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A review: Micropropagation of guava (*Psidium* Spp.)

Krishan Kumar Singh and Shiv Pratap Singh

Abstract
The rapid process for development of identical offspring is micropropagation. In this technique the plants grow from meristematic tissue or somatic cells of superior plants using suitable nutrient media under controlled environmental conditions. Micropropagation in Guava, was successfully done on the variety Allahabad safeda. After that micropropagation progress has been made in the different areas via in vitro culture of guava. MSO or MS0 (MS-zero) was mostly used as mineral medium for culturing *Psidium species*. The Woody Plant Medium (WPM) was more suitable for micro propagation of some guava genotypes. Starting material for guava culture (in vitro) is growing shoot tips or nodal segments as explants (1.0-3.0 cm size) collected during in spring. Browning of the explant and culture medium is mostly obstruct in culturing of woody plants, which is remove by avoidance of toxic substances in the medium in guava. Cytokinin (6-Benzylaminopurine) is the most frequently used for guava micropropagation. Use of dual auxins (IBA & NAA) in compare to IBA followed by NAA, resulted higher frequency of rooting, which is important for successful survival of plant. In guava, acclimatization has been experienced by gradually decreasing the relative humidity.

Keywords: guava, micropropagation, BAP, IBA, NAA

Introduction
Guava (*Psidium guajava* L.) belong to Myrtaceae family. Place of origin of guava is tropical (Singh, 2016 and Singh 2018) [27, 28] America. It is utilized as fresh fruit consumption in India along with other Asian sub-continental countries. Genus *Psidium* have about 150 species, most of which are fruit bearing trees. It is native to tropical America, stretching from Mexico to Peru (Singh, 2016) [27]. It is widely adopted and tolerate in frost, drought and saline conditions (Samson, 1986) [51]. It has good amount of ascorbic acid, dietary fibres, vitamin-A (about 250 IU/100 g), pectin, calcium, phosphorus, iron and other nutrients. The roots, bark, leaves and green fruits are used as medicine for gastrointestinal problems, diarrhoea and dysentery. (Rathore, 1976) [48]. Salad, guava jelly and pudding are made from the of ripe fruits. Besides that, Guava juice and guava pulp wines are also prepared from guava fruit (Bardiya *et al.*, 1974) [4]. It also contains high amount of antioxidants like, lycopene, carotenoids and polyphenols. (Jiménez *et al.*, 2001) [20]. Guava has the chemicals which help to reduce the incidence of diseases like arthritis, arteriosclerosis, diabetes, cancer, heart disease, inflammation and brain dysfunction. Antioxidants reported to retard aging, high concentrations of pectin content in guava fruit reduces the cholesterol of human body (Feskanich *et al.*, 2000; Gordon, 1996 and Halliwell, 1996) [12, 16, 17].
Production of Guava is overlaying the agronomical and horticultural problems including susceptibility to many pathogens, wilting, low fruit growth, short life, higher seed content and stress sensitivity. Improving guava species by traditional methods of breeding are limited because plants generally have long juvenile growth periods, and are heterozygous in nature. While, seed originated guava plantlets do not maintain the genetic purity of the variety due to the segregation and mixing of genetic characters during sexual reproduction. In addition, high internal fungi, bacterial contamination and phenolics compounds exudation tend to limit *in vitro* cultures of the guava plant. Genetic engineering technique reducing the breeding period. Due to this approach micro-propagation and regeneration produce large numbers of rooted plants from unique plants. Clonal propagation can reduce plant-to-plant variation and ensuring the uniform populations of clones.

Micropropagation
Micropropagation refers to *in vitro* plant propagation method. The advantage of micropropagation over conventional propagation technique is to reduce the time needed for ich are true to the type and disease free

Murashige (1974) [42] and Hammerschlag (1982) [18] reported three stages i.e. establishment, multiplication and rooting. Hartmann and Kester (1983) [19] added another stage i.e. acclimatization. Micropropagation is now a well-established technology which has made significant contributions to the propagation and improvement of agricultural crops in general and applicable to various crops like *Withania somnifera* L. (Ashwagandha) (Bhuria *et al.*, 2014) [6] *Saccharum officinarum* L. (Sugercane) (Shrivastava *et al.*, 2014). Greater contribution is predicted from this technology in years to come, both in its own right and as an adjunct to the application of molecular biology.

Explants

Totipotency is the ability of plant tissues/parts (excepting bark) to regeneration *in vitro.* Juvenile explants, comparatively gives batter results. New vegetative growths in *Psidium guajava* L. have been reported to be reliable source of explant (Amin &Jaiswal, 1987; Kumar, 2001) [3, 30]. The majority of workers have used actively growing shoot tips or nodal segments as explant (Loh& Rao, 1989; Papdatou *et al.*, 1990; Parkash & Tiwari, 1996; Kumar & Tiwari, 2001; Singh 7

et al., 2001; Meghwal *et al.*, 2003; Bisen, 2004; Zamir *et al.*, 2007; Rai *et al.*, 2008; Xiaomei*and* Yang, 2011; Usman *et al.*, 2012) [37, 45, 30, 7, 39, 7, 69, 46,66, 65]. A propagation culture system from germinated seedlings has been established by (Yasseen*et al.*, 1995 and Shah *et al.*, 2008) [67, 52, 53, 54]

Time of collection and size of explants

The explants of guava collected from the base of the main stem when vegetative growth vigorously establish (Amin & Jaiswal, 1987) [3]. Parkash and Tiwari, (1996) [30] reported that early spring collection of explants show less contamination in compare to late autumn and summer. Some reports by other workers (Kumar, 2001; Singh *et al*., 2001, Meghwal *et al*., 2003, Bisen, 2004) [7, 39, 40] on explants collection in spring gives best culture establishment and profuse sprouting. Generally, the size of explants best suited for in vitro propagation of guava is 1.0-3.0 cm (Amin & Jaiswal, 1987, 1988; Loh & Rao, 1989; Siddiqui & Farroa, 1996, Meghwal *et al.*, 2003; Bisen, 2004; Zamir *et al.*, 2007, Xiaomei & Yang, 2011) [3, 55, 40, 7, 69, 66].

Sterilization of explant

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Table 1

S. No.	Chemical used for sterilization	Reference
1	Rinse in 70 % ethanol + $HgCl_2$ 0.05-0.50 % (containing a few drops of Tween-20) for 2-5 min.	Amin &Jaiswal, 1987
2	70 % ethanol for 1 min + 5 % NaOCl for 5 min and treatment of shoots which were plastic wrapped with 0.05 per cent $HgCl_2$ for 5 min.	Khattak *et al.* (1990)
3	Wash apical shoots of 5-10 cm for 30-40 min in running tap water + washed with 1 % KCl + 1 % NaOCl for 5-7 min. + washed 3-4 times with sterile distilled water	Parkash& Tiwari (1996)
4	Shoot tip or nodal cuttings (3-5 cm) transferred in a solution 2 g l^{-1}bavistin, 50 mg l^{-1} ascorbic acid, 75 mg l^{-1} citric-acid and 1 ml l^{-1}liauiclean (a.i. benzolkonium chloride); cut 1-2 cm long nodal sections washed thoroughly under running tap water for 15 min.; rinsed with 0.4 % $HgCl_2$ for 10 min.	Sidddiqui&Farrooq (1997)
5	Sterilization with H_2O_2(10%), silver nitrate (0.25%) and $HgCl_2$(0.05%) one by one for 5, 6 and 3 min. respectively	Meghwal *et al.* (2001); Bisen& Tiwari, (2006)
6	Stem segments in 200 mg l^{-1} 8-hydroxy quinaline citrate + 0.1 per cent bavistin; washed in running tap water for 30 min.; stem parts were surface sterilized by dipping them serially in multi disinfectant viz., 70 % ethanol (15 sec.), 10 % (v/v) H_2O_2 (3 min) and 0.05 % $HgCl_2$ (3 min)	Singh *et al.* (2001)
6	Ethyl alcohol (70%) for 30 sec, $HgCl_2$(0.1%) for 5 min and NaOCl(1%) for 6 min	Kumar & Tiwari (2001)
7	H_2O_2 (10%, v/v), silver nitrate (0.25%) and $HgCl_2$(0.05%) for 5, 5 and 3 minutes, respectively; 2-3 times rinses with sterile distilled water	Meghwal *et al.* (2003)
8	70% ethanol; 0.05 % $HgCl_2$ and a drop of surfactant (Tween 80) was added and agitated at 100 rpm on a rotary shaker for 5 min.; shoot tips were rinsed 3 times with sterile distilled water	Zamir *et al.* (2007)
9	15% bleach solution for 20 min; culturing in MS medium with 250 mg L^{-1} polyvinyl-pyrrolidone	Xiaomei & Yang (2011)

Phenolics

Guava exude the phenolic compounds into the culture media makes difficulty in regeneration process. *Psidium guajava* L. is a recalcitrant species having high phenolic exudation that kills explants from sources outside the laboratory. Establishment of *in vitro* cultures of woody plants is greatly hampered by the browning of the explant and culture medium. Browning of explant is due to oxidation of the phenolic compounds, released from the cut ends of the explants. In vitro establishment of the guava explants was very difficult due to the exudation of phenolic compounds into cultures, by which the media turned brown or black within 12-24 hrs and most of the explants died within 2 days of inoculation (Amin & Jaiswal, 1987; Fitch et, 1990; Siddiqui & Farooq, 1996, 1997; Leon-de-Sierralta *et al.*, 1997; Kumar & Tiwari, 2001; Meghwal *et al.*, 2001; Meghwal *et al.*, 2003; Bisen, 2004; Zamir *et al.*, 2007 and Xiaomei & Yang, 2011).

Control of phenolics

Table 2

S. No	Treatment/Strategies to remove toxics from media	Reference
1.	Early spring growth which gave less contamination and browning	Kumar (2001); Singh *et al.* (2001); Meghal *et al.*(2003)
2.	Partial etiolation of stock plants of Allahabad Safeda and aneuploid No. 82 (PusaSrijan) rootstock before explant excision followed by culturing the explants in darkness resulted in early bud sprouting and significant increase in explant survival	Leon-de-Sierralta (1997); Kumar (2001); Meghwal *et al.* (2001); Bisen (2004);Joshee *et al.* (2004)
3.	Transferring the explants in fresh medium at short intervals	Amin &Jaiswal(1987, 1988); Singh & Tiwari (1998); Siddiquai& Farooq(1997); Kumar & Tiwari (2001)

4.	Dipping the explants in the antioxidant solution i.e. citric acid (75 mg/l) and ascorbic acid (50 mg/l)	Fitchet (1990); Singh *et al.*(2001); Kumar(2001); Meghwal*et al.*(2003, 2008); Josheet *al.* (2004)
5.	Use of absorbing agents such as activated charcoal, polyvinylpyrrolidone (PVP)	Amin &Jaiswal (1988); Mohammed *et al.*(1995); Fougat*et al.* (1997); Siddiqui & Farooq (1997); Kumar & Tiwari (2001); Meghwal *et al.*(2003); Concepción *et al.*(2005); Xiaomei & Yang (2011)
6.	Agitation of explants in antioxidant solution	Meghwal *et al.*(2001); Meghwal*et al.*(2003); Zamir*et al.*(2007)
7.	Air drying of explants in an air flow for 30-45 min prior to inoculation	Fitchet (1989, 1990)
8.	Coating the explants at their cut ends with commercial silicon which is a novel process was established for completely inhibiting phenol-based browning	Youssef *et al.*(2010)
9.	Regeneration of explants has been successfully achieved mainly in Murashige and Skoog's medium	Amin & Jaiswal(1987); Fitchet, (1989); Loh& Rao (1989); Khattak *et al.* (1990); Mohammed *et al.*(1995); Parkash & Tiwari (1996); Ramirez & Salazar (1997); Siddiqui &Farrooq (1997); Meghwal *et al.* (2003); Bisen, (2004); Zamir*et al.*(2007); Shah *et al.* (2008); Xiaomei & Yang (2011); Usman *et al.* (2012).

Plant growth regulators

Plant Growth regulator are such organic compounds occurring naturally in the plants as well as synthetic and promote, inhibit or modify any physiological process in small amount. The concentration of plant growth regulator varied from species to species for *in vitro* culture of guava, and type of growth to be initiated i.e. callus formation, shoot proliferation, rooting, etc. In order to support good growth of tissue and organs, it is generally required to add one or more PGR's such as auxin, cytokinin and gibberellins in the medium. 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. In guava, media supplemented with 4.5 mM BA produced 3-6 shoots in 12 weeks (Amin and Jaiswal, 1987) [3] while, according to other workers (Loh and Rao, 1989 and Papadatau, 1990) [37, 44] 0.5 to 2.0 mg/l BA produced maximum shoot proliferation. Maximum number of shoots in guava cultivar sardar (L-49) was obtained in media supplemented with 1.0 mg/l BA + 0.2 mg/l IBA. Siddiqui and Farooq (1996) [56] found that 1.0 mg/l BA was effective in stimulating the formation of axillary shoots and similar results were also reported by Fitchet (1990)]14], Meghwal *et al.* (2001) [39], Khattak *et al.* (2002) [23] and Zamir *et al.* (2007) [69], who got maximum shoot development in cv. Safeda with 1mg/l BAP and glutamine 500 mg/l cultured on MS medium. Ramirez and Salazar (1997) [47] obtained highest percentage of bud sprouting in MS medium supplemented with 4 mg/l BA in nodal segment of guava. A combination of 2 mg/l BA and 0.2 mg/l IBA was found more effective for sprouting and number of shoots per explant at establishment stage. Further increase in cytokinin reduced the explant sprouting in Chinese guava (Kumar *et al.*, 2004) [7]. Highest number of shoots proliferated on WPM supplemented with 0.5 to 1.0 mg/l BAP in different cultures of guava (Singh *et al.*, 2001 and Meghwal *et al.*, 2003) [40]. However, Usman *et al*. (2012) reported that maximum shoots were induced with 2.0 mg/l of BAP.

Root induction

Media

There is no single medium as well as constituent of medium to suit every stage of in vitro cloning. Therefore, to attain full plants, the micro-shoots must be transferred to a rooting medium which is different from the shoot proliferation medium especially in its hormonal composition (Bhojwani and Rajdan, 1992). Full strength WPM was used for rooting of guava cultivars Allahabad Safeda, Lucknow-49 and Thailand (Singh *et al.*, 2001) and Aneuploid No. 82 (Meghwal *et al.*, 2003) [40].

Plant growth regulators

Table 3

S. No.	Treatment of Chemicals for root induction	References
1	Half MS medium supplemented with 1 mm each of IBA and NAA	Amin &Jaiswal (1987); Yasseen *et al.* (1995) and Shah *et al.* (2008)
2	Shoots produced from guava explants produced roots in media containing 9.8 mM TBA	Mohammed *et al.* (1995).
3	3Shootlets cultured on half MS 0.2 mg/l IBA and incubated for one week in dark resulted in early and better rooting of guava	Parkash& Tiwari (1996)
4	Shoot explants were easily rooted in vitro using Rugini olive medium (OM) with 0.5 or 1.0 mg NAA or IBA/l	Papadatau *et al.* (1990)
5	Best rooting (66.66%) was recorded with IBA + NAA (0.2 + 0.2 mg/ l) in Chinese guava	Kumar (2001)
6	Achieved best rooting of micro shoots on half strength modified Murashige and Skoog's (MMS) medium supplemented with 4.90 mM indole-3-butyric acid along with 100 mg l-1 activated charcoal	Singh *et al.* (2002)
7	Excised proliferating shoot segments of 1.5 to 2.0 cm for in vitro rooting on WPM containing 200 mg/l activated charcoal and dual auxins (IBA and NAA) @ 0.2 mg/l each which resulted in higher frequency of rooting	Meghwal *et al.* (2003)
8	Obtained maximum (54) plants rooted with average number of roots (3.8) per plantlet in MS medium supplemented with 2.5 mg/L IAA + 2.5 mg/L IBA	Zamir *et al.* (2007)
9	Achieved maximum rooting (65%) when shoots were dipped in 4.9 mM Indole-3-butyric acid (IBA) solution for 1 min and then rooted	Xiaomei & Yang (2011)

Rooting

Among the various auxins viz., IBA, IAA, NAA etc. used for *in vitro* rooting, IBA is the most commonly used auxin. Khattak *et al*. (2002) [23] reported that the initiation of adventitious roots at 1/2MS + 1 mg/l IBA from *in vitro* derived guava shoots, whereas 2.5 mg/l each of IAA and IBA for root development was noticed by Raziuddin *et al*. (2004). The best rooting of *in vitro* desired microcutting of guava cv. Hisar Safeda with 1/2MS + 2 mg/l IBA + 200 mg/l activated charcoal (Kumar, 2003)). Biswas *et al* (2007) reported healthy root system on half strength WPM media containing 0.1 mg/l NAA. Shah *et al* (2008) observed NAA supplementation at 0.5 mg/l in 1/2MS + IBA media was found most responsive for cv. Safeda. Singh *et al*. (2001) reported the in vitro clonal propagation of guava cultivars Allahabad Safeda, Lucknow - 49 and Thailand and concluded that the rooting was noted to be the best in WPM + 0.5 or 1.0 mg/l IBA and NAA + 200 mg/l activated charcoal.

Hardening of rooted plantlets

The success of any micropropagation research depends on the success of plantlet transferring technique, where shoot or plantlets that have been growing heterotrophically under an aseptic environment of test tube (having very high humidity) become autotrophic and grows under condition of moderate to low humidity. For acclimatization rooted plantlets of guava were taken out of culture tubes, washed thoroughly to remove any remaining medium and planted in small plastic pots filled with garden soil and compost (1:1). During first 7-10 days, the potted plantlets were covered with glass beakers to provide high humidity (Amin and Jaiswal, 1988). Loh and Rao (1989) transferred the rooted plantlets to small plastic pots (containing vermiculite) and covered initially with thin plastic film to maintain high humidity and then small holes were made in the plastic to acclimatize it gradually. The plantlets were kept outdoor under 80 per cent shade for about a week, after which they were transplanted in pots with soil. More than 90 per cent of the plantlets survived after transplantation to soil. According to Khattak *et al.*, 2002 and Zamir *et al.*, 2007, the rooted plantlets were transferred to potting medium (sand, clay, compost at 1:1:1 by volume) with a layer of sand in greenhouse and initially watered with half Knop's solution. Parkash and Tiwari (1996) transferred rooted plantlets for hardening treatments 30 days after rooting and 86 per cent survival was recorded in the pots containing a mixture of sand, soil and FYM (1:1:1). Same technique was followed by Kumar (2001) for Chinese guava. Whereas, Singh et al. (2001) washed the rooted plantlets and removed the adhering agar in sterile water and transplanted in container filled with peat + perlite (1:1) and moisture with one-fourth strength MMS (Modified Murashige and Skoog) macro-salts solution (pH 5.8). The hardened plantlets (30-45 days) were then transferred to small plastic pots filled with soil: farmyard manure, sand (1:1:1) and shifted to the glasshouse. The plants were regularly misted with one tenth MMS macro-salts + 0.1 per cent bavistin with pH adjusted to 5.7 and exposed to light of high intensity (62 m mol m^2S^{-1}) with temperature maintained at 26 ± 1°C. The plantlets (after 4 weeks) were then shifted to the shade house in small plastic pots. A regular irrigation to these plantlets were practiced at 15 days' interval and later on shifted to natural environment. Xiaomei and Yang (2011) planted rooted shoots (with 3-5 fully expanded leaves) 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 ìmol 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.

Genetic fidelity

The commercial multiplication of a large number of diverse plants species represents one of the major success stories of utilizing tissue culture technology profitably. However, a major problem often encountered with the use of tissue culture techniques such as SE is the occurrence of soma clonal variation, which is often heritable as it represents induced genetic changes (Larkin & Scowcroft, 1981; Svabova & Lebeda, 2005) [34, 64]. Thus, genetic fidelity testing is an important prerequisite for in vitro regeneration protocols of many crop species, particularly if the resultant plants are to be transplanted to the field. Several strategies have been employed to assess the genetic fidelity of regenerated plants, each with their own advantages and disadvantages. Molecular markers facilitate the screening of SE regenerated plants with high precision, and since these markers are unaffected by environmental factors (that can alter phenotypes), they produce reliable and reproducible results. However, for an effective analysis of the genetic stability of in vitro regenerated plantlets, a combination of markers that amplify different regions of the genome should be used (Alizadeh& Singh, 2009; Liu & Yang, 2012) [1,1, 36]. Kamle et al. (2013) [22] have reported various DNA based molecular markers for assessment of genetic fidelity in guava. Liu and Yang (2012) [36] have reported ISSR markers for assessment of genetic fidelity in guava.

Conclusion

In vitro clonal propagation protocols for different guava spp. and varieties have been developed in all over the world. For extending the guava production in tropical and subtropical areas a rapid and efficient method for clonal propagation of elite mature genotype is necessary. During past few decades, transpiring biological techniques for micropropagation and tissue culture of predominant guava cultivars have been discussed by several researchers are effective. However, there are several problems related 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 which require to be given more attention. Understanding of the biological processes that permit the manipulation of *in vitro* morphogenesis and investigations on various physiological, biochemical and molecular aspects of plant hormones will highly improve our understanding and gives details that will necessary for addressing the problems of *in vitro* recalcitrance or *in vitro* plant growth and development.

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