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Propagation of Pineapple by Shoot Tip Culture

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Abstract: This study was conducted with the objective of modifying the composition of MS medium for the clonal propagation of pineapple using shoot tips of "Smooth Cayenne" cultivar. Modifications were made on various medium components. Results showed that both MS salts at the full or half strength were optimal, and there was no significant difference between them. Sucrose concentrations of 3% and 6% were better than other concentrations tested for growth and development of plantlets. The cultures responded positively to the increase of adenine sulfate and 80 mg/l was the optimal. As for the additions of NAA and BA, alone or in combinations, the best results were recorded with the combination of NAA at 0.01 mg/l, and BA at 3.0 mg/l where the largest number of shoots was obtained. Better explants performance was achieved on liquid medium with cotton support compared to solid medium.

Key words: *In vitro*; *Ananas comosus*; clonal propagation; tissue culture

INTRODUCTION

The pineapple (*Ananas comosus* L. Merr) is a perennial mono-cotyledonous plant, belonging to the family Bromeliaceae. It is a native of the New World but is now cultivated in frost-free areas in the tropics and sub-tropics of both hemispheres. It is one of the major tropical

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fruits entering world trade. This trade depends largely on processed rather than fresh fruit (Berrie 1977). World pineapple production was 18 million metric tons in 2005; the leading producers were Brazil, Thailand, Philippines, Costa Rica, China, India and Nigeria (FAO 2005). Pineapple production in Sudan is limited and is confined to the equatorial states. The civil war and the scarcity of planting material have impeded the rapid expansion of pineapple cultivation in other parts of the country.

Pineapple conventionally propagated by crowns, slips or suckers, methods which are time consuming, laborious and produce only two plants per mother plant per year. It would thus take some thirty years to produce enough plants from a single parent plant to cover one hectare (Rottger 1987). Tissue culture propagation techniques provide means for rapid, mass and clonal propagation of pineapple. Various explant types and sources have been used for culture initiation, including axillary buds excised from offshoots (Cabral *et al*. 1984); lateral buds obtained from crowns (Mathews *et al*. 1976; Fitchet *et al*. 1993), shoot tips of suckers, crowns and slips (Rangan 1984; Jose *et al*. 1996), leaf sections taken from *in vitro* produced plants (Dolgov *et al*. 1998) and crown buds (Fotso *et al*. 2001). Both direct (Singh and Manual 2000) and indirect (Dolgov *et al*. 1998) pineapple propagation have been achieved. Zepeda and Sagawa (1981) reported that about 5000 plants could be obtained from one single crown within a year by using tissue culture. Chemically defined (Fitchet *et al*. 1993; Jose *et al*. 1996) as well as undefined (Zepeda and Sagawa 1981; Bordoloi and Sarma 1993; Singh and Manual 2000) modifications of MS medium in agar-jelled (Kiss *et al.* 1995) or in liquid (Fotso *et al*. 2001) state have been used in pineapple tissue culture for various purposes. The success of tissue culture techniques for plant propagation is greatly influenced by the chemical constituents, and the physical state of the culture medium. This study was, therefore, initiated to determine some of the factors that are optimal for shoot proliferation and subsequent growth and development of pineapple shoot tips under *in vitro* conditions.

MATERIALS AND METHODS

The study was conducted at the Commission of Biotechnology and Genetic Engineering, Department of Tissue Culture at the National Centre

for Research, Khartoum, Sudan. The plant material was an *in vitro* stock of "Smooth Cayenne" cultivar brought from Lena Tissue Culture Company at Al- Kadaru, Khartoum North. Shoot tips 0.5-1.0 cm in length were excised and used as explant throughout this study. Murashige and Skoog (1962) MS medium, supplemented with sucrose (30 g/l), MS vitamins (1ml/l), myo-inositol (100 mg/l), adenine sulphate (80 mg/l), benzyl adenine (BA) (3.0 mg/l), naphthalene acetic acid (NAA) (0.3 mg/l) and agar (8g/l) was used as basal medium. The pH was adjusted to 5.8 using 1.0 N HCl or 1.0 N NaOH before the addition of agar. The agar was then dissolved by heating in a microwave oven for 3 min and the medium dispensed in measured amounts of 25 ml into 25x150 mm test tubes. The tubes were capped with polypropylene Bellco-Kaput closures, sterilized in an autoclave at 121°C and 1.01kg/cm² for 15 min and then slanted at 45° angle while cooling.

Ten explants were cultured for each level of treatment in separate test tubes, and each tube was considered a replicate. MS inorganic salts formula was prepared in stock solutions, concentrated 100 times; each stock was added at the rate of 10 ml/l of medium for the normal concentration (1X). In the first experiment, the following MS-salt concentrations were tested: 0.0X, 0.25X, 0.5X, 1X or 2X. In the second experiment, the commercial sugar sucrose was added to the basal medium in concentrations of 1.5%, 3%, 6% or 12%. Adenine sulphate was tested in a third experiment in the following concentrations: 0.0, 40, 80, 160, or 240 mg/l.
To determine the best combination of an auxin and a cytokinin (in mg/l) for high proliferation rates, both NAA at 0.0, 0.01, 0.03, 0.1 or 0.3 and BA at 0.0, 0.1, 0.3, 1.0 or 3.0, were tested in a factorial experiment.

The effect of the physical state of the nutrient medium on growth and development of pineapple explants was studied using a liquid medium with different supporting agents including agar, the common and most used solidifying agent in plant tissue culture. Liquid medium supported with cotton, with filter paper, 8 g/l guar with filter paper or 8 g/l agar (control) were tested in the final experiment.
The following parameters were recorded: Number of shoots, shoot length, number of leaves, and leaves length after 6 weeks of incubation. The data

were analyzed using the analysis of variance procedure (ANOVA) on Excel computer programme. Duncan's multiple range tests was used to separate treatments means.

RESULTS AND DISCUSSION

MS-salt strength

The effect of different concentrations of MS-salt strength on explants growth and development is shown in Table 1. The highest number of shoots was obtained on the medium containing the normal-or half-strength of MS salts, with no significant differences between them. The medium devoid of salts gave the lowest number of shoots and leaf length. There were no significant differences between treatments in shoot length and leaf number, and no rooting was observed in all treatments. These results are similar to the findings of Dale Visco *et al*. (2001) who reported that MS in its normal salt strength was better than all salt concentrations tested. The present data also confirm the results of Jose *et al*. (1996) who used full MS-salt strength for maximum multiplication of pineapple shoots *in vitro*. Half MS-salt strength has also been used for shoot proliferation in pineapple tissue culture by Zepeda and Sagawa (1981) and by Fotso *et al*. (2001).

Table 1. Effect of MS-salts concentrations on growth and development of pineapple shoot tips cultured *in vitro* after 6 weeks of incubation period

MS-salts conc. (X)	No. of shoots	Shoot length (cm)	No. of leaves	Leaf length (cm)
0.0	1.16b	0.43a	4.50a	0.66b
0.25	2.50ab	0.45a	4.00a	1.66a
0.50	4.16a	0.43a	5.60a	1.16a
1.0	4.16a	0.43a	4.80a	1.08ab
2.0	2.80ab	0.36a	4.30a	0.91b

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan Multiple Range Test.

Sucrose concentration

The effect of different concentrations of sucrose on pineapple shoot tips growth and development showed a progressive increase in shoot number

with increase in sucrose concentration up to 6% (Table 2). The lowest number of shoots was recorded at the sucrose level of 1.5%. However, it was noticed that shoots produced at the 12% sucrose concentration were weak and pale in colour compared to shoots produced at 3.0% or 6.0% treatments. Cabral *et al*. (1984) reported that using 3% sucrose rapidly initiates growth and development of pineapple explants cultured on MS medium. Sucrose has been used consistently at the concentration of 3% for various tissue culture purposes of the majority of plant species and varieties (Murashige 1974). In pineapple tissue culture, 3% sucrose has been used for callus induction by Bordoloi and Sarma (1993) and for rapid proliferation of shoots as well (Singh and Manual 2000). The present result confirms these reports. Cote *et al*. (1991) grew pineapple plantlets *in vitro* with or without sucrose in the medium and with normal or increased CO_2 concentration and light intensity. Plants without sucrose, but with increased CO_2 and light, showed better growth than those with sucrose, but with greater proportion of losses upon transfer to *ex vitro* conditions connoting the importance of sucrose for the production of strong vigorous plantlets that can be established successfully under greenhouse and field conditions.

Table 2. Effect of sucrose concentrations on growth and development of pineapple shoot tips cultured in vitro after 6 weeks of incubation period

Sucrose conc. (%)	No. of shoots	Shoot length (cm)	No. of leaves	Leaf length (cm)
1.5	1.16b	0.3a	1.50b	1.08a
3.0	5.00a	0.33a	3.80a	1.16a
6.0	5.50a	0.33a	2.50ab	1.16a
12.0	5.16a	0.33a	2.50ab	0.91a

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan Multiple Range Test.

Adenine sulphate

The effect of different concentrations of adenine sulphate on explant growth and development is displayed in Table 3. The highest number of shoots was obtained from 80 mg/l adenine sulphate treatment; the number of leaves and shoot length also increased significantly at this concentration. Increasing the concentration to 240 mg/l reduced shoot multiplication and shoot length. Singh and Manual (2000), however, added 50 mg/1 adenine hemisulphite to their multiplication medium for pineapple.

Thorpe and Murashige (1968) reported beneficial effects upon addition of adenine sulphate in the presence of a recognized cytokinin. Higher concentrations of adenine sulphate may be beneficial when kinetin is the cytokinin used in the nutrient medium, whereas lower adenine sulphate concentrations give better response in the presence of BA in the nutrient medium. Adenine is part of the molecule of BA; hence, the addition of adenine sulphate may increase the concentration of adenine in the medium to inhibitory levels. The optimum concentration of adenine sulphate is dependent on the type and concentration of the cytokinin added to the medium as well as other factors such as plant species and media composition. Higher concentrations than 80 mg/l might have been inhibitory due to the presence of BA in the nutrient medium.

Table 3. Effect of adenine sulphate on growth and development of pineapple shoot tips cultured *in vitro* after 6 weeks of incubation period

Adenine sulphate (mg/l)	No. of shoots	Shoot length (cm)	No. of leaves	Leaf length (cm)
0.0	1.16d	0.36ab	2.50ab	0.58b
40	2.50bc	0.36ab	2.16b	0.75ab
80	4.60a	0.43a	4.16a	0.58b
160	3.50ab	0.30b	3.16ab	0.50b
240	1.83cd	0.30b	3.33ab	0.91a

Means with the same letter(s) in the same column are not significantly different at P=0.05 according to Duncan Multiple Range Test.

Growth regulators

The effect of BA and NAA, singly or in combinations, is shown in Table 4. The highest number of shoots per explant was obtained on the medium containing 3.0 mg/l BA and devoid of NAA. Similar observations have been reported where only a cytokinin is needed for the promotion of proliferation of pineapple explant cultured *in vitro* (Aydeih *et al*. 1999; Barboza and Caldas 2001; Fotso *et al*. 2001). The BA concentration recommended by these investigators ranged from 1 mg/l to 4 mg/l. The same values of number of shoots per explant were obtained on the medium containing 3.0 mg/l BA and 0.01 mg/l NAA. Again, these results agree with previous reports that a combination of BA and NAA are necessary for an efficient proliferation rate and plantlet production (Guerra *et al*. 1999; Singh and Manual 2000). Relatively high concentrations of NAA (0.03 mg/l) combined with relatively low concentrations of BA (0.1 mg/l) or BA-free medium inhibited shoot proliferation.

Excessive callus was formed at the cut basal end of cultured explants, especially on the medium containing 3.0 mg/l BA and 0.3 mg/l NAA (data not shown). This may increase the frequency of off-type plantlets. Similar observations with papaya have been reported by Drew and Smith (1986) and Smith and Drew (1990).The medium with 0.3 mg/l BA and without NAA supported the highest values of shoot length. Increase in BA concentrations was associated with a decrease in shoot length. Contrary to our results, Jose *et al*. (1996) sub-cultured pineapple shoots on BA-free medium to enhance shoot elongation. The highest mean number of leaves was obtained on the medium containing 0.3 mg/l of each of BA and NAA. The combinations of BA and NAA, both at 0.1 mg/l and BA-free medium with 0.3 m/l NAA, produced significantly high values of mean leaf length. These two parameters denote growth vigour of pineapple explants *in vitro*.

Medium physical state

Liquid medium with cotton fibre support gave significantly high number of shoots per explant compared to the other treatments (Table 5); these results are consistent with previous findings (Cheng and Voqui 1977;

Tabor 1980; Moraes-Cerdeira *et al*. 1995). High values for shoot lengths, number of leaves and leaf length were obtained on liquid medium with filter paper platform compared to all other treatments tested. This is in general agreement with the findings of Horsch *et al.* (1980). Guar with a filter paper platform and agar gave similar results in all parameters measured. Preliminary attempts to use guar as medium support were unsuccessful (Idris 2002). Difficulties in melting and solidification of media have been encountered.

The inhibition of growth and development of plant tissues on agar medium has been attributed to toxic contaminants in the agar (Romberger and Tabor 1971; Kohlenbach and Wernike 1978) or to accumulation of toxic exudates at the cut basal end of cultured explants (De Fossard 1985). These exudates would be more readily diluted in a liquid medium than in agar. It is also possible that nutrient and other growth factors are more available to cultures in liquid medium compared to agar solidified medium.

In conclusion, the present work provides a system for the clonal propagation of pineapple, an important fruit plant that is slow to propagate by conventional means. There is good potential for large-scale propagation from a single shoot tip using this system. Further refinements of the system are necessary to maximize plantlets production *in vitro* and to determine survival and subsequent growth and development of plantlets produced under greenhouse and field conditions.

Table 4. Effect of NAA and BA on growth and development of pineapple shoot tips cultured *in vitro* after 6 weeks of incubation period

NAA (mg/l)	BA (mg/l)	No. of shoots	Shoot length (cm)	No. of leaves	leaf length (cm)
0.0	0.0	1.66fgh	0.50cdef	8.66abcde	1.66bcde
	0.1	1.50fgh	0.63bcd	10.16ab	2.25abc
	0.3	2.16efgh	0.83a	8.16bcde	2.33ab
	1.0	4.16abcd	0.58bcde	8.00bcdef	1.41cdef
	3.0	5.33a	0.50cdef	8.33abcde	1.00ef
0.01	0.0	1.16gh	0.40fg	7.50cdefg	1.50bcdef
	0.1	1.00h	0.50cdef	10.00abc	1.91bcd
	0.3	2.00efgh	0.50cdef	9.66abcd	1.58bcdef
	1.0	3.00 bcdef	0.46defg	7.33defg	1.08def
	3.0	5.33a	0.30g	6.16efg	0.75f
0.03	0.0	1.00h	0.40fg	7.83bcdef	1.66bcde
	0.1	1.33fgh	0.46defg	9.00abcd	1.91bcd
	0.3	1.50fgh	0.43efg	8.33abcde	1.25def
	1.0	2.83cdefg	0.50cdef	8.33abcde	1.08def
	3.0	4.66abc	0.50cdef	5.66fg	1.00ef
0.1	0.0	1.33fgh	0.40fg	7.66bcdef	2.25abc
	0.1	1.33fgh	0.71ab	9.83abcd	2.91a
	0.3	2.66 defgh	0.46defg	6.16efg	1.25def
	1.0	4.66abc	0.66abc	8.00bcdef	1.50bcdef
	3.0	4.83ab	0.58bcde	9.50abcd	1.08def
0.3	0.0	1.16gh	0.66abc	8.66abcde	2.91a
	0.1	4.83ab	0.50cdef	9.50abcd	1.75bcde
	0.3	3.83abcde	0.50cdef	10.83a	1.75bcde
	1.0	3.66abcde	0.33fg	5.66fg	1.08def
	3.0	3.66abcde	0.30g	5.16g	1.00ef

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan Multiple Range Test.

Table 5. Effect of the physical state of the nutrient medium on growth and development of pineapple shoot tips cultured *in vitro* after 6 weeks of incubation period

Treatment	No. of shoots	Shoot length (cm)	No. of leaves	Leaf length (cm)
Liquid+ cotton fibre	8.16a	0.50ab	8.83a	1.00b
Liquid+filter paper	2.00b	0.66a	10.16a	1.58a
Agar (8g/l)	2.83b	0.43b	8.50a	0.75b
Guar(8g/l)+ filter paper	2.50b	0.43b	8.66a	0.66b

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan Multiple Range Test.

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إكثار الأناناس بزراعة قمة الساق

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موجز البحث: أجريت هذه الدراسة بغرض تحوير مكونات وسط "موراشيقي" و" أسكوج " للإكثار الخضري لنبات الأناناس باستخدام قمم سوق صنف "كايين ناعم". تم إجراء تعديلات على مختلف مكونات الوسط الغذائي **MS.**
أوضحت النتائج أن أملاح "مورشيقى" و"اسكوج" عند التركيز الكامل أو نصفه هما الأمثل و لا توجد بينهما فروقات معنوية ، وكان تركيزي السكروز 3% و6% أفضل لنمو وتطور النبيتات مقارنة بالتراكيز الأخرى التي تم اختبارها. استجابت الزراعات استجابة إيجابية لزيادة كبريتات الأدنين وكان تركيز 80ملجرام/لتر هو الأمثل .
أما فيما يخص إختبار التواليف بين نفثالين حمض الخليك (NAA) و بنزايل ادينين (BA) فقد كانت أفضل النتائج عند التركيز 3.0 مليجرام/لتر BA، و التركيز 0.01 مليجرام/لتر NAA حيث تم الحصول على أعلى معدل لعدد السيقان. حققت البيئة الغذائية السائلة المدعومة بالقطن أفضل النتائج في معدل الإكثار داخل الأنابيب مقارنة بالبيئة الغذائية الصلبة.

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