ENHANCEMENT OF GROWTH AND CONTROL OF BROWNING OF TISSUE CULTURES OF GUAVA (*PSIDIUM GUAJAVA L.*)

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ABSTRACT

Difficulties reported on tissue culture of guava were mainly in the initiation phase due to the detrimental effect of phenolic browning beside the low multiplication rate and cultures decline in the proliferation phase. This study aimed to counteract browning and declining and to enhance growth and multiplication of cultures.

The results revealed that better establishment of *in vitro* cultures of guava was obtained by vertical positioning of shoot explants and illuminated incubation. Presoaking explants in a solution containing activat-ed charcoal (AC) counteracted the adverse effect of phenolic browning on cultures vigor especially when activated charcoal (AC) was added to media of 2 passages in initiation phase.

Axillary shoot formation was enhanced by a supplement of 160 mg/l adenine sulphate to multiplication media containing 1 mg/l 6-benzyladenine (BA). Upon addition of 1 mg/l silver nitrate to media, cultures decline was avoided. Rooting was enhanced by addition of 1 mg/l indol-3-butyric acid (IBA) to the rooting medium.

الملخص:

أجريت هذه الدراسة بهدف التغلب على بعض الإشكالات المسببة لتدنى نجاح الزراعة النسيجية للجوافة. بناء على الدراسات السابقة صممت تجارب لمعالجة الاسمرار الفينولى في الطور الإنشائي، وتدهور المزارع وانخفاض معدلات التفرع في الطور التكاثري إضافة إلى تحفيز تجذير النبيتات.

أوضحت الدراسة أفضلية الوضع الرأسي لأنسجة الجوافة في أوساط النمو مع الحضانة تحت الإضاءة الصناعية في الطور الإنشائي للزراعة في القوارير كما أن غمر الأنسجة في محلول يحوي الفحم المنشط

(AC)قبل الزراعة يساعد على تجنب الآثار السالبة للاسمرار الفينولي خاصة إذا أضيف الفحم لبيئات النمو لدورتين في الطور الإنشائي.

شجعت إضافة كبريتات الادنين بمعدل 160مجم/ل إلى بيئة الإكثار الحاوية على 1مجم/ل بنزايل أدنين (BA) التفرعات الجانبية وعند إضافة نترات الفضة لأوساط النمو بمعدل 1مجم/ل أمكن التغلب على تدهور المزارع النسيجية. وأمكن الحصول على أفضل تجذير عند إضافة أندول حمض البيوتريك (IBA) إلى وسط التجذير بمعدل 1مجم/ل.

INTRODUCTION

Common guava (*Psidium guajava L*.) is one of the five major fruit crops in Sudan, where it is grown throughout the country under irrigation or rains with minimum human attention. Wide genotypic variations in fruit quality are prevailing, as plantations were mainly raised from seeds. This necessitates selection programs for competitive marketing and clonal expansions.

Difficulties associated with conventional vegetative propagation procedures of guava were well documented (Amin and Jaiswal, 1987; Rao *et al*, 1984; Wally *et al*, 1981). The potential of tissue culture as an alternative technique for guava clonal multiplication was found promising. However, the high mortality rate of explants caused by the detrimental phenolic browning of explants is one of the difficulties reported by (Jaiswal and Amin 1987), (Fitchet 1989) and (Idris and Mahdi 1996). Low multiplication rates were reported in the proliferation phase, as well as cultures decline characterized by leaf yellowing, necrotic spots and loss of the overall vigor, especially if cultures were left for more than six weeks without transfer to fresh media (Amin and Jaiswal, 1987 and 1988; Idris and Mahdi, 1996).

The objectives of this study were to investigations on the possible means for successful initiation of cultures via preventive approaches to overcome failures caused by phenolic browning, beside growth enhancements to increase the prolific and rooting capacities coupled with counteraction to the decline of cultures.

MATERIALS AND METHODS

Shoot terminals (15 cm long) were collected from basal sprouts of a mature, field grown, 10 years old guava seedling tree of excellent fruit quality. Surface dust was removed by washing in running tap water for 30 minutes. Nodal sections (10 mm) containing two oppositely arranged axillary buds, and shoot apices were excised and temporarily submerged in 2% sodium hypochlorite solution. Groups of 10 explants were wrapped in 10cm squares cheesecloth and disinfected by immersion for 25 minutes in 15% sodium hypochlorite solution containing 2 drops of tween 20 per 100ml solution. After rinsing three times with autoclaved distilled water, the explants were excised to 5mm and transferred under aseptic conditions to media. The nutrient the cultures media contained the inorganic salts (MS) (Murashige and Skoog, 1962); 3% sucrose; 0.7% Difco-bactoagar, and (in mg/l): i-inositol, 100; thiamine-HCl 1.0; pyridoxine-HCl, 0.5; nicotinic acid, 0.5; and glycine, 2.0. Media aliquots were 20 ml per 25x150mm culture tube, and cultures were incubated under constant 27 ± 1˚C and 16 hours daily exposure to 1000lux illumination from Gro-lux fluorescent tubes.

Stage (1) (initiation phase): Nodal sections or shoot tip explants were employed as primary explants. The following factors were investigated as means for control of browning:

(a) Explants orientation: Horizontal, vertical and inverted positioning of nodal sections on medium surface.

(b) Illumination: Shoot tips were either incubated under 16 hours day length or complete darkness.

(c) Anti-browning pretreatments of explants: The immediate and extended effects of pretreatments were tested in two passages of the initiation phase. Prior to culturing, explants were soaked for 3 hours in one of the following solutions: water, 0.15% activated charcoal (AC), 0.2% polyethylene glycol (PEG), 0.3% polyvinylpyrrolidone (PVP), or a mixture of 150mg/l citric acid and 100mg/l ascorbic acid. Shoot tips and nodal sections were employed as explants in this test, but polyvinylpyrrolidone (PVP) was omitted for nodal sections. The initiation phase was composed of two passages. The basal medium in the first passage included 0.3% activated charcoal (AC) and data were collected

for this passage after four weeks. Thereafter, explants were transferred to fresh media (second passage). Unlike shoot tips, the media for nodal explants was devoid of activated charcoal (AC) in the second passage. Observations for this passage were recorded after two weeks.

(d) Preculture soaking in activated charcoal (AC): Nodal explants were preculture soaked in solutions of different concentrations of activated charcoal (AC) (0, 0.5, 1 and 5 g/l) for 2, 3, 20, 27 hours durations. Data were recorded after 4 weeks.

Stage (2) (Proliferation Phase): shoot tips produced *in vitro* were employed as explants. Raising sucrose concentration to 4.5% and addition of 1 mg/l 6-benzyladenine (BA) amended the basal medium. Adenine sulphate was tested for improve-ment of the multiplication capacity in concentrations: 0, 40, 80, 160 and 320 mg/l.

Silver nitrate in concentrations of 0, 1, 3, 10 and 30 mg/l was tested for the possibility of counteracting cultures decline.

Stage (3) (Rooting Phase): The medium was composed of half MS salts, 2% sucrose, 0.1% activated charcoal (AC), 0.7% agar, 0.1 mg/l kinetin. For enhanced rooting, idol-3-butyric acid (IBA) was tested in concentrations: 0, 0.1, 0.5, 1.0 and 5.0 mg/l.

The complete randomized design was used in all tests, where 10-20 replicates were employed per treatment. Visual recordings were set for media browning and vigor of explants. The browning scale was 1-10, where 1 represented the least and 10 the maximum browning intensity. Vigor scale was 1-5, where 1: stood for poor, 2: fair, 3: good, 4: very good, and 5: excellent. Standard errors of means were computed whenever appropriate. When data were expressed as percentage, the 95% confidence limits of binomially distributed attributes were obtained according to (Diem and Seldrup 1982).

RESULTS AND DISCUSSION

Vertical placement of explants resulted in least media browning and enhanced cultures vigor (Table 1). Nodal sections have two cut surfaces and as horizontal orientation means double contact sites with media surface, there is higher chance for phenolics leakage from the two cut surfaces of the explants to the medium. In a study on initiation phase of mango, (Thomas and Ravindra

1997) recognized the adverse effect of cut surfaces coming in contact with medium on the extent of browning.

Table (1): Performance of Guava Tissue Cultures in Response to Explants Orientation and Illumination

	Survival (%)	Vigor	Browning intensity
Orientation:			
Horizontal	40(19-64)*	1.9 ± 0.28**	6.4 ± 0.43**
Vertical	70(46-88)	3.3 ± 0.30	3.5 ± 0.34
Inverted	50(27-73)	3.0 ± 0.21	4.3 ± 0.40
Illumination:			
Light	90(69-99)	2.9 ± 0.13	2.9 ± 0.26
Dark	50(27-73)	1.4 ± 0.22	2.2 ± 0.20

* 95% confidence limit in parenthesis, ** ± Standard error.

Although dark incubation of shoot tips reduced media browning, it failed to support higher survival or vigor compared to illumination (Table 1). This may be due to the lengthy four weeks dark period. Culture initiation in dark for two weeks followed by illumination was recommended for browning control of phalaenopsis nodal explants (Pieper and Zimmer, 1976). Moreover, the mortality might be due to the high level of Potassium iodide (Kl) in MS formula that causes phyto-toxicity in dark incubated cultures as suggested by (Eriksson 1965).

The five anti-browning pretreatments of shoot tips revealed the superiority of activated charcoal (AC) in the two passages, as vigor increased with sub culturing (Table 2). Vigor enhancement was also observed with water presoak treatment (control) that ranked second. Citric and ascorbic acids, polyethylene glycol (PEG), and polyvinylpyrrolidone (PVP) depressed vigor below the control.

Table (2): Effects of Antibrowning Pretreatments on Vigor of Guava Shoot Explants in Two Passages of Initiation Phase

Vigor of Explants		
	First Passage:	**Second Passage:**
Shoot Tips:	**(+) AC**	**(+) AC**
Water	3.10 ± 0.16*	3.8 ± 0.29*
Citric+ Ascorbic	2.75 ± 0.14	2.5 ± 0.22
Activated charcoal	3.50 ± 0.17	4.8 ± 0.13
PEG	2.85 ± 0.17	1.7 ± 0.21
PVP	1.90 ± 0.18	1.9 ± 0.23
Nodal Sections:	**(+) AC**	**(-) AC**
Water	3.00 ± 0.20	2.50 ± 0.27
Citric+ Ascorbic	3.33 ± 0.19	4.50 ± 0.27
Activated charcoal	4.13 ± 0.19	3.40 ± 0.34
PEG	2.73 ± 0.21	1.90 ± 0.28

* ± Standard error.

For nodal explants, activated charcoal (AC) excelled the other treatments in the first passage, but vigor declined in the second passage upon sub culturing to media devoid of activated charcoal (AC) (Table 2). Such behavior was observed with all other treatments except the combination of citric and ascorbic acid that ranked top in the second passage. This result confirms the benefit of pretreating shoot tip explants with activated charcoal (AC) along with inclusion of 0.3% activated charcoal (AC) in the media of the two passages of the initiation phase. The decline recorded for nodal explants in activated charcoal (AC) free media of the second passage suggests the continuation of phenolics production. The combination of citric and ascorbic acids might be employed successfully in media lacking activated charcoal (AC), and the results suggest the capability of this combination in preventing phenolics production and their oxidative reaction. According to (Thomas and Ravindra 1997), activated charcoal (AC) was advantageous in control of browning of mango explants compared to other adsorbents, antioxidants, and dark incubation of cultures. (Chin *et al.*, 1988) accomplished initiation of cultures of five tropical species renounced for phenolic browning, upon incorporation of activated charcoal (AC) in the basal media.

In the succeeding test for activated charcoal (AC) optimization, presoak in a solution of 0.5 g/l activated charcoal (AC) for 3-20 hours was found to be the best (Table 3). However, in detergent-free presoak solution, the chance of

explant contamination increases with time, and as time adds to labour cost, the 3 hours period seems reasonable especially when considering the high vigor it supported.

Table (3): Means of Vigor of Nodal Explants as Affected by Pretreatments with Different Concentrations of Activated Charcoal for Various Durations

Duration (hours)	AC concentration (g/l) 0.0	0.5	1.0	5.0
2	3.71 ± 0.36*	3.43 ± 0.20	3 .29 ± 0.36	2. 57 ± 0.20
3	3.57 ± 0.20	4.00 ± 0.22	3.43 ± 0.30	3.29 ± 0.29
20	2.43 ± 0.20	4.57 ± 0.20	3.57 ± 0.30	3.43 ± 0.20
27	2.43 ± 020	4.00 ± 0.31	4.29 ± 0.29	3.57 ± 0.30

***** ± Standard error.

In the presence of 1 mg/l 6-benzyladenine (BA), 160 mg/l adenine sulphate proved to be a beneficial supplement as it increased shoot number (Table 4). (Amin and Jaiswal 1987) the pioneers of guava tissue culture-did not investigate this addendum. This may be due to the fact that addition of adenine sulphate to culture media was not always beneficial (Davies, 1972; George and Sherrington, 1993), although promotive effects were frequently reported (De Rojas and Kitto, 1988; Paek *et al*, 1987; Ziv *et al*, 1970).

Table (4): Axillary Shoot Formation as Influenced by Various Supplements of Adenine Sulphate to the Multiplication Media.

Adenine conc. (mg/l)	Shoot Number	Shoot Length (cm)
0.0	2.0 ± 0.37*	1.70 ± 0.15*
40	2.0 ± 0.21	2.03 ± 0.18
80	2.4 ± 0.27	1.85 ± 0.13
160	3.2 ± 0.39	1.84 ± 0.18
320	1.4 ± 0.22	1.72 ± 0.11

* ± Standard error.

At 1 mg/l, silver nitrate enhanced vigor and reduced leaf necrosis, whereas higher concentrations were depressive (Table 5). The decline might be attributed to the adverse effect of ethylene accumulation in old cultures as suggested by (De Proft *et al.,* 1985). Inclusion of silver nitrate in culture media was found beneficial to bypass decline in cocoa (Dublin *et al.,* 1991) and petunia (Gavinlertvatana *et al.,* 1980).

Table (5): Influence of Various Levels of Silver Nitrate on Shoots Number, Vigor and Number of Necrotic Leaves After 6 Weeks on Basal Medium.

Silver nitrate (mg/l)	Shoots number	Vigor	Necrotic leaves (No.)
0.0	1.7 ± 0.40*	3.3 ± 0.21*	1.6 ± 0.16*
1	2.5 ± 0.45	3.7 ± 0.15	1.2 ± 0.13
3	2.0 ± 0.37	3.1 ± 0.23	1.2 ± 0.13
10	2.1 ± 0.41	3.1 ± 0.18	1.1 ± 0.18
30	1.9 ± 0.39	2.7 ± 0.15	2.2 ± 0.39

* ± Standard error.

Indol-3-butyric acid (IBA) at 1.0 mg/l induced the highest rooting incidence (80%), as well as the highest number of roots per culture (Table 6). It restored vigor to a very high level. The result is in line with that of (Ahmed *et al.*, 1995), who obtained high rooting percentage by inclusion of indol-3-butyric acid (IBA) in rooting media of guava, whereas the same goal was achieved by (Amin and Jaiswal 1987) upon use of auxin in rooting media containing activated charcoal (AC).

Table (6): *In Vitro* Rooting and Vigor of Guava Propagules as Affected by Various Supplements of IBA to the Rooting Media.

IBA conc.(mg/l)	Rooting (%)	Root No.	Vigor
0.0	10(0-45)*	0.2 ± 0.20**	3.0 ± 0.21**
0.1	20(3-56)	0.4 ± 0.31	3.2 ± 0.25
0.5	60(26-88)	2.4 ± 0.67	3.8 ± 0.39
1.0	80(44-97)	3.0 ± 0.52	4.0 ± 0.21
5.0	40(12-74)	1.0 ± 0.42	4.6 ± 0.16

*95% confidence limits in parenthesis, ** ± Standard error.

On the basis of the results of the present investigation, it can be concluded that the detrimental effect of phenolic browning was avoided by presoaking of guava explants in solution containing 0.5 g/l activated charcoal (AC) for 3 hours, incorporation of 1.5% activated charcoal (AC) in the basal medium and vertical positioning of explants onto culture media. The multiplication potential of cultures was enhanced by a supplement of 160 mg/l to the media of the proliferation phase in the presence of 1 mg/l 6-benzyladenine (BA). Cultures decline was avoided by an addendum of 1 mg/l

silver nitrate. Enhanced rooting was obtained in media enriched with 1 mg/l idol-3-butyric acid (IBA).

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