Clonal propagation of guava (*Psidium guajava* L) on nodal explants of mature elite cultivar

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Abstract

Guava (*Psidium guajava* L.) is a very valuable tropical and subtropical fruit. However, guava micro-propagation are genotypes dependent, there are several problems associated with *in vitro* cultures of guava including browning or blackening of culture medium due to leaching of phenolics, microbial contamination, and in vitro tissue recalcitrance. A micropropagation system using Murashige and Skoog (MS) medium with 6-benzylaminopurine (BA), kinetin and naphthaleneacetic acid (NAA) was developed for guava (*Psidium guajava* L) from mature cultivar. As part of this research various disinfection methods and plant growth regulators were tested *in vitro*. The most effective method involved treating explants in a 15% bleach solution for 20 mins followed by culturing them in MS medium with 250 mg/L polyvinylpyrrolidone (PVP). This method maximized the percentage of bud breakage (53.3%), while producing the minimum browning rate (18.3%) for the explants. The best observed proliferation rate (71.2%) occurred on the MS medium supplemented with 4.44 μM BA, 4.65 μM kinetin (KT) and 0.54 μM NAA. It produced the highest mean number of shoots (2.2). Shoots were then rooted (65%) when dipped in 4.9 mM Indole-3-butyric acid (IBA) solution for 1 min and rooted plantlets survived (100%) after acclimatization to the greenhouse.

Introduction

Guava (*Psidium guajava* L), sometimes called *the apple of tropics*, is a very valuable tropical and subtropical fruit representing a staple food in many countries. It is a rich natural source of vitamin C as well as a good source of calcium, phosphorous, iron and pectin.[1] It also contains many high-grade antioxidants such as, lycopene, carotenoids and polyphenols.[2] These compounds are *superstar* chemicals that are believed to help reduce the incidence of degenerative diseases such as arthritis, arteriosclerosis, diabetes, cancer, heart disease, inflammation and brain dysfunction Antioxidants have also been reported to retard aging.[3-5]

Because of its widespread cultivation, guava production is facing major agronomic and horticultural problems including susceptibility to many pathogens, such as guava wilting, low fruit growth, short shelf life, high seed content, and stress sensitivity. Conventional breeding methods to improve woody species like guava are limited because these plants generally have long juvenile growth periods, experience self incompatibility, and are heterozygous. In addition, seed originated guava plantlets often do not maintain the genetic purity of the variety due to the segregation and recombination of characters during sexual reproduction, whereas high internal fungi, bacteria contamination and phenolic compounds exudation tend to limit *in vitro* cultures of the guava plant.[6]

Genetic engineering has been considered a promising production alternative since it shortens the breeding period. For this approach to gain wide acceptance an efficient micro-propagation and regeneration procedure to produce large numbers of rooted plants from unique plants is a prerequisite. Also, clonal propagation reduces plant-to-plant variation to ensure uniform populations of unique clones. Morphogenesis from explants derived from mature trees is of great commercial value because it facilitates direct cultivar improvement. However, there are several problems associated with *in vitro* cultures of these explants including browning or blackening of culture medium due to leaching of phenolics, microbial contamination, and *in vitro* tissue recalcitrance.[7]

The tendency of guava to exude phenolic compounds into the media makes the regeneration process particularly difficult. A number of approaches have been tried to get rid of phenolic compounds from guava tissue cultures. Brookdrijk,[8] for example, used silver nitrate for sterilization and achieved a 70% success rate. Concepción *et al.*[9] found that PVPP was better than citric acid and ascorbic acid for controlling exudation of phenolic compounds. Pretreatment of guava explants in 0.2% ascorbic acid also has been found effective in helping to overcome the browning problem, achieving an establishment rate of 64.4%.[10] Joshee *et al.*[11] found that dark treatments were helpful for guava *in vitro* culture establishment. Zamir *et al.*[12] investigated the effect of different surface sterilization agents and antioxidants on guava and found that mercuric chloride produced the maximum survival rate of 67%. However, because of the danger of environmental pollution mercury compounds are not recommended.

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Other workers have shown advances in guava production through tissue culture. Yasseen *et al.*[13] established a propagation culture system from germinated seedings. Rooted plantlets were acclimatized into a greenhouse. The levels and kinds of plant growth regulators included in the culture medium were found to largely determine the success of tissue culture. Cytokinin levels especially have been shown to be critical for multiplication of many tropical fruit trees. BA has been the most common cytokinin used for guava propagation.[6,13-16] However, proliferation of elite, mature genotypes or commercial cultivars is much more difficult than tissues from juvenile sources. Successful propagation from mature guava trees has been limited. Amin and Jaiswal 14 reported plantlet formation from mature tissue of guava and the best shoots multiplication rate was achieved when using BA (4.5 μM) only, but the survival and response rate of shoot tip explants were low.

In this paper, we report a protocol for rapid clonal propagation of guava using *in vitro* shoot proliferation on nodal explants of adult trees from elite cultivar.

Materials and Methods

Establishment of nodal explants cultures

Apical shoots explants, about 5-7 cm, were collected from four 10 year-old, greenhouse-growing, elite mature guava cultivar Beaumont (Figure 1A). This cultivar was selected because it produced large, pink-fleshed fruits with few seeds. Shoots apices and nodal segments of new shoots were brought to the laboratory in water where the outer leaves were removed. The shoots were washed thoroughly under running tap water,

and then samples of the explant materials were subjected to different surface sterilization applications (Table 1). The treated explants were either stirred or not stirred in 0.5% (w/v) PVP solution for 40 mins followed by a 15% bleach solution for 20 mins and then washed with sterile water for five times. Nodal sections were placed vertically in the G7 Magenta boxes (Magenta Corporation, Chicago, IL, USA.) containing 50 mL of Murashige and Skoog (MS) medium[17] with 3% (w/v) sucrose, 8.88 μM BA and 0.7% Difco-Bacto agar, with or without 250 mg/L PVP. The pH of the medium was adjusted to 5.7 before the addition of agar and autoclaved at 121°C for 20 min. Cultures were incubated at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps (80-100 μmol m^{-2} s^{-1}). The media was changed 2-3 times over the first 10 to 14 days of the study to control phenolic exudation and to establish the *in vitro* culture.

The number of explants developing new shoots was expressed in terms of percentages. Browning rate, contamination rate and clean cultures were recorded approximately 2 weeks after culture initiation. For each treatment, at least 20 explants were tested and each experiment was replicated three times. The results were quantified as a mean ± se of three independent experiments. The data were analyzed statistically using one factorial analysis of variance; significant differences between means were assessed using Duncan's multiple range test at P=0.05.

Shoot proliferation and rooting

Clean nodal segments were transferred to shoot multiplication media for shoot proliferation. Briefly, nodal explants were cultured on MS medium containing 3% (w/v) sucrose, supplemented with different concentrations of BA alone or in combination with KT and NAA. Five treatments were tested for shoot proliferation and each treatment had three replications (Table 2). The rooting protocol was modified according to Liu and Pijut.[18] Samples of 2 to 3 cm of elongated shoots were excised and cultured on a half strength MS media supplemented with 100 mg activated charcoal (AC), with or without IBA. Shoots that were to be exposed to the rooting medium without IBA were dipped in 4.9 mM IBA for 1 min before being placed in the rooting media. Shoots to be initiated into the rooting medium containing IBA were placed directly in the media after being excised without dipping. Both treatments were cultured by incubating them at 25°C under a 16-h photoperiod provided by cool-white fluorescent lamps (80-100 μ mol m^{-2} s^{-1}). Rooting rate, root number per shoot, and root length were determined 4 weeks after rooting.

Acclimatization

Rooted shoots with 3-5 fully expanded leaves were planted in 15cm diameter plastic pots containing a mixture of sterile sand and garden soil (1:3), covered with polyethylene bags for 21 days to prevent excessive water loss. The pots were watered once a week. Full strength MS macronutrient solution 10 mL was applied every other week. Plantlets were kept at 25°C in artificial light (16 h photo period and irradiance of 50 μmol mm^{-2} s^{-1}) provided by white florescent tubes for 6 weeks and were then transferred to the temperature controlled (25.6/18.3°C, day/night) greenhouse to grow under natural light. Survival rates were determined before transfer to the greenhouse.

Results

Establishment of guava culture

Some shoot apices and nodal segments exhibited browning immediately after being cultured; within two weeks, 60% of explants showed browning (Figure 1B). New shoot primordia from healthy clean nodal segments (neither browning nor contaminated) sprouted on MS medium within three weeks. The different disinfection treatments were found to significantly affect guava establishment (Table 1). There were significant differences among different treatments regarding the browning rate and the percentage of plants breaking new buds. The best establishment (53.3%) of guava explants was observed in the MS medium supplemented with PVP 250 mg/L (Figure 1C), a low browning rate of 18.4% was observed. For the treatment of 15% bleach for 20 mins, only 21.7% shoots broke new buds. The highest phenolization of explants (60%) was recorded when the explants were treated with bleach only. Adding PVP into the culture medium produced substantially lower phenolization rates of explants (18.3%) than the PVP solution stirring treatment (51.7%). Treatment with PVP stirred or PVP supplemented into the culture medium did not improve the effect. The reason for this may be related to the time the explants were exposed to the solution. For the treatment with 15% bleach and PVP in the medium, the explants exposure time to the air was the shortest before it made contact to culture medium. There was no significant difference among treatments regarding to the contamination rate with a range from 11.7-21.7%.

Micro-propagation

Of the two cytokinins tested, BA used alone produced good elongated shoots. But it should be noted that most explants either one shoot developed or no shoots. Among the various concentrations of BA tested, the highest number of shoots per explant (2.2) was observed with the medium containing BA 4.44 μM and 4.65 μM (Table 2). BA has been reported to stimulate shoot multiplication from different explants of many woody species including black cherry[18] and black walnut.[19] In the present study, the multiplication rate was almost doubled compared to those obtained with individual cytokinins, with the percentage of proliferating cultures highest for highest on dual

Table1. Different disinfection methods on guava culture establishment.

Disinfection methods*	Browning rate (%)	Contamination (%)	Percentage of plants that break new buds (%)
15% Bleach	60.0±5.0a	16.7±7.6a	21.7±3.0b
PVP solution + 15% Bleach	51.7±12.6a	11.7±2.8a	41.7±5.7ab
PVP solution + 15% Bleach + PVP in the media	28.3±7.6b	20.7±8.6a	43.3±6.0ab
15% bleach + PVP in the media	18.3±2.8b	21.7±10.4a	53.3±2.8a

*Treatment methods are explants were stirred in 15 % bleach for 20 mins, or stirred in 0.5% (w/v) PVP solution for 40 mins or 250 mg/L PVP was added into the medium. Data were taken 2 weeks after initiation of explants. Means (±standard error) within a column followed by the same letter are not significantly different by Duncan's test and P=0.05.

Table 2. Guava shoots proliferation responding to different hormone treatments.

Plant growth regulators (μM)	Percentage of proliferating cultures (%)	Mean number of shoots per culture	Mean shoot length(cm)
4.44 μM BA	38.5±15.6b	1.2±0.4a	3.0±0.9a
4.44 μM BA +0.54 μM NAA	34.9±5.5b	1.3±0.6a	2.4±0.8a
8.88 μM BA +0.54 μM NAA	45.8±9.1b	1.4±0.5a	2.2±0.7a
4.44 μM BA +4.65 μM KT +0.54 μM NAA	71.2±8.4a	2.2±0.9a	2.6±0.8a
8.88 μM BA +4.6 5 μM KT + 0.54 μM NAA	51.6±18.0ab	1.9±0.4a	2.2±0.8a

Data were taken 4 weeks after cultured in the treatment medium. Means (±standard error) within a column followed by the same letter are not significantly different by Duncan's test and P=0.05.

cytokinin media (71.2%). Despite these observations, there was no significant difference among five treatments regarding to the mean number of shoots per culture and mean shoot length (Figure 1E).

Rooting and acclimatization

The data in Table 3 revealed that dipping shoots in the IBA 4.9 mM solution not only increased the mean root number, but also enhanced the overall rooting percentage. The highest rooting rate (65%) was observed for this method (Figure 1 G), inducing the earliest rooting in 12 days. There were significant differences regarding the mean root number per shoot (5.0 *vs* 3.0 and 2.8) and the rooting rate (65% *vs* 53.3% and 33.3%) between dipping method and the medium method (IBA was added into the medium, Figuer 1 F; Table 3). AC supplemented root induction medium also helped rooting; they rooted better in comparison to with the non activated charcoal condition (*data not shown*). However, regarding mean root length, there was no significant difference between these two methods. Rooted plantlets were successfully acclimatized in the culture room and new leaves appeared 2 weeks after acclimatization (Figure 1 H) with 100% survival rate. Some of the plantlets grew to 20 cm after 10 weeks of acclimatization (Fiure 1 I). Totally 51 plantlets survived in the greenhouse after 1 year.

Discussion

The results revealed that rapid clonal propagation of mature guava is possible through nodal explants. High internal fungi and bacteria contamination along with phenolic compounds exudation are the main problems that limit *in vitro* culture of guava species. Browning of explants due to phenolic exudation has been reported as a serious problem in establishing axenic cultures of tree species, including guava.[14,20] We observed this problem only when explants were treated with bleach. Shoot tips were found to be more sensitive to browning and blackening than nodal segments exposed to the same disinfection method. Phenolic exudation from guava explants was successfully controlled by agitation in PVP solution or by adding PVP in the culture medium. Similar treatments have been reported for guava by agitating in PVP solution[11] or by adding PVP in the culture medium.[12] These results are consistent with reports of successful treatments with PVP for other woody species. For example, PVP was used successfully as a rinse for *Rosa hybrida* shoot fragment[21,22] showed that after treatment with PVP, the brown exudate from cut tissue of *Eucalyptus grandis* was not toxic to explant materials. The addition of PVP into the medium also has been reported to improve the frequency of culture initiation for *Malus* and *Quercus spp*.[23,24] Abenavoli and Pennisi[25] found that PVP treatments reduced tissue browning resulting in a better callus formation in chestnuts.

Cytokinin concentrations have been found to be the most critical factor for successful multiplication of many tree species. BA is the most common cytokinin used for guava propagation.[6,13-16,20] Superiority of BAP for shoot induction may be attributed to the ability of plant tissues to metabolize BAP more readily than other synthetic growth regulators or to the ability of BAP to induce production of natural hormones such as zeatin within the tissue.[26] Singh *et al.*[27] improved shoot multiplication by using a combination of TDZ and NAA. Other growth-enhancing medium additives including sucrose[20] and adenine sulfate[27] have also been reported to significantly effect on shoot multiplication and elongation. In our study, we found that BA was also effective for inducing shoot proliferation with frequencies of up to 71.2% and shoot elongation. We tested the protocol developed by Amin and Jaiswal[14] for mature guava proliferation and it did not work well. The reason could be that the process is more applicable for woody species, and that to be effective the protocol needs to be cultivar and genotype dependent.

Rooting of guava microshoots has been found to be effective with a low concentration of IBA.[13,14] Research has shown that adding in low levels of IBA in the medium took about 20.9 to 38.9 days for root induction.[27] In contrast to this approach we dipped the shoots in a high concentration of IBA solution and found that this method improved rooting by shortening the time for root initiation to only 12 days. According to our observation, it took 14 days for root initiation when IBA was added into the medium. Also, this method produced more roots which enhanced the survival rate at the

Table 3. Effects of concentrations of IBA on root induction and growth of guava

Rooting medium	Days to root initiation	Mean number of roots per shoot	Mean root length (cm)	Rooting rate %
1/2MS + 0.1 g/L AC+	12	5.0±1.8a	3.4±0.7a	65.0a
1/2MS + 2.45 µM IBA + 0.1 g/L AC	14	3.0±1.3b	3.5±1.2a	53.3b
1/2MS + 4.9 µM IBA + 0.1 g/L AC	14	2.8±1.0b	2.2±1.1b	33.3c

*Shoots were dipped in 4.9mM IBA solution for 1 min before put into this medium. Data were taken 4 weeks after root induction. Means (±standard error) within a column followed by the same letter are not significantly different by Duncan's test and P=0.05.

Figure 1. *In vitro* proliferation of guava (*Psidium guajava* L). (A) stock guava plants in the greenhouse; (B) nodal section became browning after sterilization; (C) new shoots break out from healthy nodal sections; (D) shoots proliferated; (E) elongated shoots; (F) rooted shoots by medium method (medium with IBA); (G) rooted shoots by dipping method; (H) guava plantlets acclimatized into the soil for 2 weeks; (I) guava plantlets acclimatized into the soil for 10 weeks.

acclimatization stage. The likely reason for the shorter rooting period is that we used different genotypes of guava and the season when we performed our experiments was different from former investigations. We observed that it took much longer time for guava shoots to develop roots in the winter (*data not shown*). AC supplementation at 100 mg/L was found helpful for guava rooting. The rooting efficiency was much lower when AC was not added (data not shown). The effect of AC is based on its capacity to adsorb toxins, excess phytohormones and other phenolics, hence, optimizing rooting conditions.

The complete protocol for micro-propagation of mature guava that we developed involved disinfection of nodal sections explants in a 15% bleach solution for 20 mins followed by culture on MS with 8.8 μM BA and 250 mg/L PVP for 2 weeks before transfer to MS medium with 4.44 μM BA, 4.65 μM KT and 0.54 μM NAA. Elongated shoots were rooted by dipping them in 4.9 mM IBA for 1 min. Rooted plantlets were then acclimatized to the culture room within 6 weeks after root induction. Acclimatized plantlets were survived in the greenhouse at 100% rate after 1 year. To our knowledge, this is the first report of a complete protocol for micro-propagation and rooting for this mature elite cultivar of guava. More research is needed to improve the regeneration efficiency that will provide a better regeneration system for micro-propagation and transformation of this mature, elite cultivar of guava.

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