Studies on Initial Shoot Tip Culture of Guava (*Psidium guajava* L.)

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Key words: *Psidium guajava*, Tissue culture

Summary

This study conducted to study the effects of pre-treatment, medium and hormones on shoot tips culture of guava (*Psidium guajava* L.) cvs. Jen-Ju, Li-Tzy, Pakistani, Shyh-Jii, and Huang.

Shoot tips about 2 cm in length were harvested from different season and were treated with 100 mg/l ascorbic acid and 150 mg/l citric acid for 20 minutes before disinfection, followed by surface sterilized with immersion in a solution of 1% sodium hypochloride for 10 minutes. Shoot apexes with 2 leaf primordia in 0.2-0.3 mm length were excised under microscope and cultured in different media.

The results showed that the survival percentage of pre-treated explants was higher than non-pretreated. All of the cultivars cultured on the filter paper bridge liquid medium had higher survival percentage than on solid medium. Moreover, the survival percentage of medium supplemented with antioxidants, 100 mg/l ascorbic acid and 150 mg/l citric acid, was higher than the medium without antioxidants. Incubating explants under darkness condition for 15 days before moved to lightness condition increased survival percentage and fresh weight. Survival percentage and fresh weight were higher in treatments of using spring and autumn shoots than summer shoots. Optimum medium for initial culture was medium containing 0.1 mg/l IBA and 1 or 2 mg/l BA.

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Introduction

Conventionally, guava is propagated through seeds; however, air layering, cuttings, stooling, and budding are also found to give appreciable success (Jaiswal and Amin, 1992) but the rate of multiplication by these methods is not very fast. Micropropagation method could assist in rapid and mass production of clonal stock of newly released improved cultivars of guava (Mishra *et al*., 2007). Clonal propagation of guava through micropropagation has been demonstrated using shoot tip (Papadatou *et al*., 1990; Lee and Yang, 1994), nodal segments (Amin and Jaiswal, 1987, 1988; Loh and Rao, 1989; Mohamed-Yasseen *et al*., 1995) and shoot buds (Amin, 1986; Amin and Jaiswal, 1988; Papadatou *et al*., 1990).

Of the several diseases affecting guava, wilt is the most important, as it occurs in endemic form and may wipe out entire orchards in a region (Singh, 2002). During the last two decades, this malady has been reported from several countries including India (Dwivedi *et al*., 1990) and South Africa (Schoeman *et al*., 1997). In this research, an efficient plant initiation *in vitro* from shoot tip of guava were studied for produced healthy plants and high quality plants.

Materials and Methods

Materials

Five years old trees of *Psidium guajava* L. cvs. Jen-Ju, Li-Tzy, Pakistani, Shyh-Jii, and Huang grown on the Horticultural Research Station, National Chung Hsing University, Wufeng, Taichung, Taiwan, were used as initial explants sources.

Culture medium

The basal medium contained half strength of MS salts (Murashige and Skoog, 1962), 30 g/l sucrose and 7 g/l agar (Bacto-agar) for solid medium or use paper bridge for liquid medium. The pH value of the medium was adjusted to 5.7 with 1N NaOH or 1N HCl and then autoclaved at 121°C, 1.25 kg/cm^2 for 20 min. The plant growth regulators (IBA and BA) were supplemented according to the experiments detailed below. Shoot tips were incubated at 25 ± 2°C under a 16 hours photoperiod and light intensity of 2,500 to 3,000 lux. The cultures were maintained for 45 days for shoot initial culture and 60 days for shoot multiplication and growth.

Methods

Active shoots were collected and leaves were removed. Shoots were cut with approximately 2 cm long and soaked into 100 mg/l ascorbic acid with 150 mg/l citric acid for 20 min to prevent accumulation of phenolic compounds. After that surface sterilization with a solution of 1% sodium hypochloride (NaOCl) plus 1 drop Tween 20 for 10 min, the shoots were

rinsed 5 times with sterilized distilled water under axenic condition in a laminar-flow cabinet. Shoot apexes (0.2-0.3 mm long) containing the meristem with two leaf primordia were excised under a microscope and cultured into the medium incorporated with antioxidants (100 mg/l ascorbic acid and 150 mg/l citric acid). After culturing, shoot tips were incubated under dark condition for 15 days then move to light condition. Survival percentage and fresh weight were recorded. Survival percentage was counted from green shoot grown out.

1. Effects of antioxidants in the medium on initial shoot tip culture

In this experiment, 'Pakistani' guava was taken in spring season. Before surface sterilization, explants were pretreated by one of the following solutions for 20 min: 0.7% PVP (polyvinyl pyrrolidone) with 2% sucrose; 100 mg/l ascorbic acid; 150 mg/l citric acid; 100 mg/l ascorbic acid plus 150 mg/l citric acid; or 0.7% PVP with 2% sucrose for 20 min and 100 mg/l ascorbic acid plus 150 mg/l citric acid for 20 min. After surface sterilization, explants were cultured on mediums which were supplemented with or without 100 mg/l ascorbic acid and 150 mg/l citric acid after autoclaving. Ascorbic acid and citric acid solutions were added by filtration through a 0.22 μm filter (Minisart, Sartorius). There were 2 treatments with 6 replications each with one treatment as one replicate. The cultures were maintained for once after 45 days of incubation. Survival percentage was recorded.

2. Effects of darkness and lightness treatment on initial shoot tip culture

In this experiment, shoot tips from different cultivars (Jen-Ju, Li-Tzy, Pakistani, Shyh-Jii and Huang) were taken in autumn and cultured on solid and liquid medium. Experiment was carried out under different environment of darkness and lightness treatments. The cultures were incubated in darkness for 15 days and then move to lightness and another one was incubated in lightness after cultured on medium. Experiment divided into 4 treatments for darkness and lightness, respectively, 5 replications each with one cultivar as one replicate. The cultures were maintained for once after 45 days of incubation. Survival percentage and fresh weight were recorded.

3. Initial shoot tip culture of guava in different growth season

In this experiment, shoot tips were taken in different seasons of 2007 (spring, summer, and autumn), five cultivars (Jen-Ju, Li-Tzy, Pakistani, Shyh-Jii and Huang) were cultured on ½ MS liquid medium contained 30 g/l sucrose, 0.1 mg/l IBA, and 0.5 mg/l BA. There were 3 treatments and 5 replications each with one cultivar as one replicate. The cultures were maintained for once after 45 days of incubation. Survival percentage and fresh weight were recorded.

4. Effects of BA on initial shoot tip culture

In this experiment, shoot tips of 3 cultivars in 2008 (Li-Tzy, Jen-Ju and Shyh-Jii) were

cultured on ½ MS liquid medium contained 30 g/l sucrose and 0.1 mg/l IBA adding different concentrations of BA at 0, 0.1, 0.5, 1 and 2 mg/l. There were 5 treatments and 3 replications each with one cultivar as one replicate. The cultures were maintained for once after 45 days of incubation. Survival percentage and fresh weight were recorded.

5. Statistical analysis

Data were subjected to analysis of CRD (Completely Randomized Design) using general linear model (GLM) procedure (Statistical Analysis System; SAS, 2007). Significant differences among treatment means were measured by Least Significant Difference test (LSD) at $P<0.05$.

Results

1. Effects of antioxidants in the medium on initial shoot tip culture

The data on the effects of antioxidants in the medium on initial shoot tip culture of 'Pakistani' guava has been presented in Table 1. There were significant different in survival percentage between medium with and without antioxidants. Survival percentage of medium with antioxidants (67.5%) was higher than medium without antioxidants (33.1%).

Table 1. Effects of antioxidants in the medium on initial shoot tip culture of 'Pakistani' guava

Pretreatment	Survival (%)	
	antioxidants	no antioxidants
Control	45.0	30.0
PVP	68.4	55.0
Ascorbic acid	80.0	57.9
Citric acid	78.9	25.0
Ascorbic acid + citric acid	87.5	10.5
PVP + ascorbic acid + citric acid	45.0	20.0
Mean	67.5 ± 18.4 a[z]	33.1 ± 19.2 b

[z] Mean ± SE, in the same row followed by the same letter are not significantly different at p=0.05 by *t*-test.

2. Effects of darkness and lightness treatment on initial shoot tip culture

The effects of darkness and lightness treatment on initial shoot tip culture in different medium type were presented in Table 2. There were significant different in survival percentage and fresh weight of solid and liquid medium between darkness and lightness treatment. Shoot tips in liquid medium and incubated under darkness treatment were the highest of survival percentage and fresh weight (35.2% and 3.8 mg, respectively). Both of solid and liquid medium incubated under lightness treatment were recorded not significant different in survival percentage and fresh weight.

3. Initial shoot tip culture of guava in different growth season

Survival percentage and fresh weight in initial culture of shoot tip in spring, summer and autumn were recorded in Table 3. There were significant different in survival percentage among growth season. Survival percentage of shoot tip in spring season (64.6%) was higher than in autumn and summer season (50.9 and 34.7%, respectively). Fresh weight were recorded not significant different among growth season. Moreover, fresh weight in spring season (2.7 mg) was higher than in autumn and summer (2.4 and 2.3 mg, respectively). Comparison in different season to take shoot tip for culturing, it was found that survival percentage and fresh weight was more effective in spring and autumn than summer, in summer season had lower survival percentage and higher browning percentage.

4. Effects of BA on initial shoot tip culture

The effects of BA on initial shoot tip culture were presented in Table 4 and 5. There were significant different in survival percentage among different concentration of BA. The maximum in survival percentage was recorded in medium containing 2 mg/l BA (78.8%) followed by medium containing 1 mg/l BA (72.1%) and the minimum survival percentage was recorded in medium containing 0.1 mg/l BA (24.6%). The results recorded that there were not significant different in fresh weight among different concentration of BA. The maximum in fresh weight was recorded in medium containing 2 mg/l (2.2 mg) followed by medium containing 0.5 and 1 mg/l (2.1 mg) and the minimum fresh weight was recorded in medium containing 0.1 mg/l (1.7 mg).

Table 2. Effects of darkness and lightness treatment on initial shoot tip culture in different medium type

Treatment	Medium type	Cultivars	Survival (%)	Fresh weight (mg)
Darkness	Solid	Pakistani	23.1	3.4
		Li-Tzy	33.3	3.4
		Jen-Ju	12.5	2.0
		Huang	26.3	1.5
		Shyh-Jii	0	–
		Mean	19.0 ± 13.0 b[z]	2.6 ± 1.0 ab
	Liquid	Pakistani	25.0	4.9
		Li-Tzy	40.0	5.9
		Jen-Ju	40.0	2.9
		Huang	41.2	2.5
		Shyh-Jii	30.0	2.6
		Mean	35.2 ± 12.4 a	3.8 ± 1.5 a
Lightness	Solid	Pakistani	12.5	1.4
		Li-Tzy	28.6	1.7
		Jen-Ju	5.0	1.1
		Huang	25.0	1.1
		Shyh-Jii	0	–
		Mean	14.2 ± 7.3 b	1.3 ± 0.3 b
	Liquid	Pakistani	25.0	3.3
		Li-Tzy	11.1	3.1
		Jen-Ju	0	–
		Huang	20.0	1.2
		Shyh-Jii	5.3	1.5
		Mean	12.3 ± 10.3 b	2.3 ± 1.1 ab

[z] Mean ± SE, in the same column followed by the same letter are not significantly different at $p<0.05$ by Least Significant Difference test (LSD).

Table 3. Initial shoot tip culture of guava in different growth season

Season	Cultivars	Survival (%)	Fresh weight (mg)
Spring	Pakistani	46.5	4.9
	Li-Tzy	56.1	1.8
	Jen-Ju	70.8	1.9
	Huang	76.3	2.2
	Shyh-Jii	73.2	2.6
	Mean	64.6 ± 12.7 a[z]	2.7 ± 1.3 a
Summer	Pakistani	11.1	4.3
	Li-Tzy	42.9	1.5
	Jen-Ju	48.8	1.5
	Huang	41.9	1.8
	Shyh-Jii	28.6	2.2
	Mean	34.7 ± 15.1 b	2.3 ± 1.2 a
Autumn	Pakistani	45.7	4.3
	Li-Tzy	43.9	1.7
	Jen-Ju	64.0	1.7
	Huang	48.6	1.9
	Shyh-Jii	52.4	2.3
	Mean	50.9 ± 8.0 ab	2.4 ± 1.1 a

[z] Mean ± SE; in the same column followed by the same letter are not significantly different at $p<0.05$ by Least Significant Difference test (LSD).

Table 4. Effects of BA on survival percentage in initial shoot tip culture of guava

BA concentration	Survival (%)			
(mg/l)	Li-Tzy	Jen-Ju	Shyh-Jii	Mean
0	33.3	10.3	41.4	28.3 ± 16.1 b[z]
0.1	23.3	7.1	43.3	24.6 ± 18.1 b
0.5	43.3	30.0	26.7	33.3 ± 8.8 b
1	63.3	90.0	63.0	72.1 ± 15.5 a
2	93.1	80.0	63.3	78.8 ± 14.9 a

[z] Mean ± SE; in the same column followed by the same letter are not significantly different at $p<0.05$ by Least Significant Difference test (LSD).

Table 5. Effects of BA on fresh weight in initial shoot tip culture of guava

BA concentration	Fresh weight (mg)			
(mg/l)	Li-Tzy	Jen-Ju	Shyh-Jii	Mean
0	1.4	2.4	1.8	1.9 ± 0.5 a[z]
0.1	2.9	0.0	2.2	1.7 ± 1.5 a
0.5	1.4	2.8	2.2	2.1 ± 0.7 a
1	2.3	1.7	2.2	2.1 ± 0.3 a
2	1.7	2.6	2.2	2.2 ± 0.5 a

[z] Mean ± SE; in the same column followed by the same letter are not significantly different at $p<0.05$ by Least Significant Difference test (LSD).

Discussion

The reduction or elimination of phenolic oxidation during culture initiation is essential for successful and efficient establishment and culture of many woody species *in vitro*. Browning is generally considered to result from the oxidation of phenolic substances released from the cut ends of explants by polyphenol oxidases or peroxidase. The commonly employed to overcome the harmful effect of browning include the use of adsorbing agents such as activated charcoal or polyvinylpolypyrrolidone, inclusion of antioxidants in the medium or soaking the explants in antioxidant solution, transfer of explants to fresh medium at frequent intervals or sealing the cut ends of the explants with paraffin wax (Broome and Zimmerman, 1978; Weatherhead *et al.*, 1978; Lloyd and McCown, 1980; Amin and Jaiswal, 1988; Bhat and Chandel, 1991). In the present study, the browning problem was overcome by soaking the explants in ascorbic acid with citric acid before culturing, and including ascorbic acid with citric acid in the medium. Explants were pretreated by ascorbic acid with citric acid and cultured on medium containing antioxidants have been found to be more effective than those in the other pretreatment and medium without antioxidants, which is according to the methods by Murashige (1974) that use of ascorbic acid and citric acid as antioxidants in the establishment stage, furthermore, Lee and Yang (1994) used 0.7% PVP adding 2% sucrose to rinsed guava shoot tip for 20 min, and then rinsed with 150 mg/l citric acid and 100 mg/l ascorbic acid for 20 min before disinfection to culture for preventing browning.

Murashige and Skoog’s (1962) medium had been used for micropropagation in several plants. Thomas and Ravindra (1997) reported that low strength medium or distilled water was

useful to initiate a primary culture. In the present study, shoot tips were cultured on ½ MS medium, this is in agreement with results obtained by Lee and Yang (1994) who found that guava shoot tip explants had the highest survival percentage and fresh weight when cultured on ½ MS medium compared with ¼ MS, MS, 2MS and WPM medium.

The liquid medium could enhance growth and multiplication rate of several plants more than solid medium in micropropagation. For example, chrysanthemum (Hahn and Paek, 2005), pineapple (*Ananas comosus* L. Merr) (Escalona *et al*., 1999), banana (Alvard *et al*., 1993), and plantain (*Musa* AAB) (Roels *et al*., 2005). In the present study, survival percentage of explants in liquid medium was higher than solid medium which is according to the results by Ichihashi and Kako (1977) investigated that *Cattleya* shoot tip explants survived better on a stationary liquid medium containing oxidase inhibitors than on a solidified medium of similar composition.

Although light is invariably essential for the growth of normal green shoots and plantlets, unorganized cell and tissue cultures can frequently be grown in its absence, and darkness may be beneficial to growth and morphogenesis. In the present study, survival percentage and fresh weight of explants in darkness treatment were higher than lightness treatment. When incubated explants in the dark after culturing for 15 days can help to prevent browning. Blackening is usually reduced or prevented by such dark treatment and sometimes subsequent growth is improved even though tissue discoloration is not observed. Applying a dark treatment *in vitro* was successful in lowering oxidation products and increasing growth in the epiphytic orchid *Phalaenopsis* (Pieper and Zimmer, 1976). The same treatment decreased the presence of oxidation products in both *Garrya* and *Hamamelis*, but also lowered explant viability and new shoot growth in *Garrya* (Marks and Simpson, 1990). Explants may be transferred to normal light after a dark treatment, although transfer to light of low irradiance may help to prevent browning. For instance, shoot cultures of *Carya illinoinensis* were kept in the dark for an initial 2 weeks, therefore in a 16h photoperiod (Lazarte, 1984).

The success of shoot meristem cultures is affected by the growth condition of the stock plant and the season during which the explant is obtained. Lee and Yang (1994) had studied on shoot tip culture of guava but they did not study about season to take shoot tip, so in this study had studied about season to take shoot tip. Guava shoot tips were taken to culture in spring, summer and autumn season, however, survival percentage and fresh weight was more effective in spring and autumn than summer, which explants in summer had high browning percentage when culturing. Moreover, Göktürk Baydar *et al.* (2006) found the highest percentages of explant survival were seen in grapevine shoot tips collected in May, while explant survival gradually declined during the summer. It was found that explant survival, shoot number per

explant and shoot length were strongly related to the month of explant collection. Roussos and Pontikis (2001) also reported that explant collection date seemed to be a major factor influencing the relative concentrations of various phenolic compounds in olive explants. Moreover, Thomas and Ravindra (1997) reported that best response of explants in mango from shoot tip was collected during June and August, in addition, *Zizyphus nummularia* explants harvested during July and August were found to be the best for culture (Rathore *et al*., 1992). Explants collected during other periods showed more medium discoloration, turned brown sooner and showed no growth response. Amin and Jaiswal (1993) reported November to January was the best season for initiation culture of jackfruit from field-grown trees.

The most important factor for successful tissue culture is plant growth regulators. Papadatou *et al*. (1990) had been reported, maximum proliferation of guava seedlings by *in vitro* shoot tip culture was achieved with 2 mg/l BA, and the optimal BA level for shoot tips of guava seedlings was 0.1 mg/l (Loh and Rao, 1989). Although some crops were observed that in addition to BA, but a low level of auxin was necessary for optimum growth. In shoot tip of 'B8' carambola was achieved when cultured on medium containing 0.1 mg/l BA in combination with 0.02 mg/l IBA (Lin and Yang, 2002). Moreover, Lee and Yang (1994) found that the best growth of 'Erse-Xizi-Pa' and 'Tai-Guo-Pa' guava shoot tip were cultured on medium containing 0.1 mg/l IBA supplemented with 0.5 mg/l BA. In the present study for autumn season, the best result was cultured on medium containing 0.1 mg/l IBA supplemented with 1 mg/l BA. In spring season, the best result was cultured on medium containing 0.1 mg/l IBA supplemented with 1 or 2 mg/l BA. Furthermore, Loh and Rao (1989) reported that guava shoot tip explants cultured with higher BA concentrations (5 and 10 mg/l) turned brown and did not grow further.

In conclusion, during shoot tip culture of guava *in vitro*, shoot tip were harvested about 2 cm long in spring or autumn, soaked with ascorbic acid plus citric acid for 20 min and surface sterilization with a solution of 1% Clorox for 10 min plus Tween 20. Shoot apices 0.2-0.3 mm long were excised under microscope and cultured on ½ MS liquid medium (paper bridge) containing 30g/l sucrose then incubated in dark condition for 15 days, after that move to light condition. The best of initial culture medium was medium containing 0.1 mg/l IBA supplemented with 1 or 2 mg/l BA. After cultured for 45 days, explants were transferred to solid medium of the same composition containing 30 g/l sucrose, 7 g/l agar and 0.01% activated charcoal.

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關鍵字: 番石榴、組織培養

摘要：以珍珠拔、梨拔、巴基斯坦、世紀拔及黃拔等五個番石榴 (*Psidium guajava* L.) 品種之莖頂做為培植材料，以探討培植體前處理、培養基組成及荷爾蒙對莖頂培養之影響。於植株生長期，採取長約 2 cm 之莖頂為培養材料。培植體於消毒前之前處理以 100mg/l 的抗壞血酸及 150mg/l 檸檬酸處理 20 分鐘後，再浸泡於 1% 次氯酸鈉中消毒 10 分鐘，完成消毒之培植體於解剖顯微鏡下切取長約 0.2-0.3 mm 帶 2 片葉原體大小之生長點，然後植於不同處理之培養基中。結果顯示培植體存活率以經過前處理者較無前處理者高，各品種存活率以植於濾紙橋上之液態培養基者較植於固態培養基者高。此外，培養基中添加 100mg/l 抗壞血酸及 150mg/l 檸檬酸較未添加者有較高的存活率。培植體先置於黑暗環境中 15 天後再移出至光照環境培養，其培植體存活率及鮮重皆較未經黑暗處理者高。春季及秋季採取之莖頂培養後的存活率與鮮重皆高於夏季。初代培養之培養基以 0.1 mg/l IBA 添加 1 或 2 mg/l BA 最合適。

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