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Full Length Research Paper

Enhanced *in vitro* multiple shoot induction in elite Pakistani guava cultivars for efficient clonal plant multiplication

Muhammad Usman*, Madiha Butt and Bilquees Fatima

Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan.

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Elite guava (*Psidium guajava* L.) strains of cv. Safeda were explored *in vitro* for multiple shoot induction. Shoot induction was enhanced up to 83% with 3.5 to 4.25 shoots per single node cutting and shoot tip explants, respectively, using higher levels of benzyl amino purine (BAP) in Murashige and Skoog (MS) medium. Enhancing sucrose supplement to 45 gl^{-1} improved plant growth and development in shoot and root length and leaf size as well (3.8, 5.6 and 0.6 cm, respectively) compared to 30 gl^{-1} of sucrose in the media. Higher root, shoot length and leaf size (6, 8 and 2.8 cm, respectively) was obtained on transplanting multiplied plants in pots containing silt and compost (1:1) compared to compost and sand. The combined effect of BAP and enhanced sucrose in MS medium is suggested to obtain efficient, cost effective, better plant growth and development in guava for year round supply of healthy plant material to growers.

Key words: Guava, shoot tip, benzyl amino purine (BAP), sucrose, *in vitro*.

INTRODUCTION

Guava (*Psidium guajava* L.) is a nutraceutically important fruit crop and is being commercially cultivated in more than 50 countries including South East Asian countries of the world (Bailey, 1960; Watson and Dallwitz, 2007). Leading producers of guava include: India and Mexico while guava is ranked fourth in Pakistan after citrus, mango and date with respect to area 63000 ha and 0.55 million tons production per annum (FAO, 2008, 2009; www.fao.org). About 80% of guava fruit production is contributed by Punjab province. Commercial cultivars of guava in Pakistan include Safeda (Gola and Surahi) and seedless while other varieties like Allahabad, Karela, red fleshed and apple color is less frequently cultivated. We are producing about 10 tons/ha of fresh guava fruit compared to leading countries producing significantly higher. The potential yield gaps with these countries range from ten to fifteen tons per hectare. These gaps are probably due to little or no research work done on indigenous guava plant material for crop improvement.

Further, in Pakistan, guava industry is seedlings based due to lack of any commercially acceptable asexual method of propagation *in vivo* as well as *in vitro* in our indigenous guava plant material (Pereira, 1990; Ali et al., 2003). Enhancing seed germination is further a challenge as it is inhibited by presence of tannins in plants leading to poor, uneven and delayed seed germination thus making guava difficult to propagate sexually (Doijode, 2001; Ali et al., 2003). It further leads to clonal degradation of varieties yielding poor quality fruit suggesting need to establish efficient *in vitro* clonal propagation system for guava. Conventional asexual propagation methods do exist but are impracticable due to long juvenility, more time and plant material requirement for propagation and season dependency (Jaiswal and Amin, 1992). Seedless strains often claimed by amateur growers also need to be genetically characterized for horticultural traits, ploidy confirmation and shall be efficiently propagated for multiplication.

In vitro propagation offers efficient and cost-effective production of healthy, disease free and vigorous plants. These plants yield was better than their progenitors as

*Corresponding author. E-mail: musman74@gmail.com.

Abbreviations: BAP, Benzyl amino purine; **MS,** Murashige and Skoog medium.

reported in banana in Africa and certain other fruit crops. Clonal propagation of commercially important cultivars of fruit crops using direct or indirect organogenesis has been established in guava cultivars like Allahbad safeda (Singh et al., 2002), pineapple guava (Canhoto et al., 1996), Feijoa (Vesco and Guerra, 2001) and other fruit crops like grapes (Das et al., 2002), sweet cherry (Matt and Jehle, 2005) and jujube (Gu and Zhang, 2005). Crop improvement through genetic transformation also needs efficient *in vitro* regeneration system (Rai et al., 2007). The proposed research venture was, therefore, focused on developing an efficient regeneration system that could be helpful in plant transformation of this nutraceutically important fruit crop.

MATERIALS AND METHODS

Media preparation

Murashige and Skoog (MS) medium (1962) was used as basal medium to raise guava seedlings for plant multiplication. Sucrose (Phytotech, USA) was added 30 gl^{-1} as carbon source, medium pH was adjusted at 5.7 and 8 g of phyto agar was added as a solidifying agent in the media. Briefly, 10, 30 and 50 ml of medium was dispensed in each test tube, Petri plate and glass jars, respectively and covered with polyethylene sheet or parafilm. Media were sterilized in an autoclave for 20 min at 121°C and 15 psi.

Plant materials and explant sources

Two commercial strains of guava cv. Safeda which includes pyriformed and rounded (locally called as Surahi and Gola, respectively) were explored for the following experiments.

Seed sterilization and in vitro germination procedures

The fully ripe guava fruits were harvested, cut; seeds were collected in sterile water and agitated to wash off the pulp. The seeds were pretreated with 20% HCl for 12 h (protocol optimized in lab for indigenous cultivars) to enhance seed germination and seeds were surface sterilized with 70% ethanol (v/v) + 1 to 2 drops of Tween-20 detergent for 3 to 5 min followed by two to three rinses with sterile distilled water. Seeds were dipped in 5% NaOCl for 5 to 10 min followed by three to four rinses with sterile distilled water. All sterilization steps were carried out in the laminar air flow cabinet. These seeds were cultured on MS medium for germination and the seedlings were used as source of explants like shoot tip (ST; 2 to 3 mm in size) and single node stem cutting (SNC; 3 to 4 mm in size).

Media sterilization and inoculation procedures: MS medium was supplemented with different levels of benzyl amino purine (BAP) (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mgl^{-1}). Media was sterilized in autoclave at a temperature of 121°C and 15 Psi for 20 min and placed in growth room facilitated with 2500 lux light intensity and maintained at temperature 25 ± 2°C.

Multiple shoot induction: ST and SNC explants were cultured on MS media modified with BAP for multiple shoot induction to develop efficient micro propagation.

Effect of sucrose on plant growth *in vitro*: Different levels of sucrose which includes 30, 45, and 60 gl^{-1} were used to enhance growth rate of *in vitro* regenerated plants that were showing poor growth on MS medium having 30 gl^{-1} sucrose. Data were collected for shoot and root length (cm) and leaf size (cm).

Effect of potting media on growth of in vitro plants

In vitro multiplied plants were transferred to green-house environment for acclimatization and plant growth in the different potting media including sand, compost and compost + silt (1:1). Their effect was noted on the percent shoot growth, root length and leaf size of guava plantlets.

Experimental layout: All the experiments were replicated thrice with at least 20 test tubes per treatment per replication having one explant per tube. The experiments (a) and (b) were laid out according to completely randomized design (CRD) and randomized completed block design (RCBD), respectively. Data were analyzed using M-Stat and significance among treatment means were compared using Duncan's multiple range (DMR) test (Steel et al., 1997).

RESULTS

Shoot induction in commercial guava cultivars using different somatic tissue explants on modified media

BAP

In vitro shoot induction was found to be independent of genotype and explants source and the results for these variables were non-significantly different from each other. Use of BAP, a shoot inducing hormone, was found to be significantly important and shoot induction was enhanced with increasing level of BAP in the MS medium. Shoot buds were initiated after seven days of culture. Maximum shoots were induced on 1.5 to 2.0 mgl^{-1} of BAP yielding 90% shoot induction in both ST and SNC explant types compared to control treatment (66%). Further increase in level of BAP in the medium indicates decline in shoot induction. Among treatments in both explants, 2 mgl^{-1} of BAP showed maximum shoot induction (91.66%) and further increase in its level decreased shoot induction (67.5%) suggesting 2 mgl^{-1} as optimum level of BAP from shoot induction in guava irrespective to the explants used (Table 1).

Similar response was obtained for number of shoots induced per explants on different levels of BAP and the results were found independent of genotype used. Number of shoots induced was significantly ($P<0.05$) enhanced when concentration of BAP was increased in the medium and maximum number of shoots 3.45 to 4.25 was found in SNC and ST explants, respectively, on 2 mgl^{-1} (Figures 1B to D). Further increase in BAP showed sharp decline in number of shoots production (1.16) even when compared with control (1.05) suggesting 2 mgl^{-1} of BAP as the optimal level for better shoot induction in indigenous guava cultivars (Table 1 and Figures 1A to E).

In our studies, both explants gave better multiplication response on MS medium containing 2 mgl^{-1} BAP but the

Table 1. Effect of BAP on shoot induction (%) and number of shoots in guava.

MS medium + BAP (mgL^{-1})	Shoot induction (%)			Number of Shoots per explant	
	ST	SNC	Means	ST	SNC
Control	66.67 ± 3.28	68.33 ± 3.20	67.3^{c}	1.05^{d}	1.15^{d}
0.5	73.33 ± 3.94	78.33 ± 3.77	75.8^{b}	$1.28^{c,d}$	1.10^{d}
1.0	76.67 ± 4.14	78.33 ± 4.00	75.0^{b}	1.83^{c}	$1.91^{b,c}$
1.5	85.00 ± 3.77	87.00 ± 2.58	86.0^{a}	3.53^{b}	3.12^{a}
2.0	93.33 ± 3.33	90.00 ± 2.58	91.6^{a}	4.25^{a}	3.45^{a}
3.0	65.00 ± 3.94	70.00 ± 4.32	67.5^{c}	1.16^{d}	0.95^{d}

Means sharing different letters in a column are significantly different from each other at P < 5% using DMR test. DMR, Duncan's multiple range test; ST, shoot tip; SNC, single node stem cutting.

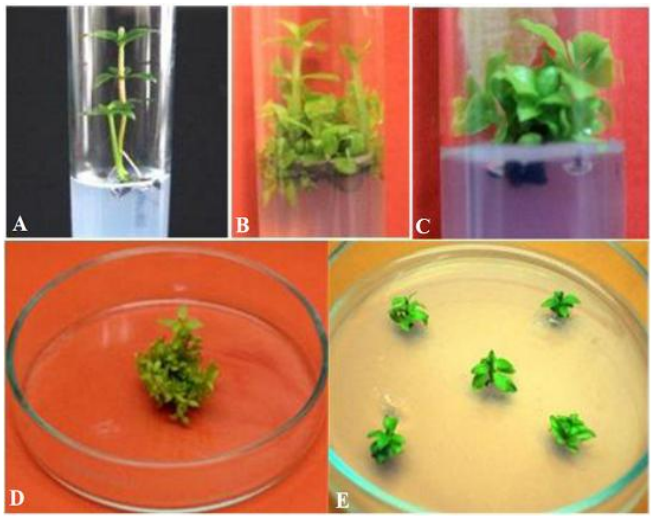

Figure 1. Schematic illustration of multiple shoot induction *in vitro* raised seedlings of guava cv. Pyriformed on MS medium. **A.** Raised guava seedlings after scarification *in vitro*. B and C. Multiple shoot induction in shoot tip and single node cutting explants, respectively, on MS media supplemented with BAP (2 mgl^{-1}). **D.** Multiple shoot induction in shoot tip explants. **E.** 2nd cycle of plant multiplication using developed shoots.

number of shoots obtained per culture vessel were more (4.25) when ST was used as an explant compared to those obtained from SNC (3.45) suggesting ST as better explant source for this purpose. During the shoot induction and micro-propagation it was realized that guava plants are showing relatively slow plant growth and development.Therefore, further studies were planned to explore effect of sucrose on plant growth and development by increasing its level than normal level (30 g) used in MS medium that was taken as control.

Sucrose

Shoot development and number of shoots were found to be significantly improved (85 to 95%) at 45 gl^{-1} of sucrose compared to 30 gl^{-1} (60 to 80%) in SNC and ST explants, respectively. Increase in the level of sucrose showed sharp decline in shoot development (46 to 68%) in SNC and ST explants, respectively, suggesting 45 gl^{-1} of sucrose as the optimum level of sucrose for better and faster growth and development of guava plants *in vitro* (Table 2). Similar trend was observed for the number of shoots per explant and the highest number of shoots (1.5) was observed at 45 gl^{-1} of the sucrose, however, the finding was not significantly different from each other. It is concluded that shoot induction increased with increase in the concentration of sucrose (45 gl^{-1}) and at higher concentration of sucrose (60 gl^{-1}) shoot induction was reduced (1.22) as shown in Table 2.

After two and four months of *in vitro* culture, plant growth and development of guava plants was observed at 30, 45 and 60 gl^{-1} of sucrose in the medium, respectively. All the three variables shoot length, root length and leaf size were found to be significantly higher after two (3.8, 5.7 and 0.6 cm, respectively as shown in Figures 2A and B) and four months (11, 8.4 and 1.94, respectively; Figures 2C and D) of culture in 45 gl^{-1} of sucrose compared to other sucrose levels suggesting significant role of sucrose in plant growth and development (Table 3). Maximum leaf size (1.9 cm) was achieved on 45 gl^{-1} of sucrose followed by 30 gl^{-1} (1.7 cm) whereas leaf size remained minimum on 60 gl^{-1} of sucrose (1.1 cm) after four months of *in vitro* culture (Figure 2C). These findings suggest that 45 gl^{-1} of sucrose is the best level for maximum leaf size in guava cultivars and the protocol may be utilized to get fully expanded leaves for direct shoot induction from leaf disc which is highly desirable for plant genetic transformation in guava.

Plant growth in *in vitro* regenerated guava plants in response to different potting media

The micro-propagated as well as regenerated plants were transferred in pots (Figure 2E) having a range of potting media which includes sand, compost and silt + compost (1:1) to get better plant growth. Plant growth was found to be independent of genotype however; growing media played an important role. Significantly (P>0.05) higher

Table 2. Effect of sucrose on shoot development (%) from different explants in guava cultivars.

MS medium + Sucrose (gL^{-1})	Shoot induction (%)		Number of shoots per explant	
	ST	SNC	ST	SNC
Control (30)	80.01[b]	60.01[b]	1.42	1.32[a]
45	95.00[a]	85.00[a]	1.50	1.45[a]
60	68.33[b]	46.67[b]	1.22	1.02[b]

Means sharing different letters in columns are significantly different from each other at P < 5% using DMR test. ST, Shoot tip; SNC, single node stem cutting.

Figure 2. Effect of sucrose (30, 45, and DE 60 gl^{-1} from left to right respectively on plant growth developed from: A, shoot tip; B, single node cuttings in Guava; C and D, Guava shoot developed on medium with 45 gl^{-1}; E, acclimatization of *in vitro* raised guava plants.

root, shoot length and leaf size (6, 8 and 2.8 cm, respectively) was observed on plants raised on silt and compost containing medium (Figures 3A to C) followed by plants raised on compost (4.8, 6 and 1.4 cm, respectively). Minimum growth was observed in plants developed on sand alone (Table 4). The developed plants were shifted to field after hardening for better growth and further studies (Figure 3D).

DISCUSSION

The study was initiated to enhance shoot induction and multiplication rate in indigenous guava germplasm for availability of healthy plant material to the commercial growers throughout the year using clonal *in vitro* propagation methods. The aforementioned results of enhanced shoot induction are in accordance with the earlier reports (Singh et al., 2002; Mishra et al., 2007; Shah et al., 2008; Rai et al., 2009) in terms of using nodal segments of guava. Maximum shoot organogenesis frequency 91% was recorded with 3.6 shoots per explant by Singh et al. (2002). Shah et al. (2008) used 1 mgl^{-1} BAP and obtained maximum shoot organogenesis (47.6%) with 3.2 shoots per explant. Rai et al. (2009) reported 2.45 shoots at 1 mgl^{-1} of BAP in nodal segments. Combination of two cytokinins, BAP and kinetin is further reported to enhance the rate of adventitious shoot induction in hypocotyls explants. It was observed that shoots obtained from ST explants were generally healthier and vigorous in growth compared to shoots obtained from SNC explants despite more number of vegetative buds leading to more competition in ST explants. This indicates that multiplication response also depends upon the nature of the explant used and shoot tip is a good source tissue for this purpose. It is worth mentioning here that the cultures derived from shoot tip on BAP 2 mgl^{-1} continued to produce shoot primordia upon transfer to fresh medium for five to six months.

In our studies, 2 mgl^{-1} of BAP showed maximum shoot induction (83.3%) with 3.45 shoots per explants in SNC and 4.25 in case of ST explant. Contrary to our findings, Ali et al. (2007) reported 3.9 shoots in nodal segments excised from *in vitro* raised seedlings and was found to be inversely proportional to the level of BAP in the medium that may be attributed to the carry over effect of cytokinin in the medium. Loh and Rao (1989) reported that BAP at low concentration is more effective for shoot proliferation compared to higher doses. There is no other report of such trend in guava as well as other crops in authors view and we have also observed the similar direct relationship of BAP in the medium to the shoots produced per explants. Hussain et al. (1987) and Singh et al. (2002) reported variation in shoot induction response using single cytokinin (BAP) and was found to be genotype dependent. Therefore higher dose requirement of BAP in the medium for enhanced shoot induction may be attributed to the genotypic difference of the cultivars used.

Among non-reducing sugars, sucrose is most common in the phloem sap of many plants (Ahmad et al., 2007) and used as energy source in efficient micropropagation studies in many fruit crops (Jain and Babbar, 2003; Faria et al., 2004). Therefore we enhanced level of sucrose in media to observe response of guava explants to sucrose availability in the medium for better growth and development.

In our studies, enhancing sucrose significantly enhanced the shoot induction (95%), number of shoots (1.42) and leaf area in guava as reported in other crops like black plum 80% (Jain and Babbar, 2003), apple rootstock (Yaseen et al., 2009) and 80% in jujube with, shoot length and fresh weight of shoots in apple (Yaseen

Table 3. Effect of sucrose in plants developed from shoot tip explant in Guava cultivars after 60-120 days of culture *in vitro*

MS medium + sucrose (gL^{-1})	Plant growth (cm)		
	Shoot length	Root length	Leaf size
After 60 days of *in vitro* culture			
Control (30)	2.61^{b}	1.25^{b}	0.12^{b}
45	3.84^{a}	5.78^{a}	0.61^{a}
60	1.45^{c}	0.70^{c}	0.04^{c}
After 120 days of *in vitro* culture			
30	9.16^{B}	7.13^{B}	1.73
45	11.03^{A}	8.46^{A}	1.96
60	5.76^{C}	4.66^{C}	1.16

Means sharing different letters in columns are significantly different from each other at P < 5% using DMR test. DMR, Duncan's multiple range test.

Figure 3. Plant growth behavior *in vitro* regenerated plants of guava cultivars in response to different potting media which includes sand, compost and silt + compost from left to right respectively (A), Root and shoot development of *in vitro* raised plants (B), plant growth after one (C) and three (D) months in green house.

Table 4. Effect of potting media on plant growth in 60 days old *in vitro* regenerated.

Potting media	Plant growth (cm)		
	Shoot length	Root length	Leaf size
Sand	4.05^{C}	5.02^{C}	0.28^{C}
Compost	4.85^{B}	6.20^{B}	1.43^{B}
Silt + compost	6.03^{A}	8.63^{A}	2.81^{A}

Means sharing different letters in columns are significantly different from each other at P < 5% using DMR test. DMR, Duncan's multiple range test.

et al., 2009) compared to other disaccharide carbon sources. Increase in sucrose concentration reduced hyperhydricity in black plum and jujube.

Conclusion

The optimized protocol using MS medium with 2 mgl^{-1} BAP and 45 gl^{-1} sucrose may therefore be used for efficient guava plant multiplication. Micropropagation consuming nodal segments from 80 plants having three to five nodes per plant and yielding 342 plants in three months proved more efficient than the previous reports. Such modes operandi could be highly useful for proficient and cost effective production of true to type healthy plant material required for higher, uniform and quality guava fruit production and to save sharply deteriorating guava industry of Pakistan.

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