GROWTH REGULATORS AND PHYSICAL STATE OF CULTURE MEDIA IN THE MICROPROPAGATION OF ORNAMENTAL PINEAPPLE HYBRIDS

REGULADORES DE CRESCIMENTO E ESTADO FÍSICO DO MEIO DE CULTURA NA MICROPROPAGAÇÃO DE HÍBRIDOS DE ABACAXI ORNAMENTAL

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ABSTRACT

The aim of this work was to evaluate the effect of different combinations of two growth regulators (6-benzilaminopurin - BA and naphthalene acetic acid - NAA) in solid and liquid culture media on the micropropagation of ornamental pineapple in order to produce shoots and seedlings in large scale. Buds obtained from pineapple's crown were used as initial explants. The explants were cultivated in MS media supplemented with 0.89 µM BAP, 0.05 µM NAA and 3% sucrose. Forty five days after culture the buds were transferred to different media. The factorial combinations were three ornamental pineapple hybrids (FRF-1392 X FRF-32, G-44 X FRF-1387 and Carauá Roxo X Ananás Tricolor); two physical culture medium states (solid and liquid); four culture media (M01 – MS control; M02 – MS + 2.68 µM NAA; M03 – MS + 2.22 µM BAP; M04 – MS + 2.68 µM NAA + 2.22 µM BAP), consisting of 24 treatments. After 45 and 90 days of culture in growth chamber, number of shoots, height of shoots and number of roots were evaluated. Data were analyzed using analysis of variance and comparisons of means were conducted using the Tukey test at 1% probability. Significant differences were observed among the three hybrids under different treatments. The highest multiplication rates were obtained with M03 and M04 liquid culture medium and the best results were obtained with the FRF-1392 X FRF-32 hybrid.

Index terms: *Ananas comosus*, tissue culture, multiplication rate, propagation.

RESUMO

Conduziu-se este trabalho, com o objetivo de avaliar os efeitos de duas diferentes combinações de reguladores de crescimento (6-benzilaminopurina - BAP e ácido naftalenoacético ANA), em meios de cultura líquido e gelificado, visando à proliferação de brotos e a produção de mudas em larga escala de três híbridos de abacaxi ornamental. Foram utilizadas gemas da coroa do fruto como explante de partida. Os explantes foram cultivados em meio de cultura MS, suplementado com 0,89 µM de BAP, 0,05 µM de ANA e 3% de sacarose. Aos 45 dias após incubação, os brotos foram transferidos para diferentes meios. As combinações fatoriais foram: três híbridos de abacaxi ornamental (FRF-1392 X FRF-32, G-44 X FRF-1387 and Carauá Roxo X Ananás Tricolor); dois estados fisicos do meio de cultura (sólido e líquido); quatro meios de cultura (M01 - MS controle; M02 - MS + 2,68 µM de ANA; M03 - MS + 2,22 µM de BAP; M04 - MS + 2,68 µM de ANA + 2,22 µM de BAP), totalizando 24 tratamentos. Após 45 e 90 dias de cultivo em sala de crescimento, avaliou-se o número médio de brotos, altura da parte aérea e número de raízes. Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Tukey a 1% de probabilidade. Houve resposta diferenciada entre os três híbridos avaliados para as características estudadas. As maiores taxas de multiplicação foram obtidas com os meios de cultura M03 e M04 e os melhores resultados foram obtidos com o híbrido FRF-1392 X FRF-32.

Termos para indexação: *Ananas comosus*, cultura de tecidos, taxa de multiplicação, propagação.

INTRODUCTION

The economic potential of tropical flowers resides in their beauty and exotic profile, which caught the attention of Brazilian and foreign consumers. In order to meet the increasing demand of tropical flowers, a genetic improvement program focused on ornamental pineapple hybrids was initiated at Embrapa Cassava and Fruits. The program aims at the production of flowers with specific traits to be used in flower bouquets, landscaping and to be grown in pots (SOUZA et al., 2009a).

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Plant tissue culture for micropropagation is a useful technique to obtain healthy plants that has been applied successfully in the large scale production of commercial varieties of pineapple (ALMEIDA et al., 2002; PASQUAL et al., 2008; SANTOS et al., 2008; CARVALHO et al., 2009; FRÁGUAS et al., 2009; SOUZA et al., 2009b). However, studies with wild varieties of the *Ananas* genus showed low multiplication rates and presence of endogenous bacteria which suggest that the protocols must be reviewed (DIAS et al., 2008; SOUZA et al., 2009b). The ornamental pineapple hybrids obtained by the genetic improvement program resulted from the crossing of wild varieties (SOUZA et al. 2009a). Therefore, in order to improve multiplication and avoid contamination, similar studies as those performed with the commercial varieties were undertaken (ARAUJO et al., 2008; FRÁGUAS et al., 2009).

The *in vitro* response of a plant depends on several factors such as species, genotype, initial explant and culture medium. However, culture medium adjustment is one of the most relevant factors to obtain high multiplication rates and healthy plants.

The aim of this research was to evaluate the effect of the addition of BAP and NAA to solid and liquid culture media on the production in large scale of ornamental pineapple shoots and seedlings.

MATERIAL AND METHODS

Plant material consisted of buds obtained from crown of three ornamental pineapple *Ananas comosus* L. F1 hybrids developed by the genetic improvement program at Embrapa Cassava and Fruits.

The buds were dipped in alcohol 70% for 5 minutes, immersed in a solution of sodium hypochlorite 1% with 3 drops of surfactant Tween-20® for 20 minutes, and rinsed three times with sterile distilled water.

In the establishment stage, the buds were inoculated into 14 x 100 mm tubes that contained 10 mL MS culture medium (MURASHIGE; SKOOG, 1962) supplemented with 0.89 µM BAP, 0.05 µM NAA and 3% sacarose solidified with 0.2% Phytagel® the pH was adjusted to 5.8. The cultures were incubated in growth chamber at 27º C ± 1º C, under a 16 h photoperiod and photon flux density of 30 µEmol $m^{-2} s^{-1}$ for 45 days. After this period the explants were transferred to the experimental flasks. Culture procedures were the same as in the establishment stage. A 3x2x4 factorial experiment with a completely randomized design was then conducted. The factorial combinations were three ornamental pineapple hybrids: FRF-1392 [*A. comosus* var. *erectifolius* (L.B. Smith) Coppens & Leal] X FRF-32 [*A. comosus* var. *bracteatus* (Lindl.) Coppens & Leal], G-44 [*A. comosus* var. *ananassoides* (Baker) Coppens & Leal] X FRF-1387 [*A. comosus* var. *erectifolius*] and Carauá Roxo [*A. comosus* var. *erectifolius*] X Ananás Tricolor [*A. comosus* var. *bracteatus*]; two culture medium physical states (liquid and solid), and four culture media (M01 = MS control; M02 = MS + 2.68 µM NAA; M03 = MS + 2.22 µM BAP; M04 = MS + 2.68 µM NAA + 2.22 µM BAP). Each of the 24 treatments was replicated 12 times in two flasks with six explants each. The cultures established in liquid medium were agitated at 120 rpm. They were inoculated in growth chamber at 27º C ± 1º C, under a 16 h photoperiod and photon flux density of 30 µEmol $m^{-2} s^{-1}$.

After 45 and 90 days of incubation in growth chamber, average number of shoots, shoot height (mean of all shoots height) (cm) and number of roots (mean of the number of all roots per shoots) per explant were measured. The potential number of plants that could be produced after appropriate treatment of each genotype was estimated according to $T = y\,x^n$, where T= number of seedlings; y = initial number of explants; x = propagation rate; n = number of recultures (ESCOBAR, 2002).

Data were analyzed using analysis of variance and comparisons of means were conducted using the Tukey test at 1% probability. Statistical analysis was performed with the SAS software (SAS INSTITUTE, 2004).

RESULTS AND DISCUSSION

In the establishment phase, 51 and 26 % of buds from FRF-1392 X FRF-32 and G-44 X FRF-1387 hybrids,

respectively, were contaminated with bacteria, which suggest the disinfection process of the explants was not efficient or the explants contained endophytic bacteria.

Preliminary tests performed in the Tissue Culture Laboratory at Embrapa Cassava and Fruits showed that during the establishment stage bacterial contamination of micropropagated wild pineapple varieties was higher than the contamination observed in micropropagated commercial varieties (SILVEIRA et al., 2006).

Endophytic bacteria can cause severe losses to *in vitro* cultivated plant material, and depending on their concentration in the tissues, these microorganisms can hinder micropropagation by affecting all the stages of the process (SILVEIRA et al., 2006; SOUZA et al., 2009a). Therefore, better disinfection procedures should be developed when working with ornamental pineapple explants originated mainly from wild varieties.

Twenty five days after establishment bud intumescence and leaves were observed. Average number of shoots, shoot height and root number were significantly different (P<0,01) among the three factors (genotype, culture medium physical state and culture medium) and among the interactions of two or three of them.

The statistical analysis of the data obtained after 45 and 90 days of incubation is shown in Table 1, respectively.

TABLE 1 – Average number of shoots, shoot height (cm) and number of roots after 45 and 90 days of *in vitro* culture of ornamental pineapple hybrids submitted to different treatments.

Treatments		Average number of shoots			Shoot height (cm)			Number of roots		
		FRF1392 X FRF32	G44 X FRF1387	Carauá X Tricolor	FRF1392 X FRF32	G44 X FRF1387	Carauá X Tricolor	FRF1392 X FRF32	G44 X FRF1387	Carauá X Tricolor
45 days										
Liquid	M01	0.75aC	0.75aB	1.83aB	5.27aA	4.18aA	5.23aA	3.91bA	3.33bA	4.91aA
	M02	2.18bB	1.50cB	3.25aB	4.54aA	3.73aA	4.33aA	2.36bB	1.50bB	3.91aB
	M03	5.16aA	3.91bA	5.50aA	4.26aAB	1.01bC	3.35aB	0.50aC	0.16aC	0.58aC
	M04	7.76aA	3.66bA	5.25aA	3.27aB	2.43aB	1.30bC	0.25aC	0.00aC	0.00aC
Solid	M01	0.16aB	0.16aA	0.41aB	3.52aA	3.24aA	2.21bA	2.58aA	2.91aA	2.00aA
	M02	0.33aB	0.41aA	0.50aB	2.81baA	2.42abA	2.33aA	2.50aA	2.50aA	2.50aA
	M03	3.58aA	0.66bA	2.41aA	2.11aB	1.51abB	0.88bB	0.00aB	0.00aB	0.66aB
	M04	4.41aA	0.66bA	1.36bA	2.49baA	1.98abB	0.94bB	0.25aB	0.00aB	0.00aB
CV(%)			30.13			38.76			69.66	
90 days										
Liquid	M01	1.25aB	1.66aC	2.50aB	8.66aA	6.80bA	8.53aA	7.16aA	5.66aA	6.91aA
	M02	2.45aB	2.83aB	3.91aB	6.77aB	6.08abA	6.68aB	3.27bB	2.75cB	5.08aA
	M03	8.58aA	5.75bA	7.08aA	6.56aB	1.68cC	4.55bC	1.41aC	0.16aC	1.00aB
	M04	11.16aA	5.25cA	8.75bA	5.37aB	4.40aB	1.78bD	0.50aC	0.00aC	0.00aB
Solid	M01	0.91aB	0.33aA	0.75aA	5.45aA	5.20aA	3.57bA	4.33aA	4.00aA	4.08aA
	M02	0.58aB	0.83aA	1.08aA	4.85aAB	3.65aB	3.41aA	4.25aA	3.66aA	3.91aA
	M03	4.91aA	0.91bA	3.00aA	3.65aB	2.50abC	1.06bB	0.16aB	0.00aB	0.83aB
	M04	5.91aA	1.25bA	2.18bA	4.14aAB	3.02aC	1.26bB	0.83aB	0.16aB	0.00aB
CV(%)			38.11			37.02			69.44	

Means followed by the same lowercase letter in the row and uppercase letter in the column within the same factor are not significantly different according to the Tukey test ($p \leq 0,01$).

In general the largest number of shoots was observed in M03 and M04 liquid media in all micropropagated genotypes after 45 and 90 days of culture. This finding suggests that the addition of BAP is relevant for axillary shoot production. Number of shoots, shoot height and number of roots were higher in liquid than in solid medium for all genotypes and treatments, as shown in Figure 1. Similar results, which liquid was considered better than solid medium have also been reported for micropagation of pineapple plants and are probably due to the homogeneity of the liquid medium, which allows faster diffusion and absorption of nutritional elements by the tissues (COSTA; ZAFFARI, 2005). Moreover, liquid media do not require gelling agent to solidify medium, reducing the production costs (ALMEIDA et al., 2002; BORGES et al., 2003).

Despite the absent of significative difference between M03 and M04 the first one was selected to estimate seedlings productions. The projection for the potential production of seedlings from an *in vitro* established bud with 6 recultures would be 218,358.60 plants for the cross FRF-1392 X FRF-32; 2,403.73 for the hybrid G-44 X FRF-1387 and 20,938.99 for the cross Carauá Roxo X Ananás Tricolor (Table 2). According to Souza et al. (2009b), to avoid somaclonal variations, the number of subcultures should be limited to a maximum of six or to the initial production of 3,000 plants/explants.

Low number of shoots per explant was observed after 45 and 90 days in all the varieties cultured in media without BAP (M01). However at almost all the time, M02 also presented a lower number of shoots when compared with results from M03 and M04. Although shoot height and root formation of explants was satisfactory, culture media without BAP were considered not efficient for the multiplication of the three varieties of ornamental pineapple studied. Similar results were reported by Santos et al. (2008) who worked with *A. comosus* var. *bracteatus*, a variety of ornamental pineapple. Our results are consistent with previous works that showed that best height and root formation in pineapple seedlings is achieved in medium without BAP (ZEPEDA; SAGAWA, 1981; KISS et al., 1995; PASQUAL et al., 2008; FRÁGUAS et al., 2009).

Micropopagation in medium that contain BAP and NAA has been studied in several species of the Ananas genus (BORGES et al., 2003; COSTA; ZAFFARI, 2005; FRÁGUAS et al., 2009). However, the ideal combination of BAP and NAA to maximize morphogenetic response is genotype-dependent. The results of the current research indicate a strong effect of genotype on the in vitro morphogenetic response of the pineapple varieties evaluated. An implication of this finding is the need to develop specific protocols for each genotype in order to obtain satisfactory multiplication rates.

Furthermore, the present study found that the addition of BAP is essential for *in vitro* shoot induction. Cytokinins act on several developmental processes of plants such as cell division and culture differentiation (REDIG et al., 1996), morphogenetic responses (CENTENO et al., 1996), and organ development (AUER; COHEN, 1993).

Shoot height and number of roots are important variables to estimate plant development. The best shoot height values for all the micropropagated hybrids were obtained in plants cultured in M01 (MS) and M02 (MS + 2.68 µM NAA). The largest height values (8.53 and 8.66) were observed in the hybrids Carauá X Ananás Tricolor and FRF-1392 X FRF-32, respectively, cultured in liquid MS medium after 90 days (Table 1). On the other hand, the largest number of roots was observed in the hybrids Carauá Roxo x Ananás Tricolor (4.91 roots/explant after 45 days). It was not observed statistical difference among the genotypes cultivated in liquid MS medium. The FRF-1392 X FRF-32 and G-44 X FRF-1387 hybrids presented the highest number of roots in the liquid MS medium without any growth regulators (M01).

Although the addition of low concentrations of auxins results in biomass development in bromeliads (SANTOS et al., 2008), in regard to pineapple, MS media without growth regulators have been used mainly during *in vitro* shoot elongation and root formation (SOUZA et al., 2009b). Fráguas et al. (2009) and Pasqual et al. (2008),

who worked with the variety cv. 'IAC Gomo-de-mel' and ornamental pineapple, respectively, found that best responses regarding shoot height were obtained in MS medium without growth regulator. Their findings are consistent with the results obtained in the present study. Zepeda and Sagaya (1981) and Kiss et al. (1995) also used MS medium without growth regulator for *in vitro* root formation of pineapple plants and they considered this

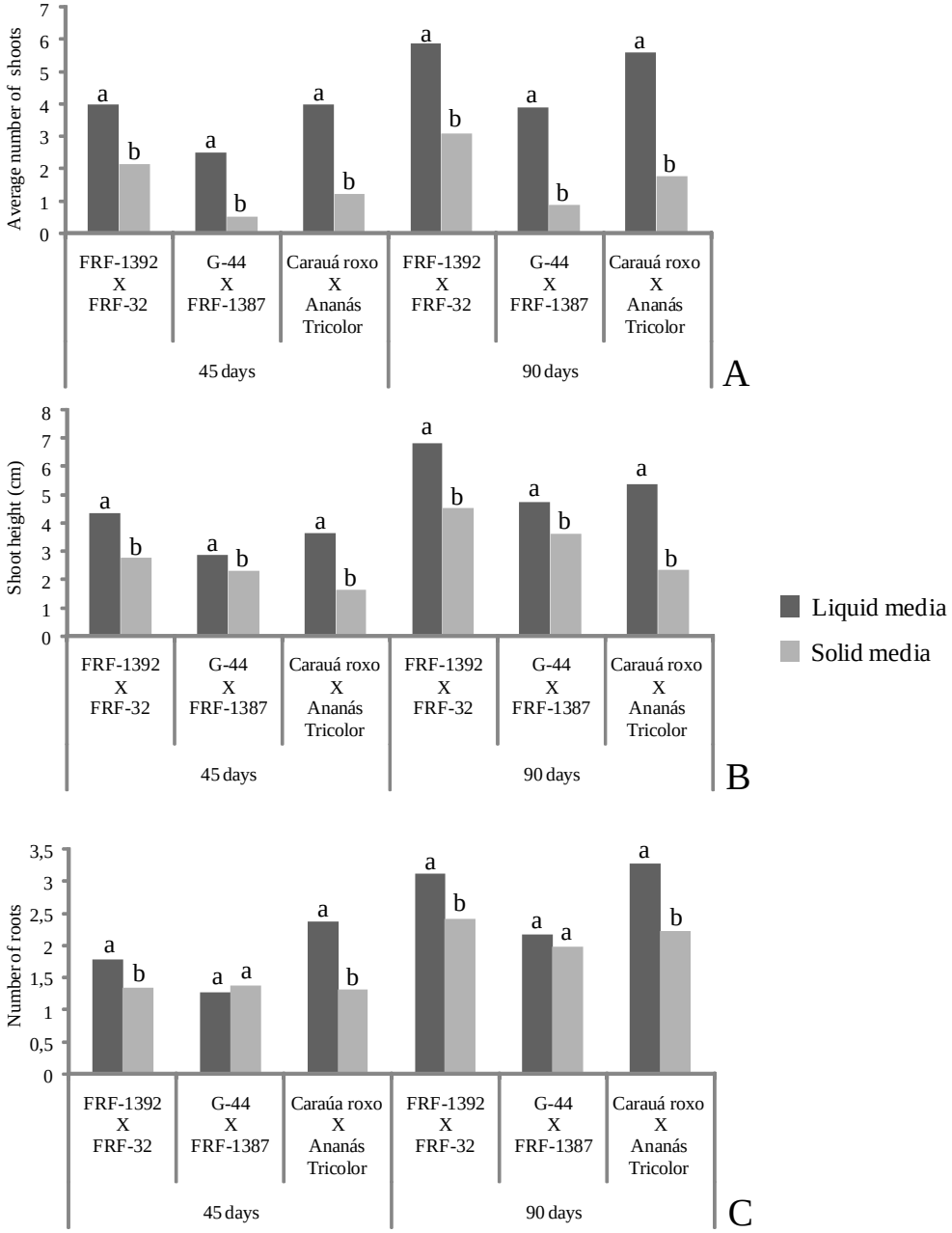

FIGURE 1 – Average number of shoots (A), shoot height (cm) (B) and number of roots (C) produced by ornamental pineapple hybrids after 45 and 90 days of culture in solid and liquid media. Means followed by the same letter are not significantly different according to the Tukey test ($p \leq 0,01$).

procedure suitable for automation in pineapple micropropagation. Besides, it reduces expenses with growth regulators, which in a commercial-scale production might result in a significant price reduction of the seedlings. Furthermore, the study of Macedo et al. (2003) with axillary buds of cv. Pérola confirmed that the presence of BAP in the culture medium inhibits root formation, while NAA stimulates it.

The development of the root system and aerial parts is essential for successful transplantation. Short roots are preferred, because they facilitate washing and removal of adhered culture medium, as well as transplanting (SOUZA et al. 2009b).

In order to ensure plant survival during the acclimatization stage a large number of roots are required. With regard to pineapple plants, old roots have to be removed to promote the development of new and functional ones (SOUZA et al., 2009b).

Finally, we would like to note that was not observed any morphological variation in all the treatments. The differences among micropropagated genotypes in various solid and liquid culture media and the morphology of the plants after 90 days of culture can be seen in Figure 2.

TABLE 2 – Estimated production based on the number of seedlings produced in six recultures in the best culture media for each ornamental pineapple hybrid [(1)].

Reculture	FRF-1392 X FRF-32	G-44 X FRF-1387	Carauá X Tricolor
1	7.76	3.66	5.25
2	60.21	13.40	27.56
3	467.29	49.03	144.70
4	3,626.16 [(2)]	179.44	759.69
5	28,140.00 [(3)]	656.76	3,988.38 [(2)]
6	218,358.60 [(3)]	2,403.73[(2)]	20,938.99 [(3)]

[(1)] Liquid culture medium M04 (MS + 2.68 µM de ANA + 2.22 µM de BAP) after 45 days of culture; [(2)] suggested limit; [(3)]Not recommended.

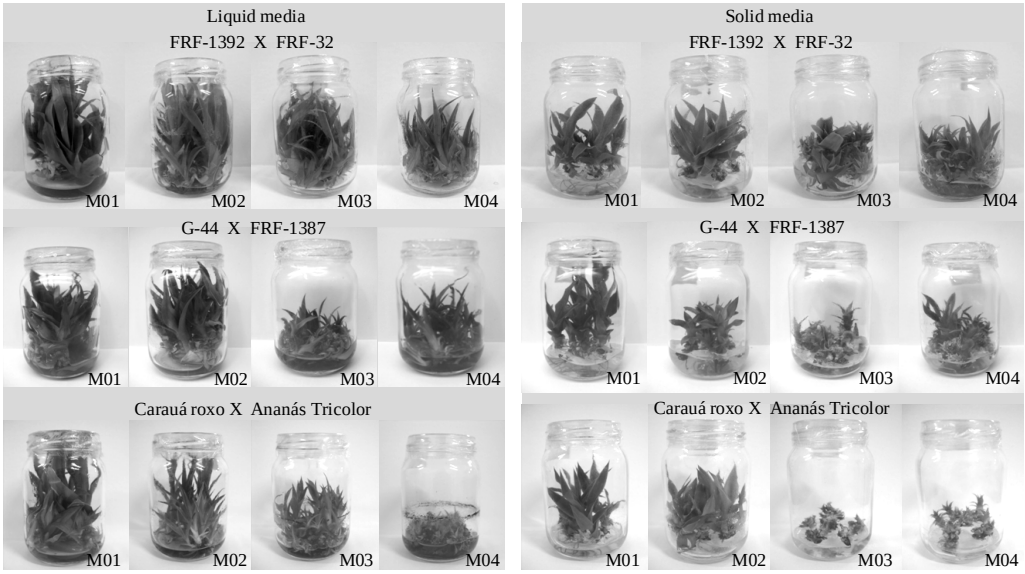

FIGURE 2 – Effect of different treatments on *in vitro* multiplication of three ornamental pineapple hybrids after 90 days of incubation.

CONCLUSIONS

The addition of BAP in the MS culture medium was relevant for the *in vitro* production of large number of shoots in the three hybrids evaluated. This growth regulator was not needed to improve rooting and growth.

Liquid culture medium is the best medium for the micropropagation of ornamental pineapple hybrids.

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REFERENCES

ALMEIDA, W. A. B. et al. Optimization of a protocol for the micropropagation of pineapple. **Revista Brasileira de Fruticultura,** v.24, n.2 p. 296-300, 2002.

ARAUJO, R. F.; SIQUEIRA, D. L.; CECON, P. R. Multiplicação *in vitro* do abacaxizeiro 'Smooth Cayene' utilizando benzilaminopurina (BAP) e ácido naftalenoacético (ANA). **Revista Ceres,** v.55, n.5, p.455-460, 2008.

AUER, C. A.; COHEN, J. D. Identification of a benzyladenine disaccharide conjugate produced during shoot organogenesis in Petunia leaf explants. **Plant Physiology,** v.102, n.2, p.541-545, 1993.

BORGES, N. S. S.; CORREIA, D.; ROSSETTI, A. G. Influência do meio bifásico na multiplicação de gemas e no alongamento de brotos *in vitro* de *Ananas lucidus* Miller. **Revista Brasileira de Horticultura Ornamental,** v.9, n.1, p.37-44, 2003.

CARVALHO, A. C. P. P. et al. Multiplicação *in vitro* de abacaxi ornamental por estiolamento e regeneração de brotações. **Horticultura Brasileira,** v.27, n.1, p.103-108, 2009.

CENTENO, M. L. et al. Relationship between endogenous auxin and cytokinin levels and the morphogenic responses in *Actinidia deliciosa* tissue cultures. **Plant Cell Reports,** v.16, n.1-2, p.58-62. 1996.

COSTA, T. ZAFFARI, G. R. Micropropagação de *Ananas bracteatus* (Schultz) var. *striatus* Hort. **Revista Brasileira de Horticultura Ornamental**, v.11, n.2, p.109-113, 2005.

DIAS, G. M. G. et al. Micropropagação de abacaxi ornamental (*Ananas comosus* var. *ananassoides*) por estiolamento e regeneração de plântulas. **Plant Cell Culture & Micropropagation**, v.4, n.1, p. 1-54, 2008.

ESCOBAR, R.H. **Sistemas de propagación rápida in vitro de la yuca.** CIAT. Cali. Colômbia. 2002.

FRÁGUAS, C. B.; DORNELLES, C. M. V.; LIMA, G. P. P. *In vitro* bud induction and multiplication of cv. 'IAC Gomo-de-mel' pineapple fruit with benzyl amino purine and naphthalene acetic acid. **Ciência Rural,** v.39, n.6, p.1682-1687, 2009.

KISS, E. et al. A novel method for rapid micropropagation of pineapple. **HortScience,** v.30, n.1, p.127-129, 1995.

MACEDO, C. E. C. et al. Concentrações de ANA e BAP na micropropagação de abacaxizeiro L. Merrill (*Ananas comosus*) e no cultivo hidropônico das plântulas obtidas *in vitro*. **Revista Brasileirade Fruticultura**, v.25, p. 501-504, 2003.

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

PASQUAL, M. et al. Micropropagação do abacaxizeiro ornamental. **Horticultura Brasileira**, v.26, n.1, p.45-49, 2008.

REDIG, P. et al. Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during cell cycle of synchronized tobacco BY-2 cells. **FEBS Letters**, v.391, n.1-2,p.175-180, 1996.

SANTOS, M. D. M.; RIBEIRO, D. G.; TORRES, A. C. Brotações adventícias de abacaxizeiro ornamental sob o efeito de benzilaminopurina, ácido naftalenoacético e períodos de subcultivo. **Pesquisa Agropecuária Brasileira**, v.43, n.9, p.1115-1120, 2008.

SAS INSTITUTE. **SAS user's guide**: statistic: version 6.12. Cary: SAS Institute, 2004. 846p.

SILVEIRA, D. G. et al. *In vitro* production of wild pineapple seedlings with ornamental value. *In:* XIX CONGRESSO BRASILEIRO DE FRUTICULTURA. **Frutas do Brasil: saúde para o mundo.** Cabo Frio, p. 367. 2006.

SOUZA, F. V. D. et al. Evaluation of F1 hybrids between *Ananas comosus* var. *ananassoides* and *Ananas comosus* var. *erectifolius*. **Acta Horticulturae**, v.822, p.79-84, 2009a.

SOUZA, F. V. D. et al. **Microprogagação do Abacaxizeiro e outras Bromeliáceas.** *In:* JUNGHANS, T. G.; SOUZA, A. S., (eds.) Plant micropropagation: practical issues. Embrapa CNPMF: Cruz das Almas, p.177-205. 2009b.

ZEPEDA, C.; SAGAWA, Y. *In vitro* propagation of pineapple. **HortScience**, v.16, p.495. 1981.