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Effect of Medium Components on *in vitro* Shoot Formation and Rooting of Papaya (*Carica papaya* L.)

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Abstract: The effect of modification of some selected MS nutrient medium on *in vitro* growth and development of papaya (*Carica papaya* L.) shoot tips was examined with the objective of optimizing shoot regeneration and rooting efficiencies. Modifications were made on selected components of MS medium. Shoot tip explants were obtained from *in vitro*-grown plantlets. The results showed that MS salts at the twice full strength was optimal for all parameters measured compared with other strengths tested. Sucrose concentrations of 0.75% and 1.5% were better than the other concentrations tested for growth and development of cultured shoot tips, and there was no significant difference between them. The results revealed the importance of inclusion of BA and NAA in the nutrient medium at relatively low concentrations, the best results for shoot and leaf formation was recorded with the combination of BA at 1.0 mg /litre and NAA at 0.01mg /litre, where the greatest number of shoots and leaves was obtained. The combination treatment of 0.3 mg/litre BA and 0.00 mg/litre NAA recorded the best results for mean shoot elongation, compared with the other combination treatments tested. However, the produced roots were morphologically abnormal. The study attested to the essentiality of IBA for rooting of *in vitro* produced papaya shoots, and the data suggested the conduction of additional studies for determination of optimal concentration for rooting.

Key words: *Carica papaya;* micro-propagation; growth regulators

INTRODUCTION

Papaya (*Carica papaya* L.) is a common fruit grown in tropical and subtropical countries. It is a soft-wooded, typically unbranched, erect, quick-growing small tree. Papaya's fruit is economically useful for its food value and for its valuable proteolytic enzymes, (papin and chymopapin).

Papaya remains a vastly under researched subject of fruit trees in Sudan. It is grown in the Blue and White Nile States in small monoculture gardens or multiple-cropping systems and much of the harvest is consumed or traded locally due to long distances to markets and the difficulty in handling the large fruit size. The value of papaya as a source of income is underestimated as well as its nutritional uses.

Papaya is usually propagated by seeds. Seed germination is frequently slow, erratic and incomplete. Germination inhibitors in the papaya seeds play an important role during their dormancy and germination (Chow and Lin 1991). Being essentially cross-pollinated and seed propagated, papaya seedlings show considerable variability in commercial plantation; and being dioecious, a large number of males is produced at the expense of females (Hagagy *et al.*1999). The plant's sex remains indiscernible until flowering.

The non-branching habit of the plant has greatly hampered its vegetative propagation. Tedious, impractical and labour intensive conventional vegetative propagation methods exist (Ramkhelawan *et al.* 1999), but none is suitable for rapid production of large number of clonal papaya transplants from a single stock tree. Emphasis is now placed on micro-propagation for the clonal multiplication of desired papaya plants, where both greater propagation rates and plants that are disease-free can be obtained.

Papaya appears to be amenable to rapid and efficient tissue culture propagation (Agnihotri *et al.* 2004). *In vitro* shoot proliferation in papaya is rather easily produced, but the rooting of these shoots is difficult to achieve (Drew 1988; Teo and Chan 1994). The use of rooting media

containing reduced MS salt formulation (Drew 1987), exclusive of nutrients (Teo and Chan 1994) or amended with relatively low concentrations of IBA (Rajeevan and Pandey 1986; Yu *et al.* 2000), has been found beneficial for rooting of *in vitro* produced papaya shoots.

The objective of this research was to determine the optimal concentration of some components of media for *in vitro* proliferation and rooting of papaya's shoot tips.

MATERIALS AND METHODS

This study was conducted at Leena Tissue Culture Laboratory, El-Kadaro, Khartoum North, Sudan. Attempts to culture explants harvested from 7-month old papaya seedlings raised in a lath house proved to be difficult to disinfect. High contamination rates have led to loss of all cultures. Shoot tips that were free of micro-organisms were obtained from aseptically established seedlings. Seeds were soaked in 5.25% sodium hypochlorite (100% Clorox) and a few drops of Tween-20 emulsifier for 15 min. It was necessary to remove the sarcotesta to ensure completeness of disinfestation. The disinfested seeds were rinsed 3 times with autoclaved distilled-water prior to sowing in germination media. The seeds were germinated in 25x150-mm glass culture tubes at a rate of 1 seed/tube. Germination occurred in darkness and at 25±2°C. Excisable papaya shoot tips were obtained within 3 weeks.

A minimal organic medium was used in germinating papaya seeds. The medium contained the inorganic salts of Murashige and Skoog (MS) (1962) medium supplemented with 3% sucrose, 100 mg/litre myo-inositol and 7g Gibco Phytagar/litre. The freshly excised shoot tips, 0.5-1 cm long, were aseptically placed (one per tube, proximal portion down) into 25x150-mm glass culture tubes containing 25 ml modified MS medium. The basal composition of this medium was the same as that used in seed germination in addition to 0.4 mg/litre thiamin-HCl, 0.5 mg /litre benzyladenine (BA), and 0.01 mg/litre α-naphthalene acetic acid (NAA) designated hereafter as stock plant medium. The substances and concentrations were chosen on the basis of preliminary investigations in

our laboratory with shoot tips of a diverse number of fruit species including papaya. Multiple-shoot induction and plantlet regeneration were developed with shoot tips collected from *in vitro* produced seedlings. Proliferating shoot culture was established by repeatedly sub culturing the original seedling shoot tip on stock plant medium after each harvest of newly formed shoots. When sufficient numbers of propagules were obtained, various concentrations of MS inorganic salt formula, various levels of sucrose and growth regulators were tested for determining the optimum medium for promoting greatest shoot prolificacy and vigour.

Due to plant material limitations, all combinations were not tested at the same time but were divided into four experiments as follows: In experiment 1, shoot tip explants excised from *in vitro* grown plantlets were cultured either on 1/4X-, 1/2X-, 1X-, 2X- or 4X-MS-salt strength to test the effect of dilute and concentrated solution of MS inorganic salts formula. MS salt mixture was prepared in five inorganic salt stock solutions, concentrated 100 times the final medium concentration, and each stock was added to the nutrient medium at the rate of 10 ml/litre of medium for the normal concentration (1X). Experiment 2 consisted of the addition of different concentrations of sucrose at 0.75%, 1.5%, 3.0%, 6.0% or 12.0%. To determine the best combination of cytokinin and auxin (in mg/l) for high shoot proliferation rates, both BA at 0.0, 0.1, 0.3, 1.0 or 3.0 and NAA at 0.0, 0.01, 0.03, 0.1 or 0.3, were tested in different combinations in experiment 3. For the rooting of *in vitro* generated shoots (experiment 4), shoots 3-4 cm long or longer were excised from the multiplication cultures and placed on stock plant medium amended with IBA at concentrations of 0.0, 1.0, 2.0, 3.0 and 4.0 mg/litre.

The pH of each medium was set at 5.7±0.1 with 0.1N NaOH and/or 0.1N HCl. Agar was added and melted by heating on a stirring hot plate, and the medium was dispensed in aliquots of 25 ml into 25x150 mm glass culture tubes. The tubes were capped with polypropylene Bellco Kaput closures and autoclaved at 1.01 kg/cm^2 and 121ºC for 15 min. Culture tubes were placed vertically in 4x10 stainless steel rack slanted at 45º angle while cooling.

The primary explants and subsequent cultures were maintained slanted in 4x10 stainless steel racks in an incubation room at 25±2°C with a 16-h photoperiod provided by two vertical interior door-mounted 40-W Phillips broad-spectrum cool-white fluorescent lamps. The culture tubes were positioned 15 to 40 cm from the lamps.

Data were collected after 6 weeks of incubation (experiment period). The number of shoots per explant, shoot length, leaf number, root number, root length and callus formation were recorded. Callus rating was evaluated by assigning – for no callus, + for a small amount of callus, ++ for an average-sized callus and +++ for a large amount of callus. All experimental treatments were arranged in completely randomized design. Each treatment in an experiment was replicated ten times of one explant each. Each experiment was repeated at least three times. Data were subjected to analysis of variance procedures of the SAS Institute (1990) and Duncan's Multiple Range Test was used to separate treatment means.

RESULTS AND DISCUSSION

MS salt-strength

The requirement for inorganic salts among diverse plant genotypes, organs, cell types and research applications is rather constant. The inorganic salt mixture of Murashige and Skoog (1962) is most frequently used for culture initiation and shoot proliferation in fruit trees and has served as bases for further modifications (Murashige 1974).

The data of the effect of MS salt- strength on growth and development of papaya shoot tip explants are depicted in Table 1. The effect of MS salt-strength on number of shoots, shoot length and number of leaves was highly significant. Among MS salt levels tested, 2X was optimum for shoot proliferation, leaf formation and shoot elongation. The response to the total salt strength of the medium increased with increasing salt strength up to 2X (twice the full MS strength) and then sharply decreased with increasing salt strength in the medium to 4X. The highest number of shoots (9.14), the longest shoots (1.8 cm) and the greatest number of leaves (31.86) that developed from a single shoot tip explant grew on medium supplemented with twice the normal MS-salt strength (2X).

Plantlets produced were vigorous with large green leaves, strong and long shoots and no rooting was noted in all treatments. The medium containing the lowest salt strength (1/4X) gave the least values of all parameters measured and formation of pale yellow friable callus was perceived at the cut basal end of explants cultured on medium containing 1/2X MS salt-strength. Four-times (4X) MS salt-strength resulted in the death of all explants during the first two weeks of culture initiation.

It is apparent, from this study, that papayas are plants of high mineral salts requirements. Similar results were obtained with papaya (Suksa *et al*. 1997) and other plant species (Mamiya and Sakamoto 2000; Abdalla 2005). The results, however, differed from those of Hagagy *et al.* (1999), where the normal concentration (1X) has been found optimal for papaya tissue culture. Furthermore, MS salt-strength lower than normal have been used successfully for establishment and shoot proliferation of several woody plants *in vitro* culture of papaya for various research purposes (Cheng 1978; Siddiqui *et al*. 1999). These inconsistencies in results could be due to differences in plant species and varieties, type of explants, media constituents or incubation conditions. The detrimental effects of 4X MS salt-strength in this study were attributed mainly to the toxicity of high levels of mineral salts.

Sucrose concentrations

The values of all measured responses were influenced by the inclusion of sucrose in the culture medium. Table 1 depicts a gradual decrease in values of all responses measured as sucrose concentration was increased. Concentrations of sucrose of less than 3% resulted in increases in shoot number, shoot elongation and number of leaves produced up to a maximum of 7.6, 2.2 cm and 26.8, respectively, on medium containing 0.75% sucrose concentration and were significantly reduced on medium containing 6% or 12% sucrose concentrations. Shoot and leaf numbers at 0.75% sucrose were highest but not significantly different from that at 1.5%. Significant differences were, however, obtained between these two treatments for number of shoots and leaves and the other sucrose concentrations tested. Shoot elongation was not significantly increased on medium containing 0.75%, 1.5% or 3% sucrose concentrations but a significant difference between these treatments and the two elevated

sucrose concentrations was obtained. Formation of pale yellow water soaked callus was evident at the cut basal end of explants cultured on media containing 0.75% or 1.5% sucrose concentrations, and no roots were formed in any treatment.

Table 1. Effect of MS salt-strength and sucrose concentration on growth and development of papaya shoot tips after 6 weeks of incubation period

	No. of shoots	Shoot length (cm)	No. of leaves	Callus
		MS salt-strength		
MS salt-strength (X)				
1/4X	0.43b	0.51b	5.8b	–
1/2X	3.14b	1.26a	13.29b	++
1X	2.86b	1.77a	15.29b	–
2X	9.14a	1.80a	31.86a	–
		Sucrose concentration		
Sucrose conc. (%)				
0.75	7.60a	2.20a	26.80a	++
1.5	6.20a	1.86a	25.80a	++
3.0	0.80b	1.38ab	6.80b	–
6.0	1.40b	1.16b	7.40b	–
12.0	0.00b	0.32b	5.00b	–

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan's Multiple Range Test.
Callus rating: – no callus; ++ an average-sized callus.

The results showed that the optimum concentration of sucrose for shoot formation and elongation was between 0.75% and 1.5% which is much lower than that (3%) usually used in plant tissue culture (Murashige 1974). These results are comparable to those of Drew and Smith (1986) and Drew (1992), where 2% has been found optimum for *in vitro* propagation of papaya. The results, however, differed from those of Wilna (1988) and Suksa *et al*. (1999) who reported that 3% sucrose or more are optimal for *in vitro* growth and development of papaya tissues. Differences in results were attributed to variety, type of explant, media components and incubation conditions.

An unexpected result was the production of excessive callus tissues at the cut basal end of cultured explants with media containing 0.75% and 1.5% sucrose concentrations. One can only speculate on the potential carry-over effect of the growth regulators used in the stock plant medium.

Growth regulators

Table 2 illustrates the results of an experiment designed to determine which combination of BA and NAA is most conducive to growth and development of shoot-tip explants of papaya. The magnitude of response to BA and NAA varied with combinations and concentrations. All treatments stimulated some growth of cultured shoot-tip explants with significant differences. Shoot and leaf counts increased with increasing BA concentration up to 1.0 mg/litre. The highest number of shoots (3.2) and the largest number of leaves (21.2) were obtained on the medium containing 1.0 mg /litre BA plus 0.01mg /litre NAA compared with other combination treatments. These results are comparable to those reported by others (Drew and Smith 1986; Drew 1988; 1992; Rady 2004) who found that both auxins and cytokinins in relatively low concentrations are needed for best shoot growth. Media without BA gave the least value for shoot and leaf numbers per shoot tip explants. On the other hand, NAA had a repressing effect on shoot and leaf formation when applied without BA. NAA may have augmented natural apical dominance, hence retarding dormant axillary bud-break and consequently shoot proliferation. Since neither BA nor NAA alone was able to induce shoot formation, there appear to be a synergistic effect between BA and NAA on shoot growth and development.

Table 2. Effect of BA and NAA on growth and development of papaya 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	Callus
0.0	0.0	0.00b	0.28c	4.40c	–
	0.1	0.25b	0.48c	5.50c	–
	0.3	0.40b	1.70a	12.80bc	–
	1.0	1.60b	1.06bc	14.60bc	++
	3.0	1.0b	0.53c	11.50bc	+++
0.01	0.0	0.00b	0.90bc	4.30c	–
	0.1	1.50b	0.60c	5.80c	–
	0.3	2.80ab	0.80bc	17.75ab	–
	1.0	3.20a	0.80bc	21.20a	+++
	3.0	0.00b	0.60c	5.20c	+++
0.03	0.0	0.00b	0.40c	3.80c	–
	0.1	0.00b	0.48c	4.20c	+
	0.3	2.40ab	1.08b	18.80ab	+
	1.0	0.40b	0.46c	5.80c	+
	3.0	1.25b	0.60c	7.75c	–
0.1	0.0	0.00b	0.36c	3.80c	–
	0.1	0.00b	0.33c	5.67c	–
	0.3	0.20b	0.48c	4.80c	–
	1.0	1.60b	0.52c	9.80bc	–
	3.0	0.20b	0.46c	3.80c	+
0.3	0.0	0.00b	0.34c	4.00c	–
	0.1	0.40b	0.42c	4.80c	–
	0.3	0.60b	0.64bc	8.80bc	+
	1.0	0.60b	1.02bc	6.00c	–
	3.0	0.00b	0.32c	4.40c	–

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan's Multiple Range Test.
Callus rating: – no callus; + a small amount of callus; ++ an average-sized callus and+++ a large amount of callus

Shoot elongation, on the other hand, was greatly enhanced on medium containing 0.3mg BA/litre and devoid of NAA, compared with all other combination treatments tested; concentrations of BA greater than 0.3 mg /litre, repressed shoot length. It is apparent that induction of shoots was less sensitive to BA than shoot length; the best BA concentration for shoot formation was 1.0 to 0.3 mg /litre, a wider range than optimal (0.3 mg/litre) for shoot elongation.

Concentrations of BA greater than or equal to1.0 mg/litre alone or in combination with 0.01 mg/litre NAA induced excessive callusing, consistent with data reported by other investigators (Drew and Smith 1986; Rajeevan and Pandey1986; Smith and Drew 1990), whereas NAA concentrations had little effect on callus production. Contradictory reports to these data (Rahaman *et al.*1992; Hossain *et al.*1993) advocated adding relatively high concentrations of BA and NAA to media for survival and better growth of papaya explants. BA alone failed to induce callus on papaya explants. Such discrepancy could be due to differences in type and source of explants, media composition, purpose of culture and incubation conditions.

Formation of varying amounts of intermediate callus in combination treatments that were optimal for shoot proliferation may have a bearing on the genetic uniformity of *in vitro* produced papaya plantlets. The chance for somaclonal variation is likely to be high. Similar speculations and conclusions have been reported by others (Drew and Smith 1986; Smith and Drew 1990) who stressed the importance of avoiding callus formation so as to ensure the genetic fidelity of regenerated papaya plantlets. Although the shoot multiplication rates were low (2.8 shoots per explant) on the medium containing 0.3 mg/litre BA and 0.01 mg/litre NAA, the produced shoots are expected to be clonal because no callus was noted at the cut basal ends of cultured explants. The rate of shoot multiplication in this treatment is of practical interest, since it was produced from freshly cultured explants. Sequential sub culturing of *in vitro* produced shoots after each harvest onto the same medium will, it is hoped, enhance the rate of shoot multiplication in a similar manner to the findings of Rajeevan and Pandey (1986).

IBA and rooting

Root induction was the principal morphogenic response in experiment 4. However, all IBA concentrations tested resulted in substantial increase over the control in the values of all parameters measured (Table 3). The values of all measured responses increased progressively with increasing IBA with significant differences between treatments except for shoot and root lengths. Neither shoot nor root length differed for the IBA levels examined. The larger number of roots per explant (4.9), the longest roots (2.13 cm) and the highest rooting percentage (60%) were obtained on medium containing 4.0 mg /litre IBA, the highest IBA concentration tested in this study. Though IBA had no effect on root length, it did stimulate shoot length, and no rooting was obtained on media devoid of IBA.

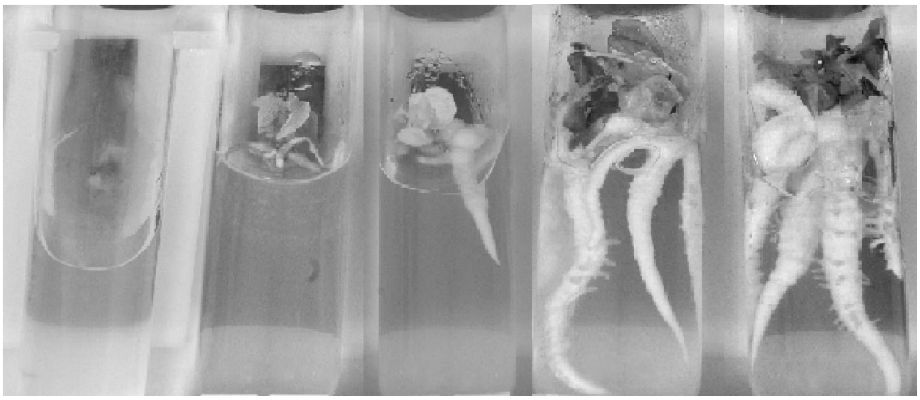
Table 3. Effect of indole-3-butyric acid (IBA) on growth and development of papaya shoot tips after 6 weeks of incubation period

IBA conc. (mg/l)	No. of shoots	Shoot length (cm)	No. of leaves	No. of roots	Root length (cm)	Callus	Rooting %
0.0	0.00b	0.34b	2.88b	0.00b	0.00a	–	0
1.0	0.50b	0.58ab	3.00b	1.60b	0.22a	–	30
2.0	1.90ab	0.65a	6.50ab	2.00ab	1.41a	–	60
3.0	2.00ab	0.70a	9.30a	2.30ab	1.22a	–	40
4.0	2.70a	0.84a	7.40a	4.90a	2.13a	–	60

Means with the same letter(s) in the same column are not significantly different at P=0.05, according to Duncan's Multiple Range Test.
Callus rating: – no callus

The findings of this research and previous ones (Rajeevan and Pandey 1986; Drew 1987) indicate that IBA is essential for root initiation in papaya. Media containing 2.0 mg /litre resulted in the formation of, more or less, normal root growth, in a very similar manner to that reported by

other investigators (Drew 1987; Rahman *et al*., 1992; Yu *et al.* 2000) where relatively low concentrations of IBA has been used consistently for root initiation and growth in papaya tissue culture. Non-significantly higher values of root number were obtained with IBA concentration higher than 2 mg /litre, but the resultant roots were stubby and thickened. The intensity of this abnormal growth and development increased with increasing IBA concentration (Fig.1). These observations are consistent with those reported by others (Drew and Smith 1986; Drew 1988; Teo and Chan 1994) who found that inclusion of high concentrations of IBA in the rooting medium caused the formation of thickened and stubby roots accompanied by excessive callusing. No callus was, however, detected in any of the IBA treatments in this study. This is likely due to differences in genotypes, media composition, explants source and incubation conditions.



0.0 1.0 2.0 3.0 4.0
mg/litre

Fig.1. Rooting behaviour of *Papaya carica* shoot tips as affected by IBA concentrations in root initiation medium. Photographed after 6 weeks of culture

The responses to IBA treatments were linear, with 4.0 mg /litre being more effective than 0.0 and 3.0 mg/litre. Accordingly, the optimum concentration of IBA for rooting of *in vitro* produced papaya shoot could not be determined from a straight line relationship. The effects of IBA on rooting of *in vitro* produced papaya's shoots warrant further studies.

It could, therefore, be concluded, that the manipulation of the chemical components of the culture medium can give important contributions to the optimization of shoot proliferation and improvement of the quality of papaya plants and, as a consequence, of papaya production. Additional studies of rooting and genetic stability of plants produced are necessary before micro-propagation can be a satisfactory method for mass production of papaya.

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تأثير مكونات الوسط الغذائي على تكوين وتجذير سيقان الباباي(*Carica papaya* L.) في الانابيب

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المستخلص: أُجريت دراسة لتأثير تحوير بعض المكونات المختاره لوسط "موراشيقي واسكوج" الغذائي على حفز نمو وتطور قمم سوق الباباي (*Carica papaya* L.) في الانابيب بغرض تحسين كفاءة تكوين السيقان وتجذيرها. أُجريت التحويرات على بعض مكونات وسط "موراشيقي" و"اسكوج" الغذائي، وأُستخدمت قمم سوق مفصوله من نبيتات منتجه في الانابيب كاجزاء للإستزراع. اوضحت النتائج ان املاح "موراشيقي واسكوج" عند ضعف التركيز الكامل هي الامثل لكل القياسات المرصوده مقارنةً بالتراكيز الاخرى التي تم إختبارها، وكان تركيزي السكروز 0.75 % و1.5 % هما الافضل لنمو وتطور القمم المزروعه ولاتوجد بينهما فروقات معنويه. دلت النتائج على اهمية إضافة تراكيز منخفضه نسبياً من كلٍ من BA و NAA لزيادة عدد السيقان والاوراق ، فقد سُجلت افضل النتائج عند التركيز 1.0 مليجرام/ لتر BA +التركيز 0.01 مليجرام/ لتر NAA حيث تم الحصول على أعلى معدل لعدد السيقان والاوراق. وحققت معاملة التوليفه 0.3 مليجرام/لتر BA مع 0.00 مليجرام/ لتر NAA افضل النتائج لمتوسط طول السيقان مقارنةً بالتوليفات الاخرى التي أُختبرت، غير ان نمو وتكشف الجذور المنتجه كان مشوهاً. اثبتت الدراسه ضرورة إضافة ال IBA لتجذير سيقان الباباي المنتجه في الانابيب وإقترحت البيانات إجراء دراسات إضافيه لتحديد التركيز الامثل منه.