

Factors Affecting *in vitro* Establishment of Guava (*Psidium guajava* L.) Explants

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Abstract: Guava (*Psidium guajava* L.) is cultivated in many tropical regions of the world including southern parts of Iran. High internal fungi and bacteria contaminations and also phenolic compounds exudation are the main problems that limit *in vitro* culture of this species. Phenolic exudation from nodal segments was successfully controlled by agitation in a solution containing antioxidants, ascorbic and citric acids each with concentration of 100 mg l⁻¹. For disinfection of explants three groups of disinfectants namely: antibiotics, fungicides and leaf extracts of myrtle and eucalyptus were tested. Among antibiotics gentamycin at 500 mg l⁻¹ was the most effective treatment since decreased the percentage of contamination from 86.18% in control to 2.1% and also had positive effect on average shoot elongation. All fungicides and also mercuric chloride treatments were effective in controlling fungal contaminations, so that no fungal growth was observed in cultures. Myrtle leaf extract significantly decreased contamination percentage from 100% in control explants to 68.4% in 'Local-1'.

Key words: Guava • Ascorbic and citric acids (ASCA) • antibiotic • fungicide • antioxidant

INTRODUCTION

Guava (*Psidium guajava* L.) is an important tropical fruit tree and is commonly propagated by layering, cutting and grafting but the number of plants obtained by these methods is small [1, 2]. Guava is cultivated in many tropical regions of the world including southern parts of Iran, in the provinces of Hormozgan and Sistan and Baluchistan. In these areas guava is exclusively propagated by seeds which may have genetic variation, low productivity and low fruit quality when the plant is mature. Propagation by cutting is also troublesome because of low rooting. Tissue culture methods offer the means for clonal propagation of the elite guava genotypes. However, contamination by fungi and bacteria is one of the problems that limit the aseptic establishments of guava. Difficulties in the establishment of aseptic culture using field-grown mature trees of woody plants due to endophytic microorganisms have been described by Pence and Sandoval [3].

Different methods using various disinfectants with a range of doses and exposure times and also different disinfectants agents were suggested for different species and cultivars of guava explants sterilization [4-8]. Ramirez *et al.* [9] tested 3 concentrations and 3 exposure times of 3 surface disinfectants: silver nitrate, mercuric chloride and sodium hypochlorite to reduce adult guava

nodal segment contamination. They reported that all 3 disinfectants at the doses and times used did not control contamination of the explants. They suggested the use of 70% ethanol for 1 min and treatment with a solution of benomyl at 2 g l⁻¹ and rifampicin at 100 mg l⁻¹ after washing the explants for 16 h in tap water in addition to above treatments. Meghwal *et al.* [10] reported the highest frequency of contamination-free explants in the treatments with H₂O₂ (10% for 5 min), AgNO₃ (0.25% for 6 min) and HgCl₂ (0.05% for 3 min). When NaClO (10% for 5 min) was used either alone or with alcohol, all the cultures were contaminated. Numerous microbial contaminants were detected in guava cv. 'Enana Roja' and the application of 7.5 g l⁻¹ mancozeb and 4 g l⁻¹ Benlate was not effective in eliminating the pathogens [11].

Another problem in explant establishment of guava in tissue culture techniques is the exudation of phenolic compounds in culture media which hampers growth and kills the explants. Various agents such as Polyvinyl Pyrrolidone (PVP), activated charcoal and antioxidants (ascorbic and citric acids) have been used to prevent or reduce the destructive effects of phenolic compounds in plant tissue culture [4, 12-14].

Although a number of investigations are carried out to control microorganisms and exudation of phenolic compounds, still researches are needed to have pathogen

and exudation free explants in different cultivars of guava. Preliminary studies using nodal segments from field grown mature trees showed high exudation of phenolic compound and high contamination rates of bacteria and fungi that revealed between 1 to 6 weeks after culture when using NaClO, ethanol and benomyl suggested by other researchers [4, 11, 12]. This research was aimed to complete control of contaminants in culture of elite Iranian guava cultivars.

MATERIALS AND METHODS

Current season shoots (12-20 cm) were collected from field grown adult trees of guava (*Psidium guajava* L.) in Minab Agricultural Research Center in Hormozgan province (Fig. 1A). Two cultivars, a white-fleshed cultivar named 'Local-1' and a red-fleshed cultivar named 'Local-2' were used. Shoots 2-2.5 cm long consisting of two lateral buds were used. The leaves were removed so that 2-3 mm of petioles remained to protect lateral buds. The explants were washed for at least 45 min with tap water containing a few drop of dish washer detergent.

Controlling the phenolic exudation: Nodal segments of cv. 'Local-1' were treated in 1 g l^{-1} benlate for 30-45 min and rinsed 2 times with distilled water, then they were placed in solutions of: a) ascorbic acid and citric acid (ASCA) (each acid with concentration of 100 mg l^{-1}), b) 5% PVP. The explants were shaken in the above solution using an orbital shaker (60 g) for 45 min. Then the nodal segments were treated in 200 mg l^{-1} mercuric chloride (MC) in vacuum for 2 min followed by 15% Golrang (a commercial detergent with 5.25% NaClO) for 15 min. After 4 rinses with sterile water, they were trimmed to 1.5 cm long portions and cultured on a half-strength Murashige and Skoog (MS) medium [15] containing different concentrations of 6-benzylaminopurine (BA), (0, 0.5 and 1.5 mg l^{-1}). Intensity of phenolic compounds exudation was evaluated after 3 days of culture. Five categories were established (from 1=low exudation to 5=high exudation) and data were subjected to statistical analysis.

Elimination of bacterial contamination: Since it has been shown that many plants have antimicrobial properties [16-20], fresh leaf extracts of myrtle (*Myrtus communis*) and eucalyptus (*Eucalyptus maculata*) were tested. The leaves of both species were washed thoroughly with tap

water containing dish washer detergent and were dried with blotting paper. Twenty grams leaves of each species was grinded in 100 ml distilled water and kept in a dark place for 12 h and then 20 ml of each solution was added to MS medium containing 1.5 mg l^{-1} BA and 0.25 mg l^{-1} α -naphthaleneacetic acid (NAA). The nodal segments were surface sterilized as mentioned above and were cultured.

In another experiment antibiotics cephaloxin, amoxycilin and gentamycin at concentrations of 0, 250, 500 mg l^{-1} were used with 'Local-2'. Antibiotics were sterilized by filtration before to add to MS medium containing 1.5 mg l^{-1} BA and 0.25 mg l^{-1} NAA, when the temperature of medium was around 60°C . After 2 weeks, percentage of contaminations were evaluated. After 4 weeks, average shoot number and shoot length per explant were recorded.

Elimination of internal fungus contaminations: In a preliminary work, after two weeks of culture the fungus grew from the explants we named them internal fungus for their elimination, after washing the explants of 'Local-2', they were placed in a solution containing ascorbic acid and citric acids each with concentration of 100 mg l^{-1} for 45 min. Then, the following treatments were tested:

T₁ = The explants were surface sterilized in 15% Golrang for 15 min under aseptic condition inside a laminar flow chamber as control.

T₂ = 300 mg l^{-1} MC for 45 min + T₁.

The explants were also treated with different fungicides solution at different concentrations for 2 h as follow:

T₃ = Benlate at 4 and 8 g l^{-1} + T₁

T₄ = Flocunazole (Flo) at 25 and 50 mg l^{-1} + T₁.

T₅ = Ketoconazole (Ket) at 100 and 200 mg l^{-1} + T₁.

T₆ = Clotrinazole (Clo) at 100 and 200 mg l^{-1} + T₁.

T₇ = Griseoflurin (Gri) at 62.5 and 125 mg l^{-1} + T₁.

The treated explants were cultured in MS medium containing 1.5 mg l^{-1} BA and 0.25 mg l^{-1} NAA. After 2 weeks, percentage of contaminations were evaluated. After 4 weeks, average shoot number and shoot length per explant were recorded.

MS medium supplemented with 3% sucrose (MERCK, LGaA 64271 Darmstadt, Germany) and different concentrations of BA and NAA as mentioned above and

solidified by 0.7% agar-agar (MERCK, LGaA 64271 Darmstadt, Germany) were used in all above experiments. The pH of the media was adjusted to 5.7±0.05 prior to autoclaving at 1.2 atm pressure, 121°C temperature for 20 min. All cultures were maintained at 25±2°C with 16 h photoperiod of 35-40 μ mol m⁻² s⁻¹ provided by cool white fluorescent lights.

Each experiment was carried out as a completely randomized design with different replications and number of explants per each treatment specified in the bottom of each table. Data were analyzed using SPSS statistical software (SPSS Inc. Chicago. USA). The means were compared using Duncan's Multiple Range Tests (DMRT).

RESULTS

Controlling the phenolic exudation: Use of Ascorbic and Citric Acids (ASCA) was beneficial for controlling exudation of phenolic compounds and explants survival, but PVP as an adsorbent agent had not any advantage. ASCA significantly prevented medium discoloration in comparison to PVP and control (Table 1, Fig. 1D, E). The intensity of discoloration was 1.69 in ASCA treatment in comparison to 3.69 and 4.15 in PVP treatment and control respectively and also in all BA concentrations ASCA treatment was more effective than PVP.

Elimination of bacterial contaminations: The addition of both myrtle and eucalyptus leaf extract in culture media retarded the appearance of internal contamination and also decreased the percentage and the intensity of contamination in both guava cultivars (Table 2, Fig. 2A). In 'Local-1' myrtle leaf extract significantly decreased contamination percentage from 100% in control to 68.4%.

Table 1: Effect of ASCA and PVP on intensity of phenolic compounds exudation of nodal segments of cv. 'Local-1'[†]

Antioxidant	BA mg l ⁻¹				Mean
	0	0.5	1.0	1.5	
Control	4.00a ^{††}	4.50a	4.00a	4.00a	4.15a
PVP	4.00a	3.25ab	3.75a	3.75a	3.69a
ASCA	2.00bc	1.50c	2.00bc	1.25c	1.69b
Mean	3.33a	3.10a	3.25a	3.00a	

[†]Results based on 4 replications each with 3 explants

^{††}Means in each column and row (small letters for treatments and capital letters for means) with the same letters are not significant at 5% level of probability using DMRT

Table 2: Effects of myrtle and eucalyptus leaf extracts on internal contamination control of guava nodal segments of two guava cultivars[†]

Cultivar	'Local-1'	'Local-2'
Treatments	Percentage of contamination	Percentage of contamination
Control	100.00a ^{††}	100a
Eucalyptus	80.32ab	95a
Myrtle	68.34b	95a

[†]Results based on 4 replications and 4 nodes in each culture vessel

^{††}Means in each column with the similar letters are not significant at 5% level of probability using DMRT

Table 3: Effect of different antibiotics on percentage and intensity of contamination, shoot number and length of cv. 'Local-2'[†]

Antibiotic	Percentage of contamination	Average shoot no. per explant	Average shoot length per explant (cm)
Control	86.18a ^{††}	1.29a	1.5c
Amoxycilin			
250	91.66a	0.63bc	0.64c
500	89.75a	0.29c	0.19c
Cephaloxin			
250	77.47a	1.08ab	0.98c
500	2.74b	0.88ab	0.76c
Gentamycin			
250	24.5b	1.15ab	5.80b
500	2.1c	1.17ab	8.9a

[†]Results based on 4 replications each with 3 explants

^{††}Means in each column with the same letters are not significant at 5% level of probability using DMRT

The effects of different antibiotics on percentage and intensity of contamination showed that gentamycin with concentrations of 250 and 500 mg l⁻¹ significantly decreased the percentages of contamination from 86.18% in control to 24.5 and 2.1% in 250 and 500 mg l⁻¹ respectively in 'Local-2' (Table 3). Cephaloxine only in concentration of 500 mg l⁻¹ controlled contamination and only 2.47% explants were contaminated in comparison to 86.18% in control. Both concentrations of amoxycilin did not affect the contamination. The effect of antibiotics on growth of explants showed that the explants in presence of gentamycin had the greatest number of shoots among 3 kinds of antibiotics used. The length of shoot in presence of gentamycin was significantly higher than other treatments and also with increasing the concentration of gentamycin from 250 to 500 mg l⁻¹ the shoot length significantly increased from 5.8 to 8.9 cm (Table 3). However this was accompanied by pale green leaves which was corrected by transferring the shoots on the same medium without gentamycin (Fig. 2B, C).



Fig. 2: Effect of gentamycin on bacterial control; rooting and acclimatization of plantlets from *in vitro* nodal culture of guava cv. 'Local-1'. A: Explant growth on medium with leaf myrtle extract. B: Decontaminated explant on medium with 500 mg/l gentamicin C: Explant growth on the same medium without gentamicine, D: Explant rooting E: Acclimatization of explant under plastic in greenhouse, f: An acclimatized plant after transplanting

Table 4: Effects of different fungicides on control of bacterial contamination and growth of nodal segments of cv. 'Local-2'[†]

Fungicide (mg l ⁻¹)	Percentage of contamination	Average shoot no. per explant	Average shoot length per explant (cm)
Benomyl 4	100a	0.8ab	3.51ab
Benomyl 8	100a	0.65b	3.64ab
Clotrinazole 100	100a	1.57a	4.32ab
Clotrinazole 200	68c	1.04ab	1.98b
Flocunazole 25	71b	1.46ab	4.19ab
Flocunazole 50	100a	0.7ab	1.98b
Griseflurin 125	71bc	0.71b	2.24b
Griseflurin 62.5	100a	1.04ab	3.08ab
Mercuric chloride 300	77bc	1.60a	5.80a
Ketoconazole 100	75bc	1.13ab	3.1ab
Ketoconazole 200	87ab	1.10ab	3.25ab

[†]Results based on 5 replications each with 4 explants

^{**}Means in each column with the same letters are not significant at 5% level of probability using DMRT

The effects of fungicides on external and internal contaminations:

Fungal contaminations appeared on control explants of 'Local-2' (disinfected with 15% Golrang for 15 min), in 24 h of culture and after 48 h all the explants were contaminated and therefore were eliminated (Fig. 1B). All fungicide and also MC treatment were effective in controlling fungal contamination, so that no fungal growth was observed during 6 weeks after cultures. The effects of fungicides and MC treatments on percentages of bacterial contamination and also on growth of explants are summarized in Table 4. The best internal contamination control was obtained by using 200 mg l⁻¹ Clotrinazole which reduced contamination to 68%. The best shoot proliferation (1.6 per explant) and elongation (5.8 cm per explant) was observed when 300 mg l⁻¹ MC was used.

The plantlets obtained in all experiments were successfully rooted, acclimatized and transferred to the soil (Fig. 2D, E, F).

DISCUSSION

Plant regeneration from woody plants via *in vitro* culture encounters several problems that limit successful micropropagation and biotechnological manipulations. In this research, exudation of phenolic compounds from nodal segments of two guava cultivars was successfully controlled by agitation the explants in a solution of ASCA. Similar results was reported by Rajesh Kumar and Tiwari [21], using the same antioxidants on another species of guava.

High intensity of contaminations in mature woody plants especially in tropical and humid regions has been reported [3]. These investigators controlled, the external contamination by using Benlate, MC and sodium hypochlorite, but after controlling the external contamination, bacterial contamination was appeared. The use of antibiotics as a second agent in controlling bacterial contamination was reported by other researchers [22-27]. It is also reported that antibiotics have adverse effect on growth of some explants in culture [28, 29]. Interesting point was that, in present experiment not only gentamycin at used concentrations reduced percentage of contamination but also it had positive effects on the average shoot length that was accompanied with pale green leaves. This was corrected by transferring the explants to the same medium but without gentamycin. Addition of myrtle leaf extract in culture media is cultivar depended. It significantly reduced percentage of internal contamination without any negative effects on growth of explants. Using leaf extract of myrtle as a natural agent and as an alternative to antibiotics in controlling contamination may be suggested. However, more investigations on the methods of extraction seems to be necessary. Fungal contaminations were successfully controlled using different fungicides. After controlling fungal contamination, it seemed that bacterial contamination has been significantly reduced and the most of the fungicides, similar to observation of Thyngan *et al.* [30] and especially Clotrinazole (200 mg l⁻¹) controlled the bacterial contamination.

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