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# IN VITRO CULTURE OF SOME GUAVA CULTIVARS 1-SURFACE-STERILIZATION AND PROLIFERATION STAGE

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**ABSTRACT:** *The experiment was carried out in the Plant Tissue Culture Lab.of the Tissue Culture Lab.of Hort.Dept., Faculty of Agric., Minoufiya Univ., Shebin El-Kom in cooperation with Tissue Culture Lab., of Plant Biotechnology Dept., Genetic Engineering and Biotechnology Research Institute, Sadat City University, during the years 2010 to 2013 to investigate the procedures of tissue culture technique for clonal propagation of Banaty, Sabahy and Mobaker (guava cultivars). After surface-sterilization, the plant materials (shoot tips) were cultured in Murashige and Skoog (MS-1962) medium supplemented with various plant growth substances that favour shooting.The best disinfestation procedure obtained when the plant materials were immersed in 0.5% sodium hypochlorite for 6-8 minutes, followed by dipping in 70% ethanol for 1- 4 minutes, then in 0.3% mercuric chloride for 1 minute. Varietal differences appeared by applying different treatments of the three guava cultivars. BA at 1.0 mg/l gave the best effect on shoot production per explant of the three guava cultivars. The better effects on shoot production were obtained when IBA at 0.02 to 2.0 mg/l was combined with BA at 1-2 mg/l. Varietal differences appeared by changing the concentrations of the growth substances used.*

***Key words:*** *Surface-sterilization, Proliferation, Guava, Tissue culture.*

## INTRODUCTION

One of the keys to the profitable production of horticultural crops in an efficient propagation system. Many recent developments have taken place by adopting the most advanced techniques. One of these techniques to be used is plant tissue culture.

The term “tissue culture” encompasses a wide range of techniques and culture systems including *in vitro* culture of protoplasts, cells, tissues, organs (meristems, shoot tips, root tips, anthers, etc.), ovules and embryos.

Fruit crops, are very heterogeneous group of plants, have a long life cycle, including trees, shrubs, climbing vines and perennial herbs.

They inhabit different climates, ranging from tropical to subarctic zones and crops also differ considerably with respect to their origin, taxonomy and breeding systems (Zagaja, 1983).

Guava (*Psidium guajava* L. Myrtaceae) as economically important fruit trees in Egypt, is one of 150 species of *Psidium* most of which are fruit bearing trees native to tropical and subtropical countries. The common guava is a diploid (2*n* = 22), but natural and artificial triploids (3*n* = 33) and aneuploids exist.

The fresh fruit is a rich source of vitamin C (up to 1000 mg per 100 g fresh wt.) and important for home marketing in most countries of the tropics (Loh and Rao, 1989; Zamir *et al.,* 2007; Manoj *et al.,* 2009; Youssef *et al.,* 2010 and Kareem, *et al.,* 2013). In addition, its root bark and young leaves are used in traditional local medicine. The hard wood is also used for engraving and for tool handles.

Sexual propagation is the main method of guava multiplication in Egypt, producing plants of different genotypes with high diversity in physical and chemical properties.

To get identical trees with high uniform fruit quality, vegetative propagation of well plants as well as selected seedy trees must be performed. The commercial vegetative method of multiplication is by the grafting approach. This method yields 85-95% of success when preformed carefully.

Though a high percentage of success is obtained but the method is expensive and troublesome (Chandra, 1965; Meghwal *et al.,* 2010; Liu Xiaomei and Yang Guochen, 2011 and Manisha *et al.,* 2014). Micropropagation is the rapid asexual multiplication *in vitro* of a desired plant. This method offers the fruit breeder the opportunity to quickly increase new selections so that can be evaluated sooner than would be possible using conventional propagation techniques (Zimmerman, 1983; Nayak *et al.,* 2010; Hussien *et al.,* 2011; Chiruvella *et al.,* 2011 and *Jin et al.,* 2014).

Selection of desire plants by farmers, depending on economic value and some important characteristics like early or late fruiting and high yield and quality, for several generations has helped fruit breeders to use the selected plants in their breeding program.

*In vitro* plant propagation from shoot tips or nodal explants of mature tree of guava has many advantages such as rapidness, production of disease-free plants, field trails can be quickly established, great savings in fuel costs, greenhouse space, etc., precise timing eliminates effect of the seasons and enabling year round production, and the propagation *in vitro* on its own root system makes grafting and budding onto root stocks unnecessary, saving a great deal of work.

The objective of this research was to determine the feasibility of using tissue culture techniques propagation for (proliferation stage) of three promising guava cultivars namely: *Banaty, Sabahy* and *Mobaker.* Establishing tissue culture techniques for raising these promising cultivars was also a main objective of this investigation.

## MATERIALS AND METHODS

This research work was carried out during the period 2010-2013, at the Plant Tissue Culture Lab. of Hort.Dept., Faculty of Agric., Minoufiya University, Shebin El-kom in cooperation with Tissue Culture Laboratory of Plant Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City University, Egypt, with some promising guava cultivars in a trial to apply the tissue culture techniques as a mean of rapid clonal propagation.

Some newly introduced guava cultivars were chosen to be the source of explants throughout this study. They are namely: Banaty, Sabahy and Mobaker. Explants were taken from adult trees grown at the orchard of the Hort. Res. Inst., Giza. The plant materials were collected during the active growth period (especially in May and June for those explants taken from the adult trees).

### I. Preparation and Surface-Sterilization of Explants:

Two experiments were carried out using different surface- sterilization treatments. The first included a combination of immersing in 0.5% sodium hypochlorite (Na O Cl) for 10 mins., followed by dipping in zero-5 mins. in 70% ethanol. To reduce the time of surface- sterilization, a second experiment was set up that included using mercuric chloride ($Hg Cl_2$) at 0.3% for 1 min. after decreased periods of immersing in 0.5% (Na O Cl) for 4, 6 or 8 mins. with dipping in 70% ethanol for 1, 2 or 4 min.

The procedure adopted by Amin and Jaiswal (1987) was nearly used. Thus actively growing shoots (10 cm) were taken and put into plastic bags, then were soaked in running tap water for 2 hr. Shoot tip explants were prepared by removing lateral leaves and excising 5-10 mm of the terminal shoot containing the apical meristem and few leaf primordia.

The apices were then immersed in 0.5% sodium hypochlorite with 0.1% Tween 20 for 10 min., rinsed 2 times for 5 min. each in SD water, then soaked for 5 min. in 70% ethanol and rinsed twice for 5 min. in SD water.

It is worth to mention that the explants of guava cultivars were cultured after disinfestation in Murashige and Skoog basal medium (MS) (1962), without growth substances for 15 days. Such medium was supplemented with 2 mg/l glycin, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine Hcl, 0.1 mg/l thiamine Hcl, 100 mg/l myoinositol and

30 g sucrose. The pH was adjusted to 5.7-5.8 with 0.1 N NaOH or HCl prior to adding the agar (7 g/l) and the medium was dispensed in 25×150 mm test tubes (each containing 20 ml of the mentioned medium) and stoppered with polypropylene caps and autoclaved at 121C° at 1.5 kg/cm² steam pressure for 20 min.

## II- Proliferation:

The following two experiments were carried out. The cytokinin (BA) was used with or without the auxin (IBA):

(a) Benzyladenine (BA) was used at three concentrations: 0.1, 1.0 and 5.0 mg/l with the guava cultivars included in this study.

(b) The auxin indole-3-butyric acid (IBA) was added at 0.02 and 2.0 mg/l to different concentrations of BA (0.2, 1.0 and 2.0 mg/l).

## III. Experimental Determinations:

**(a) Establishment**: The number of alive explants after surface-sterilization procedures was recorded and consequently the percentage of aseptic cultures was calculated.

**(b) Proliferation**: The average number of shoots per explant was calculated in all experiments 6 weeks after culturing.

**(c) Subculturing:** It is worth to mention that the cultures were subcultured to fresh media at six-week-intervals during multiplication as they turned pale when they were left for longer periods.

Guava cultures were incubated under 16/8 hr. light/dark cycle. Illuminance was provided by standard white fluorescent which produced about (2000-3000 Lux) at the explant level. A temperature of 25±1 C° was kept constant.

The completely randomized design was adopted. Each treatment consisted of 12-15 replicates. Each explant was cultured in an individual tube.

The obtained data for the investigated treatments were subjected analysis of variance according to Snedecor and Cocharn (1972). The percentages were transformed to angles. Means of the evaluated treatments were differentiated using Duncan multiple rang test at 5% level (1955).

## RESULTS AND DISCUSSION

## I. Surface-Sterilization:

Data concerning the effect of various methods of surface sterilization on asepsis of different guava cvs. (Banaty, Sabahy and Mobaker) are presented in Table (1).

Data of these guava cultivars, generally indicate that increasing the time of dipping in 70% ethanol from zero to 2.5 to 5.0 minutes increased the percentage of aseptic cultures significantly (with the time of previous immersion in 0.5% sodium hypochlorite being fixed at 10 minutes) in all tested varities expect for all the Banaty in which the addition showed a decrease with raising the time of dipping in 70% ethanol, though the difference was insignificant.

Data concerning the effect of combinations of various substances on asepsis of different guava varities are shown in Table (2).

**Table (1): Effect of various methods of surface-sterilization on asepsis of guava cultivar cultures. I.Effect of combining sodium hypochlorite with ethanol.**

| Time (min.) of | | Percentage of aseptic cultures (15 days after treatments) | | | | | |
|---|---|---|---|---|---|---|---|
| Immersion in 0.5% NaOCl | Dipping in 70% ethanol | Banaty | | Sabahy | | Mobaker | |
| | | % | Angle | % | Angle | % | Angle |
| 10 | 0.0 | 30 | 33.1 b | 40 | 39.2 b | 32 | 34.4 c |
| 10 | 2.5 | 66 | 54.4 a | 75 | 60.0 a | 66 | 54.4 b |
| 10 | 5.0 | 60 | 50.8 a | 79 | 62.9 a | 78 | 62.2 a |

Mean separation within columns by L.S.D. (5% Level).

**Table (2): Effect of various methods of surface-sterilization on asepsis of guava cultivars cultures. II.Effect of adding mercuric chloride to sodium hypochlorite and ethanol combinations.**

| Time (min.) of | | | Percentage of aseptic cultures (15 days after treatments) | | | | | |
|---|---|---|---|---|---|---|---|---|
| Immersion in 0.5% NaOCl | Dipping in 70% ethanol | Dipping 0.3% $HgCl_2$ | Banaty | | Sabahy | | Mobaker | |
| | | | % | Angle | % | Angle | % | Angle |
| 4.0 | 1 | 1 | 47 | 43.3e | 50 | 45.0d | 37 | 37.5 e |
| 4.0 | 2 | 1 | 49 | 44.4e | 63 | 52.5c | 45 | 42.1 d |
| 4.0 | 4 | 1 | 65 | 53.8cd | 72 | 58.1bc | 56 | 48.5 c |
| 6.0 | 1 | 1 | 61 | 51.4 d | 62 | 52.0c | 45 | 42.1de |
| 6.0 | 2 | 1 | 75 | 60.0ab | 73 | 61.3b | 57 | 49.0 c |
| 6.0 | 4 | 1 | 77 | 61.5 a | 76 | 58.7bc | 66 | 54.3 b |
| 8.0 | 1 | 1 | 71 | 57.5ab | 86 | 68.3a | 84 | 66.4 a |
| 8.0 | 2 | 1 | 69 | 56.2bc | 67 | 56.3bc | 82 | 65.4 a |
| 8.0 | 4 | 1 | 29 | 32.6f | 48 | 43.9d | 67 | 55.0 b |

Mean separation within columns by L.S.D. (5% Level).

Surface-sterilization methods included a combination of immersion in 0.5% sodium hypochlorite (4, 6, 8 minutes), dipping in 70% ethanol (1, 2, 4 minutes) and 0.3% mercuric chloride (1 minute).

Data obtained clearly indicate that sterilization for 8 minutes in 0.5% sodium hypochlorite followed by dipping for 1 minute in 70% ethanol and 0.3% mercuric chloride successively gave the most marked effect for Sabahy and Mobaker cultivars.

With Banaty, the best effect was obvious with immersion for 6 minutes in 0.5% sodium hypochlorite and dipping for 4 minutes in 70% ethanol then dipping for 1 minute in 0.3% mercuric chloride since increasing the time of immersion in sodium hypochlorite resulted in an obvious decline especially with increasing the time of dipping from 1 to 4 minutes in 70% ethanol.

The results of the present investigation regarding this item reveal a varietal difference between cultivars to the disinfestation procedures. In order to reduce the time of disinfestation and to increase the efficiency of such treatment, guava explants were treated with mercuric chloride ($Hg\ Cl_2$) as it increasing the percentage of aseptic cultures obtained.

Mercuric chloride was originally recommended by Nekrosova (1964) and Khattak *et al*., (1990).

In conclusion, one can say that using 0.5% sodium hypochlorite for 8 minutes followed by 70% ethanol for 1 minute then 0.3% mercuric chloride for 1 minute for Sabahy and Mobaker cvs., while 6 minutes in 0.5% sodium hypochlorite followed by 4 minutes 70% ethanol then 1 minute 0.3% mercuric chloride for Banaty cv. cultures could be recommended.

## II. Proliferation:

Clonal propagation or rapid asexual multiplication can be obtained through enhanced precocious growth of lateral growing points on the explant at the nodes below the apical meristem.

Growth from these axillary shoots provides a rapid multiplication system in which the number of potential plants is increased exponentially. Such proliferated shoots are excised at interval periods and transferred to fresh media containing various growth substances where further axillary shoot production occurs.

## 1. Effect of adding cytokinin BA on proliferation:

Table (3) show the effect of different BA concentration (0.1 to 5.0 mg/l) on three cultivars namely: Banaty, Sabahy and Mobaker.

It is evident that increasing the concentration from 0.1 to 1.0 mg/l exerted a pronounced significant increase in average shoot number per explant. Nevertheless, increasing BA concentration to reach 5.0 mg/l, caused a marked significant drop in shoot number especially with Mobaker cv.

## 2. Effect of combining IBA with BA on proliferation:

It is clear from Table (4) that increasing BA concentration from 0.2 to 1.0 mg/l (with IBA fixed at 0.02 mg/l) gave a marked increase almost about four times the value obtained with the lower concentration with the three guava varieties.

The three genotypes responded differently when BA was doubled to 2.0 mg/l with a constant concentration of IBA (0.2 mg/l). Therefore, Mobaker shoots gave the highest value with a concentration of 1.0 mg/l BA plus 0.2 mg/l IBA, while the highest value was obtained with 2.0 mg/l BA plus 0.2 mg/l IBA in the case of Banaty guava cv. concerning Sabahy cv., the highest value was obtained with 1.0 mg/l BA plus 0.02 mg/l IBA.

Such finding is in harmony with that reported by Fuenmayor and Montero (1997) who found that BA at 1.0 mg/l consistently was the most satisfactory for multiplication of almond and almond-peach hybrid shoots. Generally, the tendency of better shooting was observed for Mobaker cv than Banaty or Sabahy, thought the plant material of the former cv was taken from adult trees.

Such unexpected behavior could be explained by the fact that though mature tissues to not do as well as juvenile tissues *in vitro,* the differences in growth rate may be negated with time as the explants slowly or rapidly revert to juvenility *in vitro* as reached by Hackett (1985).

The present data further indicate that adding IBA to BA yielded better values of shooting of the three guava cultivars explants. Yet, varietal differences were evident.

**Table (3): Effect of adding BA to the culture media on average number of new shoots produced per explant of the three guava cvs.**

| Cultivar | BA (mg/L) | | |
|---|---|---|---|
| | 0.1 | 1.0 | 5.0 |
| Banaty | 3.5 b | 4.6 a | 2.5 c |
| Sabahy | 2.6 c | 6.1 a | 4.6 b |
| Mobaker | 4.3 b | 7.2 a | 1.2 c |

Mean separation within rows by L.S.D. (5% Level).

**Table (4): Effect of adding combinations of IBA and BA to the culture media on average number of new shoots produced per explant of the three guava cvs.**

| Cultivars | BA + IBA (mg/l) | | | |
|---|---|---|---|---|
| | 0.2+0.02 | 1.0+0.02 | 1.0+0.2 | 2.0+0.2 |
| Banaty | 2.9 d | 8.1 c | 11.6 b | 14.7 b |
| Sabahy | 2.4 c | 7.8 a | 3.9 b | 3.1 bc |
| Mobaker | 3.5 d | 13.8 c | 20.4 a | 15.3 a |

Mean separation within rows by L.S.D. (5% Level).

The present data are in harmony with the original elegant experiments of Skoog and Miller (1957) who found that combining auxin with cytokinin exhibits a striking quantitative relationship in the regulation of morphogenesis. They showed that organ formation in tobacco pith tissue cultures could be controlled by the concentration and ratio of auxin and cytokinin in the medium.

Thus, a relatively high auxin: cytokinin ratio led to extensive root formation, whereas a low ratio of the same substances led to spectacular changes in the growth and differentiation of the tissue. Intermediate concentrations of both, produced only callus growth.

The concentrations and ratios of auxin to cytokinin required to elicit a particular morphogenetic response, vary widely with the plant species used and the constitution of the growth medium (Horgan, 1984).

Thus, the striking mature of the above phenomenon has resulted in suggesting that the endogenous cytokinin: auxin ratio can control growth and morphogenesis in whole plants.

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# الاكثار النسيجى لبعض أصناف الجوافه
# ١- التطهير السطحى وانتاج الأفرع

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## الملخص العربى

كان الهدف من هذا البحث دراسه التطهير السطحى وإنتاج الأفرع كمرحله من مراحل إنتاج ثلاثه من أصناف الجوافه (البناتى – الصباحى – المبكر) بواسطه تكنيك زراعه الأنسجه النباتيه وقد أجريت هذه الدراسه فى معمل زراعه الأنسجه النباتيه بقسم البساتين – كليه الزراعه – جامعه المنوفيه بالتعاون مع معمل زراعه الأنسجه النباتيه بقسم بيوتكنولوجيا النبات – معهد الهندسه الوراثيه – جامعه مدينه السادات خلال الاعوام ٢٠١٠–٢٠١٣ و ذلك بعمل تجربتين كالآتى:

الاولى : إجراء التطهير السطحى للمنفصلات النباتيه (قمم طرفيه) و كانت المعاملات كالآتى:

أ- نقع المنفصلات النباتيه فى محلول هيبوكلوريت الصوديوم (٠,٥٪) لمده ١٠ دقائق ثم الغمس فى كحول الايثيل ٧٠٪ لمده (صفر-٥ دقائق).

ب- إستخدام كلوريد الزئبقيك (٠,٣٪) لمده دقيقه بعد نقع المنفصلات النباتيه فى محلول هيبوكلوريد الصوديوم (٠,٥ ٪) لمده (٤-٦-٨ دقائق) ثم الغمس فى كحول الايثيل ٧٠٪ لمده (١–٢-٤ دقائق).

الثانيه : تمت زراعه المنفصلات النباتيه فى بيئه موراشجى و سكوج (١٩٦٢) و المضاف اليها العديد من منظمات النمو وذلك لدراسه تشجيع إنتاج النموات وكانت المعاملات كالآتى:

أ- تأثير إضافه البنزيل أدنين بتركيزات (٠,١ – ١ – ٥ ملجرام/لتر)

ب- تأثير إضافه اندول حامض البيوتريك بتركيزات (٠,٠٢ – ٠,٢ ملجرام/لتر) الى البنزيل أدنين بتركيزات ( ٠,٢ - ١ - ٢ملجرام/لتر).

و قد تم تحليل النتائج إحصائيا و كانت أهم نتائج هذه الدراسه كما يلى:

أ- التطهير السطحى :

كانت أفضل النتائج المتحصل عليها هى عند النقع فى محلول هيبوكلوريت الصوديوم (٠,٥٪) لمده ٦–٨ دقائق يليها الغمس فى كحول الايثايل (٧٠٪) لمده ١–٤ دقائق ثم محلول كلوريد الزئبق (٠,٣٪) لمده دقيقه وكانت هناك اختلافات فى الاستجابه بين الاصناف عند اجراء المعاملات المختلفه.

ب- إنتاج الافرع :

١–أعطى تركيز ١ مللجرام/لتر فى البنزايل أدينيين أفضل تأثير من حيث زياده الأفرع الناتجة للثلاثة الأصناف المستخدمة.

٢– أمكن الحصول على نتائج أفضل عند اضافه اندول حامض البيوتريك بتركيز ٠,٠٢- ٠,٢ مللجرام/لتر إلى البنزايل ادينيين بتركيز ١–٢ مللجرام/لتر.

٣– كانت هناك اختلافات واضحه بين الأصناف عند تغيير تركيزات المواد المستعملة.