OPTIMIZATION OF A PROTOCOL FOR THE MICROPROPAGATION OF PINEAPPLE[1]

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ABSTRACT - The present work aimed at maximizing the number of plantlets obtained by the micropropagation of pineapple (*Ananas comosus* (L.) Merrill) cv. Pérola. Changes in benzylaminopurine (BAP) concentration, type of medium (liquid or solidified) and the type of explant in the proliferation phase were evaluated. Slips were used as the explant source, which consisted of axillary buds obtained after careful excision of the leaves. A Sterilization was done in the hood with ethanol (70%), for three minutes, followed by calcium hypochlorite (2%), for fifteen minutes, and three washes in sterile water. The explants were introduced in MS medium supplemented with 2mg L^{-1} BAP and maintained in a growth room at a 16h photoperiod (40 $mmol.m^{-2}.s^{-1}$), 27 ± 2°C. After eight weeks, cultures were subcultured for multiplication in MS medium. The following treatments were tested: liquid x solidified medium with different BAP concentrations (0.0, 1.5 or 3.0 mg L^{-1}), and the longitudinal cut, or not, of the shoot bud used as explant. The results showed that liquid medium supplemented with BAP at 1.5 mg L^{-1}, associated with the longitudinal sectioning of the shoot bud used as explant presented the best results, maximizing shoot proliferation. On average, the best treatment would allow for an estimated production of 161,080 plantlets by the micropropagation of the axillary buds of one plant with eight slips and ten buds/slips, within a period of eight months.

Index terms: *Ananas comosus*, tissue culture, *in vitro* culture, *in vitro* clonal propagation.

OTIMIZAÇÃO DO PROTOCOLO DE MICROPROPAGAÇÃO DO ABACAXIZEIRO

RESUMO - O objetivo do trabalho foi maximizar o número de brotações, buscando adequar o protocolo de micropropagação do abacaxizeiro-'Pérola', pela manipulação de concentrações de BAP, do estado físico do meio de cultura e do seccionamento das brotações na fase de proliferação. Mudas do tipo filhote foram utilizadas como fonte de explantes, que se constituíram de gemas axilares extraídas após a eliminação das folhas. A desinfestação procedeu-se com álcool 70% (três minutos) e, posteriormente, com hipoclorito de cálcio 2% (quinze minutos). Em seguida, os explantes foram estabelecidos em frascos contendo 15 mL do meio de cultura MS sólido, suplementado com 2,0 mg L^{-1} de BAP e permaneceram por 60 dias em câmara de crescimento. Na fase de proliferação, estudaram-se os meios de cultura MS sólido e líquido, as concentrações de BAP (0,0; 1,5 e 3,0 mg L^{-1}) e o seccionamento longitudinal das brotações. Concluiu-se que o meio MS líquido favoreceu a indução de brotações. O seccionamento das brotações mostrou-se fundamental no aumento da taxa de multiplicação, e a concentração 1,5 mg L^{-1} de BAP promoveu a melhor resposta para o número de brotações. O trabalho demonstrou que se pode obter até 161.080 plântulas de abacaxi, no final de oito meses, partindo de uma única planta com oito filhotes e dez gemas axilares/filhote.

Termos para indexação: *Ananas comosus*, cultura de tecidos, cultivo *in vitro*, propagação clonal *in vitro*.

INTRODUCTION

In the world scenario, Brazil occupies the third position in pineapple production, following by Thailand and Philippines (FAO, 2001). Among the Brazilian states, Paraíba, Minas Gerais, Pará, Bahia and Rio Grande do Norte are the lead producers (IBGE, 2001).

The two most important pineapple varieties (*Ananas comosus* (L.) Merrill) cultivated in Brazil are 'Pérola' and 'Smooth Cayenne', which are highly susceptible to fusarium wilt (*Fusarium subglutinans* WR), the most serious disease of this crop in Brazil, causing considerable production losses (Cunha et al., 1994).

Besides fusarium wilt, other problems affect the commercial production of pineapple in Brazil, such as the lack of high quality propagules, low rate of multiplication of plants by conventional methods and the lack of matrix plants have been limiting for pineapple culture in Brazil (Ruggiero et al., 1994). The need to solve these problems, producing better and clean propagules, improving the rate of plant multiplication and a faster multiplication of elite genotypes, led to the development of tissue culture techniques for the pineapple (Almeida, 1994).

Many authors have reported success in the micropropagation of pineapple. According to Drew (1980), is possible to produce 1.250.000 plantlets of pineapple in eight months, starting with 30 explants. Almeida et al. (1997) studying the influence of BAP on *in vitro* proliferation of pineapple, determined the possibility of obtaining 6.575 plants of pineapple in

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seven months from one plant.

The success of the micropropagation procedure depends to several factors, which should be observed during the process. Some authors have reported a optimization procedures for the micropropagation of pineapple. Almeida (1994) using different growth regulators, observed that 4.0 or 5.0 mg L^{-1} BAP in the culture medium were responsible for high rates of explant death, and that 2.0 mg L^{-1} BAP led to the best differentiation response. Later, Almeida et al. (1997) verified that 3.0 mg.L^{-1} BAP, combined with 2.0 mg L^{-1} indole acetic acid (IAA), during the establishment phase, was the combination that gave the best results in the later development of plantlets. Kiss et al. (1995) cultivated *in vitro* plantlets in the dark for 30 to 40 days, followed by the cultivation of etiolated nodal segments in culture medium supplemented with BAP and kinetin, obtaining the best result for a medium with 5.0 mg L^{-1} kinetin (KIN).

The objective of this work was to define an efficient protocol for the micropropagation of pineapple, cv. Pérola, by the manipulation of BAP concentrations, type of culture medium and sectioning of the axillary buds used as explant, aiming at the maximization of the rate of multiplication for this cultivar.

MATERIAL AND METHODS

Slips of pineapple, cv. 'Pérola' were used as explant sources, after removing the leaves carefully one by one, following their philotaxy, for the exposure of the axillary buds. The buds were then excised with a segment of the subtending stem tissue, forming a cube shaped explant of approximately $5mm^3$.

In the aseptic hood, disinfestation was done by a immersion of the explants in 70% of ethanol solution, for three minutes, three rinses in sterile distilled water and immersion in 2% of calcium hypochlorite solution for fifteen minutes, followed by four rinses in sterile distilled water. Under aseptic conditions, the most external tissue was eliminated and the explants were individually introduced in to 100-mL-flasks containing 15 mL of MS medium (Murashige & Skoog, 1962) supplemented with 30.0 g L^{-1} of sucrose, 7.0 g L^{-1} of agar and 2.0 mg L^{-1} of BAP. The cultures were maintained in the growth room at 27ºC ± 2ºC and a 16h photoperiod (40 $mmol.m^{-2}.s^{-1}$) during 60 days.

To define the best conditions for the proliferation of shoots, different treatments were defined, varying the type of medium (liquid or solidified MS medium), the concentrations of BAP (0.0; 1.5 or 3.0 mg L^{-1}), and the type of explant (entire shoot, or shoot longitudinally sectioned into halves). The experiment was laid out in a completely randomized factorial design (2x3x2) with 2 types of media, 3 concentrations of BAP and 2 types of explant, with four replications.

During the proliferation stage, five subcultures were done at intervals of four weeks, to the same proliferation medium, and the cultures were maintained in the growth room under the same conditions of illumination and temperature used during the introduction stage. After the final cultivation cycle, the shoots were individualized and the number of shoots per initial explant in each treatment was counted. The data obtained were transformed using $(x + 1.0)^{1/2}$, and the means compared using the Tukey test at 5% of probability.

The shoots were transferred to MS medium with half the concentration of salts, supplemented with 30 g L^{-1} sucrose, without growth regulators, for 30 days. The rooted plantlets were then transferred to disposable plastic cups (200 mL) with sterile substratum and maintained in a shaded greenhouse for 30 days, when they were transferred to the field.

RESULTS AND DISCUSSION

The analysis of the data showed significant effects for all the parameters tested, type of explant, type of culture medium and BAP concentration, and also for the interaction explant x culture medium x BAP concentration. The Table 1 shows the isolated effect of the culture medium, with a better response when liquid medium was used. The mean number of shoots per

TABLE 1 - Mean number of shoots of pineapple, cv. Pérola, produced per explant after five subcultures, under different treatments[1]. Cruz das Almas-BA, 2000.

Explant	Medium type and BAP concentration (mg L^{-1})						
	Liquid medium			Solidified medium			
	0.0	1.5	3.0	0.0	1.5	3.0	Mean
Whole bud	0.25aB	250.75aA	145.5aA	1.0aB	14.0aB	3.5aB	69.1a
Bud sectioned longitudinally	24.5aCD	2013.5bA	890.75bB	8.75aD	527.0bB	218.0bBC	613.7b

	Liquid medium	Solidified medium
Mean	554.2A	128.7B

	BAP concentration (mg L^{-1})		
	0.0	1.5	3.0
Mean	8.6C	701.1A	314.4B

Means followed by the same letter do not differ significantly (Tukey, 0.05) (lower-case in columns and upper-case in line)
[1] The original data was transformed using $(x + 1.0)^{1/2}$

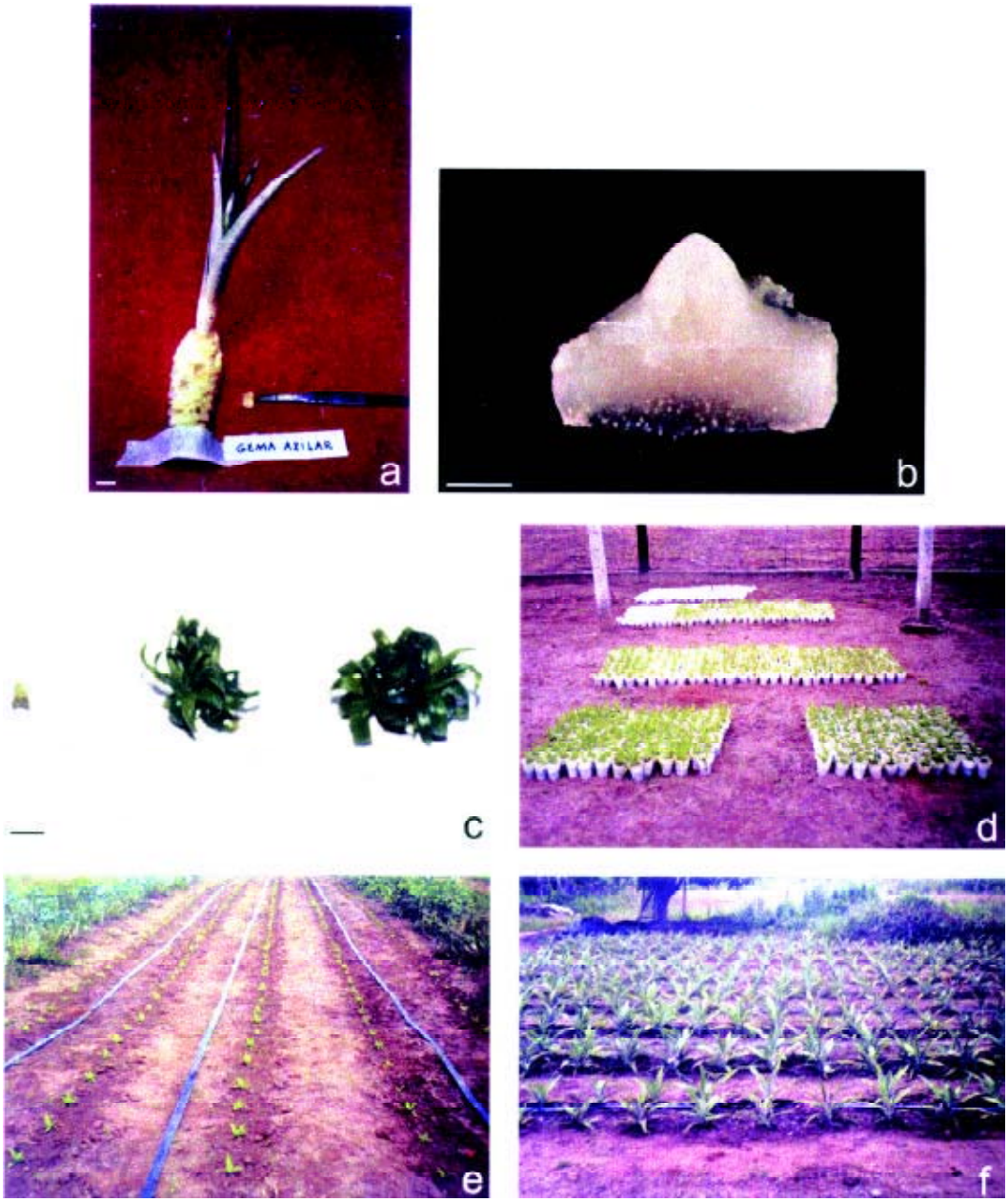

FIGURE 1 - Micropropagation of pineapple, cv. Pérola. a) slip used as explant source after removal of the leaves to expose the axillary buds; b) axillary bud used as explant; c) shoot multiplication *in vitro*; d) plant acclimatization in the greenhouse; e-f) plants outplanted in the field, with ten (e) and fourteen (f) months. Bars = 1.0 cm (a, c); 1.0 mm (b).

explant in liquid medium was 554.2, which was significantly higher than in solidified medium, with an average of 128.7 shoots per explant. According to Gupta et al. (1981), the use of liquid medium facilitates the nutrient uptake by the plant due to its distribution in the culture medium. The higher shoot production in liquid medium was probably favored by the better nutrient uptake. Similar results were obtained by Guerra et al. (1999) with higher regeneration rates in liquid medium with pineapple, cvs. Perolera and Primavera.

The longitudinal sectioning of the shoots used as explants proved to be a very good technique to improve the multiplication of shoots. The average number of shoots per explant was 613.7, compared to only 69.1 shoots/explant when the whole shoot was used as explant (Table 1). Sectioning in meristematic regions can induce cell division, due to the high mitotic ability of these cells (George, 1993). The stimulation of cell division, caused by the sectioning of the shoot, probably contributed for the differentiation of a higher number of adventitious buds and consequently a higher number of shoots produced per explant.

The use of 1.5 mg L^{-1} BAP during the proliferation stage was the best treatment. An average of 701.1 shoots per explant was obtained in medium with 1.5 mg.L^{-1}, as shown in the Table 1. The effect of BAP levels on the micropropagation of pineapple has been reported (Pescador & Koller, 1992; Kiss et al., 1995;

Almeida et al., 1997; Guerra et al. 1999). According to Albuquerque et al. (2000), the use of BAP in MS medium was essential for the regeneration of plants from shoot apices of pineapple, aiming at plants free of *Fusarium*. Paiva et al. (1998) obtained the best results in the shoot induction of pineapple, cv. Skay, with either 1 mg L^{-1} BAP or 0.1 mg L^{-1} TDZ. Barbosa & Caldas (2001) working with etiolated segments for micropropagation of the pineapple hybrid PE x SC-52, observed that BAP promoted the highest number of plants per shoot and per nodal segment, when compared with KIN, or a combination of BAP and naphtaleneacetic acid (NAA). Grattapaglia & Machado (1998) cited BAP as the best cytokinin for the multiplication of aerial plant parts and for the induction of adventitious shoots.

The combined analysis of the effects of the type of explant, type of medium and BAP concentrations (Table 1) demonstrated that the use of the longitudinally sectioned shoot bud as explant associated with the multiplication in liquid MS medium with 1.5 mg L^{-1} BAP, provided the best response in terms of *in vitro* proliferation. An average of 2013.5 shoots per explant was obtained after five subcultures, with this result being significantly superior to all the other treatments tested.

According to the number of plantlets obtained in this treatment, it can be calculated a production of 161.080 plants after eight months, starting from only one plant with an average of eight slips and ten axillary buds each. This would be enough for cultivating about 2.3 ha in high planting density (70.000 plants/ha) (Santana et al., 2001). Using the conventional method of pineapple propagation, it would take seven years and six months to obtain 32.700 plants from one initial plant, which would be enough to plant approximately 0.5 ha (Matos et al., 1988). This comparison demonstrates the advantage of micropropagation of pineapple over the conventional propagation method. The micropropagation not only provides higher rates of multiplication, but also the time and area needed is much smaller when compared to *in vivo* vegetative propagation methods. The plants obtained in this experiment were rooted *in vitro* and transferred to the greenhouse and when the plants were approximately 15-20 cm high and 150 g fresh weight were transfered to the field, with good adaptation, as shown in Figure 1.

CONCLUSION

In the pineapple cv. Perola micropropagation, the use of liquid MS medium during the multiplication stage increases the production of adventitious buds. Sectioning the shoot agglomerates at subculturing increases the adventitious shoot production. The use of BAP at 1.5 mg L^{-1} during the multiplication stage is fundamental to maximize the number of adventitious buds formed.

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