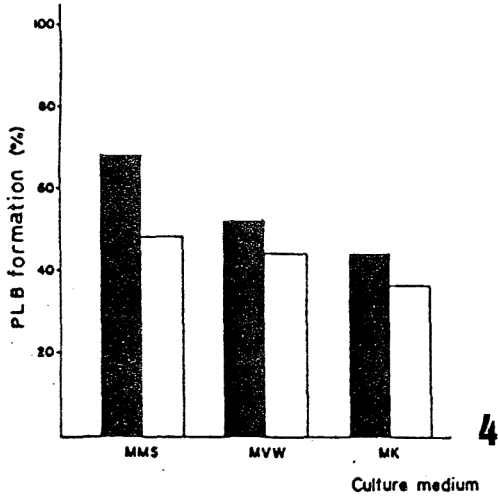
PLB - root tip with protocorm-like body
 R - root tip without protocorm-like body
 D - dead root

It is clear from the present study that, in root of *Catasetum pileatum*, the percentage of regeneration and also the type of regeneration (single or multiples), varied highly in accordance with explant size. Similar root behaviour *in vitro* was observed by Torrey (1954), Bonnett and Torrey (1965), Ballade (1970), Sauton *et al.* (1982) and Lazzeri and Dunwell (1984a). The low capacity of regeneration presented by explants 0.25 mm long seems to indicate that they are probably formed mostly by root caps. Explants 0.5 mm long - including thus cap and root meristem - showed a higher percentage of regeneration. Explants of this size also showed intense PLB proliferation with multiple regeneration, which may reflect the breakage of endogenous correlation between tip meristem and surrounding mature tissues. This hypothesis appears to be substantiated by the fact that longer explants (16 and 32 mm length) gave rise predominantly to single PLB at their apices. These explants also formed lateral PLB, which were formed in the mature parts of the explant, corresponding to the region of lateral root formation.

Among the three media used, the modified Murashige and Skoog (1962) was the most effective for PLB regeneration. Increasing or decreasing the concentration of salts of this medium, resulted in a decreased percentage of PLB formation. The efficiency of this relatively higher concentrated medium was also proved by bud formation on roots of *Brassica* (Lazzeri & Dunwell 1984a) and PLB on roots of *Vanilla planifolia* (Philip & Nainar 1986). This improved regeneration performance

Figs. 4-6 - Root explants of *Catasetum pileatum* Reichb.f. after 30 days of culture. 4. Effects of three different media composition (with modifications) on PLB formation: MMS - Murashige and Skoog (1962); MVW - Vacin and Went (1949) and MK - Knudson (1946); 5. Influence of root explant length, on the frequency of PLB formation. 6. Effect of sucrose concentration on the frequency of PLB. Presence ■ and absence □ of bacto-peptone and activated charcoal.

Figs. 4-6 - Explantes radiculares de *Catasetum pileatum* Reichb.f. mantidos em cultura por 30 dias. 4. Efeitos de três meios diferentes e modificados sobre a formação de protocormóides: MMS - Murashige e Skoog (1962); MVW - Vacin e Went (1949) and MK - Knudson (1946). 5. Influência do tamanho do explante radicular sobre a frequência de regeneração de protocormóides. 6. Efeito da concentração de sacarose sobre a frequência de formação de protocormóides. Presença ■ e ausência □ de bacto-peptona e carvão ativado.



found with Murashige and Skoog (1962) medium, strongly suggests the beneficial effects of low water potential of the media for PLB regeneration.

Modulatory effects of bacto-agar in the medium culture have been observed on both growth and morphogenetic process of materials grown *in vitro* (Margara & Bouniols 1972, Kohlenbach & Wernicke 1978, Debergh 1983, Lazzeri & Dunwell 1984a). The results obtained with *C. pileatum* showed that in gelled medium the percentage of PLB regeneration was higher than in liquid medium. Similar results were also observed by Lazzeri and Dunwell (1984a) with roots of *Brassica*. The greater proliferation of PLB (multiple regeneration) observed in liquid media may be a consequence of the greater surface for absorption of the roots in contact with the nutritive solution. The yellowish colour observed in the root explants and PLB is a consequence of reduced penetration of light due to activated charcoal in suspension in the liquid medium. Roots of *Solanum khasianum* gave rise to somatic embryos in a liquid medium and to shoot buds in a solid medium (Chaturvedi & Sinha 1979). Moreover the use of liquid medium for PLB formation is a common orchid micropropagation practice (Morel 1974).

The favourable effects of activated charcoal on different aspects of the development of plant *in vitro* have been extensively observed (Ernst 1974, Wang & Huang 1976, Reynolds & Murashige 1979, Martineau *et al.* 1981, Johansson 1983). Charcoal adsorption of growth inhibitory substances has been suggested (Johansson 1983, Wang & Huang 1976).

Taking into account the fact that root tips are active sites of protein synthesis (Street 1966), the effect of bacto-peptone in promoting direct PLB formation, as a source of amino-acids and peptides, would be understandable. On the other hand, bacto-peptone showed to be highly inhibitory for PLB regeneration in callus of *Oncidium varicosum*, and for the indirect regeneration process (Kerbauy 1984b, 1988).

Of the different carbohydrates used, sucrose, glucose, and fructose proved to be the most efficient source of carbon for PLB regeneration. Maltose permitted a reasonable level of PLB regeneration. Almestrand (1957) showed that these sugars may be used for growing roots of a great variety of monocotyledons. Sugars such as trehalose and polyalcohols such as mannitol, found in the hyphae of fungi, were not efficient for PLB regeneration, in spite of the fact that, in natural conditions, the roots of orchids were arranged in associations with certain fungi (mycorrhiza). Failure of regeneration in a medium containing ribose suggests similarity, at least in part, with the results of Ernst (1967), who also failed to develop protocorms of zygotic origin with this source of carbohydrate. The high level of explant death and the failure to regeneration in a medium with galactose prove the inhibitory effect of this sugar on root growth (Hughes & Street 1974).

The process of PLB formation was highly dependent on an exogenous supply of sucrose, an important source of carbon. According to Van't Hof (1968), root apices of *Pisum sativum*, excised and cultivated in a medium without sugar, showed meristematic cell at stages G1 (90%) and G2 (10%). However, high sucrose levels may lead to an increasing proportion of quiescent cells and in the duration of mitosis in the root apex (Scadeng & MacLeod 1976). Another consequence of the addition of sucrose could be an increase in the osmotic potential of the medium or even an alteration of the endogenous hormonal balance (Meir *et al.* 1985).

Bearing in mind that the control of organogenetic processes depends fundamentally on the synergism of auxin and cytokinin (Skoog & Miller 1957), it is reasonable to assume that the change from a root pattern to a somatic embryo (PLB) in *C. pileatum* is the result of the alteration of the endogenous level of hormones after excision. In fact, the involvement of plant hormones in the direct conversion of root tips into PLB was clearly shown by Colli (1989) in *Catasetum fimbriatum*. According to this author, exogenous cytokinins and auxins presented a noteworthy effect on the control

of this particular process of orchid embryo regeneration. PLB regeneration was conspicuously stimulated by the former growth regulators, while the presence of the latter promoted a long period of inhibition. Colli's results suggest, at first sight, that the maintenance of root meristem pattern or its conversion into PLB depends ultimately on an adequate synergism between both substances.

Structural changes in root apices of *C. pileatum* cultured *in vitro*, resulting in the formation of PLB were studied by Kraus and Monteiro (1989). Unfortunately, enough information is not available in the literature regarding the effects of chemical and physical factors, the culture conditions used in the study of root tip conversion into PLB, as well as for other process of plant embryo regeneration. However, it is plausible to consider that the effects of the conditions used in this study may reflect the endogenous hormonal balance. Thus, the next step in this study would be the determination of the endogenous nature and levels of the plant hormones involved in the root tip conversion *in vitro*.

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