Development and Application of Biotechnology in Guava: a Nutraceutical Fruit

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Abstract

Fruits and vegetables have specific healthy attributes. Guava is the most nutritious of all edible fruits. High pectin contents make guava suitable for jelly making. Guava has antidiarrheal, antibacterial, antiamoebic and antispasmodic activities. High concentrations of several vitamins, dietary fiber, carotenoids, lectins, saponins, tannins, phenols, triterpenes, and flavonoids altogether make guava therapeutically an important fruit. Ascorbic acid in guava fruit far exceeds that in citrus. Consumption of guava fruit is reported to lower blood cholesterol, triglycerides, hypertension and some cardiac problems. Due to high quercetin contents, guava leaves are used to develop a phytomedicine for diarrhea. Since guava plant is susceptible to frost, genetic improvement to enhance cold hardiness of guava germplasm is necessary for adapting and cultivating it in temperate climates. Efficient and reliable biotechnology using in vitro plant regeneration amenable to genetic transformation will ensure cold hardiness enhancement in guava. Development of biotechnology protocols for morphogenesis and somatic embryogenesis along with production of edible quality guava fruits and their nutritional analysis has been achieved using various tissue explants from mature guava trees growing at Fort Valley State University. For guava micropropagation, we have developed and germinated 'synseeds' from somatic embryos. Successful preliminary work on genetic transformation has paved the way for incorporating cold hardy genes into the guava genome.

INTRODUCTION

Guava (*Psidium guajava* L., family Myrtaceae) is an important nutraceutical fruit from warm climates. It is an evergreen small tree reaching an average height of 25 ft, with spreading branches. It is found in India, tropical Americas, Africa, and many other tropical countries. Due to high frequency of cross-pollination (about 35%, Menzel, 1985), guava has numerous naturally developed varieties. Some of the important guava cultivars growing at the Agricultural Research Station, Fort Valley State University, are Golden, Red Fleshed, Lucknow 49 or Sardar, Allahabad Safeda, Ka Hua Kula, Beaumont, Ruby x Supreme, Thailand Seedless, Gushiken Sweet, Klom Amporn, Klom Sale and Pear. Guava varieties differ widely in flavor and the flesh color which may be white, cream, pink, or red.

Along with valuable nutrients, guava provides a number of important health benefits. In traditional use, guava fruits are specially provided for eating to sick people to recover very fast after suffering from a prolonged illness. Published literature reveals that this fruit is a rich source of vitamin C, iron, calcium, carotenoids, pectin, and dietary fiber (Yadava, 1996).

For many years guava was a plant of the backyards. But observing its commercial value and future prospects some countries started commercial production of guava. Now, the largest guava producing countries are India, Brazil, Mexico, Venezuela, Africa, Colombia and the USA. Despite its importance in the international trade and domestic economy of more than 50 countries (Menzel, 1985), majority of the US guava acreage is confined to Hawaii, California, and Florida (HASS, 1999). The reason behind this trend is that guava is a crop suitable for the planting zone 10 in the USA, since it cannot withstand

hard frost and/or freezing weather. Therefore, to grow guava in the temperate USA, genetic improvement to enhance cold tolerance of guava germplasm is important.

Medicinal Importance

Researchers working for the United States Department of Agriculture (USDA) have found that guava may be among the fruits richest in antioxidants, suggesting that there may be significant health benefits for people who regularly include guava in their diets. Guava tree bark, leaves, roots and young shoots are being used in traditional medicine to treat many human ailments. Scientific research is validating traditional uses of guava. Due to high quercetin contents, guava leaves are used to develop a phyto-medicine for diarrhea. In addition to treating diarrhea, gastroenteritis and dysentery (Purseglove, 1968), guava reduces levels of LDL cholesterol, triglycerides, and hypertension but increases HDL cholesterol (Singh et al., 1992). The guava leaves and shoots possess medicinal values as they remedy many human ailments involving throat, chest, skin and cerebral tissues (Morton, 1987).

Most Nutritious Fruit

Comparing the daily nutritional value from the USDA data source, guava has been found to be the most nutritious fruit (www.cspinet.org/nah/fantfruit.htm).

Other Uses

There are many recipes for using guava fruit in pudding, cake, pie, sauce, butter, sherbet, marmalade, jam, jelly, chutney, relish, catsup, bakery, and several dairy products and others (Rathore, 1976; Menzel, 1985; Morton, 1987).

Although guava has global potential to meet nutritional needs and health benefits, yet it is an underrated fruit crop that has received little attention from researchers. A reliable gene transfer system for adding few desirable traits would catalyze the genetic improvement of guava and thus its appeal to the US farmers. Advancement towards isolation and introduction of cold hardy gene(s) into cold susceptible plant species is in progress in different research laboratories (Gilmour et al., 1998; Hsieh et al., 2002), and efforts are underway to make plant adaptation much easier in future.

Goal of Research

Hence, this investigation emphasizes the need to develop an efficient in vitro plant regeneration system coupled with a suitable gene transfer method to produce transgenic guava plants with enhanced cold hardiness. The research focused on (1) evolving an efficient in vitro plant regeneration system amenable to gene delivery techniques and (2) developing reliable protocols for gene transfer into guava genome by *Agrobacterium*-mediated genetic transformation approach.

In Vitro Study

The in vitro manipulation of somatic cells or tissues for guava plant regeneration, has not been adequately explored although some success has been reported. Early work on the in vitro culture of guava used fruit mesocarp (Schroeder, 1961; Chandra et al., 2004), anthers (Babbar and Gupta, 1986), mature explants from specific genotypes of guava (Amin and Jaiswal, 1987; Biswas et al., 2004a, b) and seedling explants (Loh and Rao, 1989; Papadatou et al., 1990; Yaseen et al., 1995; Joshee et al., 2004). Micro-propagation protocols using juvenile explants faces limitation to produce true-to-type plants (Ahuja, 1993). A protocol using mature nodal explants instead of seedling explants is more appropriate to obtain true-to-type plants (Harry and Thorpe, 1990). Therefore, the purpose of this investigation was to develop reproducible micropropagation protocols using nodal explants from mature guava trees growing in the specialty plant house (SPH) at Fort Valley State University, Fort Valley, GA, which could be helpful for genetic transformation to develop cold hardy guava genotype(s). For in vitro regeneration, various types of guava explants were explored for organogenesis and somatic embryogenesis.

MATERIALS AND METHODS

All plant materials used in this investigation were collected from mature trees grown in the SPH at Fort Valley State University, Fort Valley, Georgia, USA.

Explants

1. Nodal Explants. Ten- to 15-cm-long twigs were collected from three-year-old guava trees developed from air-layering (Fig. 1a). Twigs were collected in water and kept under running tap water for 2 h followed by surface sterilization with 10% (v/v) commercial Clorox (6% sodium hypochlorite) bleach for 10–15 min. Clorox solution was discarded and explants were rinsed 3–4 times with autoclaved deionized distilled water (DD H_2O). All twigs were cut into 1.0 to1.5-cm-long nodal segments consisting at least one node per explants for inoculation into different culture media. This operation was performed in the laminar airflow cabinet. Explants were incubated in culture room set at 25 ±2°C temperature and 16 h photoperiod produced by cool white fluorescent lamps.

2. Zygotic Embryos. Zygotic embryos of guava were isolated from immature seeds from green developing guava fruits and were cultured on various media formulations to observe their response. Guava fruits were collected at their right stage of embryo development. Then they were sterilized by plunging them into 10% (v/v) Clorox detergent (6% sodium hypochlorite) solution for 15 min. The fruits were washed three times in sterile distilled water (DD H_2O). After sterilization, the fruits were put into a sterile Petri dish and were dissected into two halves using sterile scalpel blade. One ovule was detached from the placenta and put into a drop of liquid medium to prevent desiccation. By pressing micropylar region of the ovule using fine tip forceps and needle viewing through a binocular microscope in the sterile flow cabinet, the embryo was ejected. Then the embryo was taken with the tip of scalpel blade and put on the culture medium in the Petri dish. The Petri dish was sealed with parafilm. The development of embryos was observed frequently. Cultures were incubated at 10 h photoperiod at 25 ±2°C temperature under reduced light.

Culture Media

1. For Organogenesis. MS (Murashige and Skoog, 1962) medium was used with three concentrations (0.0, 0.1, 1.0 $mg.L^{-1}$) of BAP and KIN, each separately, to select their best concentrations for shoot induction. Two shoot multiplication media were prepared with 0.1 $mg.L^{-1}$ each of BAP and KIN (M-1) and 0.1 $mg.L^{-1}$ each of BAP, KIN and Ad. S. (M-2). For root induction, one-half and full strengths of Woody plant basal medium (WPM) were tried (Lloyd and McCown, 1981) to select the optimum basal medium. Whereafter, half strength WPM supplemented with 0.1 $mg.L^{-1}$ IBA, IAA, and NAA separately was used to select the best suitable medium for healthy roots. All media were prepared using 3% sucrose, 2.5 $g.L^{-1}$ phytagel and pH was adjusted to 5.8 before autoclaving. Phytagel was added to the media after pH was adjusted. The media were autoclaved at 121°C for 20 min. Filter-sterilized plant growth regulators were added to the autoclaved media and poured 16 ml medium into each test tube.

Only one or two explants were inoculated in each test tube. The in vitro grown shoots were excised when they were at least two cm long and were used as explants for root induction. Well rooted shoots were transferred to pro-mix (Premier Horticulture Inc., PA, USA) after washing off agar from their roots and maintained under reduced light with high relative humidity for 4–7 days before transferring them to the soil. Number of shoots per explant was counted by visual observation. Shoot length (approximate length of shoot without leaf length) was measured visually from outside the culture vessel.

2. For Embryogenesis. A new medium (GSEM, Guava somatic embryogenesis medium, Table 3) has been formulated during this investigation and were supplemented with 10 $mg.L^{-1}$ indole 3-acetic acid with 400 $mg.L^{-1}$ glutamine and 130 $g.L^{-1}$ sucrose (grocery grade sugar).We tried other media too to obtain embryogenesis but except this medium we didn't get success. MS basal medium with 30 $g.L^{-1}$ sucrose was used to germinate somatic embryos. Notable variations were used in this medium than common basal

medium are lower amount of macro nutrients and higher amount of micro nutrients. Synthetic seeds (synseeds) of guava were developed using somatic embryos coating them with 3% sodium alginate with or without activated charcoal and dropped them into 0.5M $CaCl_2$ for 30 min. They were germinated either on MS medium or on pro-mix compost.

RESULTS

Organogenesis

Budbreak (Fig. 1b) was observed on MS basal medium as well as on MS medium supplemented with only BAP or KIN alone. We obtained multiple shoots on nodal explant only when BAP and KIN both were used together. An average of 3.4 shoots per nodal explant were produced on M-1 medium (MS + 0.1 $mg.L^{-1}$ BAP + 0.1 $mg.L^{-1}$ KIN), after 8 weeks of culture (Table 1). The best response for guava shoot multiplication obtained in this investigation was recorded on MS medium containing 0.1 $mg.L^{-1}$ each of BAP, KIN and Ad.S. (M-2 medium), where an average of 6.3 shoots per explant were obtained after 8 weeks of culture (Table 1) and as many as 25 shoot buds were induced from a single node (Fig. 1c). Well-elongated shoots (Fig. 1d) were excised and cultured on different media combinations for rooting (Table 2). Half strength WPM medium supplemented with 0.1 $mg.L^{-1}$ NAA supported healthy root system wherein an average of 3.08 cm long roots were produced in 92% explants within 5 weeks of culture (Table 2). The in vitro grown plantlets (Fig. 1e) were removed from agar medium and were washed thoroughly. All plants transferred in pro-mix compost have survived. Potted plants were acclimatized at room temperature with high relative humidity for 4–7 days. The acclimatized plants were planted to the soil in specialty plants house at FVSU, where they are growing well.

Embryogenesis

Numerous somatic embryos (Fig. 2a) were induced from zygotic embryo tissues within 21 days of culture on GSE medium (Table 3). In this study it was observed that zygotic embryos no more than 40 to 50 days old (post anthesis) were at the best stage to induce somatic embryogenesis on this medium. More than 100 tiny somatic embryos were recorded along with light brown calluses in each explant. Most of the embryos induced directly from the explant tissue surface without any intervening callus phase. Similar response (spontaneous embryogenesis) was obtained again if these somatic embryos were used as fresh explants (Fig. 2b). It was observed that the somatic embryos were induced from the abaxial surface of the initial leaf of the cultured embryo. Typical stages of embryo development (i.e. globular, heart, torpedo and dicotyledonary) were observed in most of the cases. Almost 100 percent embryo germination was recorded on MS basal medium. Development of plantlets from guava 'synseeds' (Fig. 2c) were also obtained (Fig. 2d) on both MS medium as well as on pro-mix compost. The plants developed from guava synseeds are now growing in the specialty plants house at FVSU-ARS.

DISCUSSION

Organogenesis

MS medium was selected as the optimum basal medium since it was used earlier for in vitro culture of guava by Amin and Jaiswal (1987), Loh and Rao (1989), Yaseen et al. (1995) and Joshee et al. (2004), whereas MS and B5 media were used by Babbar and Gupta (1986a, b), and Rugini olive medium (OM, Rugini, 1984) was used by Papadatou et al. (1990). Amin and Jaiswal (1987) reported multiple shoot induction in nodal explants from mature guava trees on MS medium containing single cytokinin. They also reported shoot induction from guava leaf and seedling explants on medium containing 0.1-5.0 $mg.L^{-1}$ BAP. Multiple shoots were also obtained by Loh and Rao (1989), Papadatou et al., (1990), Yaseen et al. (1995) and Joshee et al. (2004), in seedling explants of guava on medium containing single cytokinin. In this study we found MS medium as the most

suitable medium for nodal explant culture which was similar to previous findings. However, in this study we did not obtain multiple shoots on the medium containing single cytokinin even after repeated attempts were made. Multiple shoots were developed from nodal axils only in presence of two cytokinins (BAP and KIN) in combination. No shoots were obtained from leaf tissues but numerous roots and callus were observed in this investigation. According to our findings it seems that the synergistic effect of two cytokinins may be the reason for the induction of multiple shoots. Our investigation also revealed that the number of shoots per explant increased significantly when the multiplication medium was fortified with adenine sulfate. Use of two cytokinins in combination for multiple shoot induction has been reported earlier in the in vitro culture of *Azadirachta indica* (Biswas, 1994) and *Cajanus cajan* (Mohan and Krishnamurthy, 1998). Similarly, the use of adenine sulfate to enhance organogenesis was also observed by these researchers. In this investigation, healthy in vitro rooting system in guava was obtained on the half strength WPM medium containing 0.1 $mg.L^{-1}$ NAA. A similar response was observed earlier in rooting of olive shoots (Rugini and Fontanazza, 1981). In this study, we found that open compost was good for in vitro raised guava plantlets for acclimatization before transferring them to field. Furthermore, George (1996) reported that woody plants often require more open compost than herbaceous species. We also found that initial 4–7 days during acclimatization of in vitro raised guava plantlets required a relatively high humidity. Similar observations were also reported by George (1996).

Embryogenesis

Induction of somatic embryos in guava mesocarp tissues has been reported earlier by Akhtar et al. (2000) and Chandra et al. (2004). In our investigation, we tried all those combinations reported by former researchers; however, we achieved no success with our plant species in our laboratory. We also tried other combinations with various basal media but no success was achieved. However, the medium we developed and used in this study were found to be reproducible since they responded repeatedly for the successive year with 100% success rate. These protocols were found to be equally reproducible when the work was carried out by a totally new worker in our laboratory. Thus, the reliable protocols for morphogenesis and somatic embryogenesis have been achieved in this investigation utilizing tissue explants from guava trees grown in Fort Valley, Georgia.

Genetic Transformation in Guava

Guava nodal explants were co-cultivated with *Agrobacterium tumefaciens* (LBA 4404) harboring a binary vector *p*BI121 having selectable markers (*nptII* and GUS) with CaMV 35S promoter gene. The resultant plants showed kanamycin resistance. Efforts are underway to optimize co-cultivating conditions using embryogenic tissues. Introduction of cold tolerance genes (CBF1, CBF2, and CBF3) are planned to be attempted in both organogenic and embryogenic explants.

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Tables

Table 1. Response on shoot induction in nodal explants of *Psidium guajava* on different semisolid media, after 8 weeks of culture.

Media composition ($mg.L^{-1}$)	Number of shoot per explant (Number ±S.D.)	Mean shoot length (cm ±S.D.)
MS basal (control)	1.00 ±0.00	0.50 ±0.29
MS + BAP (0.1)	1.00 ±0.00	1.05 ±0.46
MS + BAP (1.0)	1.00 ±0.00	0.93 ±0.18
MS + KIN (0.1)	1.00 ±0.00	1.23 ±0.39
MS + KIN (1.0)	1.00 ±0.00	1.00 ±0.40
MS + BAP (0.1)+KIN (0.1)	3.43 ±1.55	2.20 ±1.43
MS + BAP (0.1)+KIN (0.1)+Ad.S. (0.1)	6.33 ±6.89	3.23 ±1.19

Table 2. Response on in vitro root induction in excised shoots of guava on different media.

Media ($mg.L^{-1}$)	Rooting (%)	Mean root length (cm ±S.D.)
WPM basal (control)	72	0.89 ±0.47
WPM + IBA (0.1)	84	2.18 ±0.76
WPM + IAA (0.1)	85	2.84 ±1.03
WPM + NAA (0.1)	92	3.08 ±1.33

Table 3. Composition ingredients of guava somatic embryogenesis medium (GSEM) developed at FVSU for somatic embryo induction in guava.

Compound	$mg.L^{-1}$	Compound	$mg.L^{-1}$
KNO_3	125.00	Myo-inositol	1000.00
$MgSO_4.7H_2O$	125.00	Glutamine	400.00
KH_2PO_4	125.00	Nicotinic acid	50.00
$Ca(NO_3)_2.4H_2O$	500.00	Glycine	20.00
$MnSO_4.H2O$	22.30	Pyridoxine HCl	
$ZnSO_4.7H2O$	8.60	Thiamine HCl	5.00
H_3BO_3	6.20	Folic acid	5.00
$Na_2MoO_4.2H_2O$	0.25	Biotin	5.00
$CuSO_4.5H_2O$	0.05	Phytagel	0.50
$CoCl_2.6H_2O$	0.05	IAA	2000.00
Na_2EDTA	37.3	Sucrose	10.00
$FeSO_4.7H_2O$	27.8	(pH) = 5.8	130,000.00

Figures

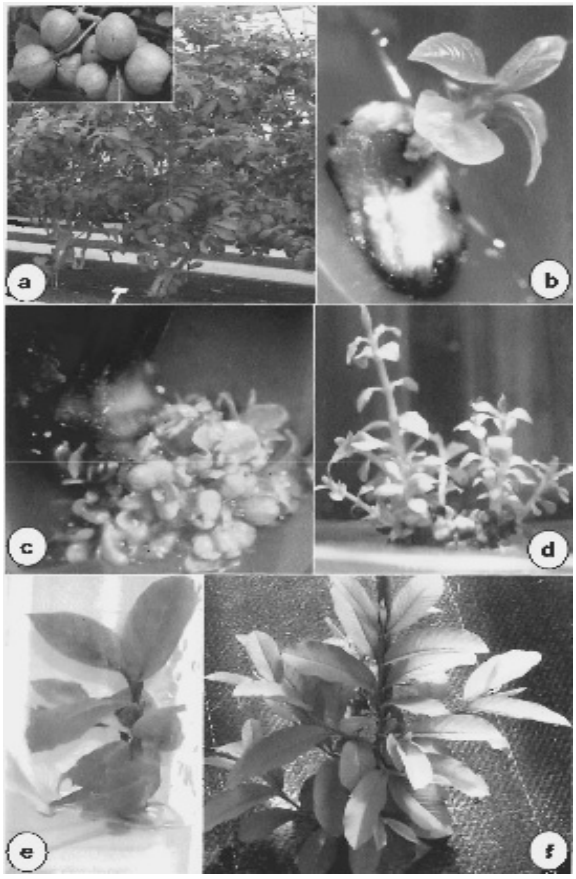

Fig. 1. (a) Three year old guava tree with fruits (inset) in the Specialty Plants House at FVSU,
(b) Budbreak on basal medium, after 8 weeks of culture,
(c) Induction of multiple shoot-buds (25+) from one node on M-2 medium,
(d) Development of multiple shoots on M-2 medium after 12 weeks of culture,
(e) Rooted shoot on WPM + 0.1 mg.L^{-1} NAA, after 5 weeks of culture,
(f) In vitro produced guava plant growing in the soil.

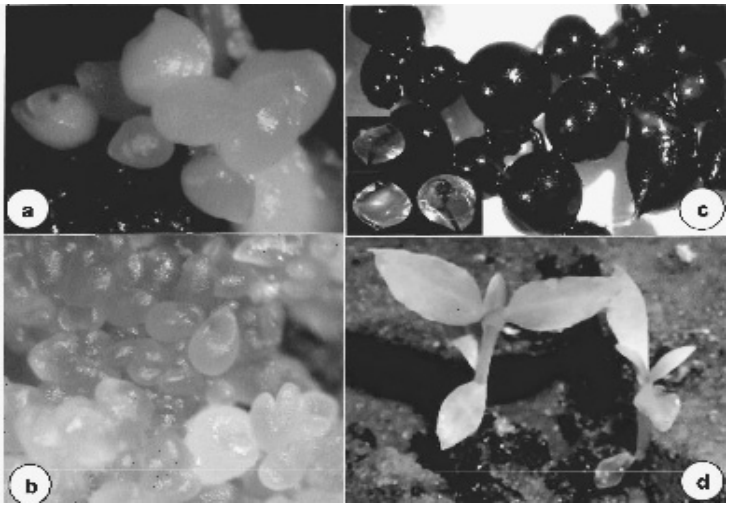

Fig. 2. (a) Induction of numerous somatic embryos on guava zygotic embryonic tissues,
(b) Induction of numerous somatic embryos on primary somatic embryonic tissues,
(c) Synthetic seeds of guava (synseeds),
(d) Germination and plant development from ‘Guava synseeds’.