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MICROPROPAGATION OF GUAVA (*PSIDIUM* SPP.) : A REVIEW

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Abstract

Guava (*Psidium guajava* L.) is one of the most common and important commercial fruit crops cultivated in both tropical and subtropical regions of the world. In India major share of the fruits production is utilized for fresh consumption. Micro propagation is a rapid process to develop identical progeny from offspring. This is the growing of plants from meristematic tissue or somatic cells of superior plants on suitable nutrient media under controlled environmental conditions. In guava, micro propagation was achieved for the first time in 1987 by M. N. Amin and V.S. Jaiswal on Allahabad safeda. Since then significant progress has been made in the different areas of *in vitro* culture of guava since the first reports. In most cases, Murashige and Skoog (MS) revised medium (1962) was used as mineral medium for culturing *Psidium* species and cultivars for regeneration and/or proliferation, sub culturing and subsequent rooting. The lower concentration of major elements in WPM (Woody Plant Medium) was more suitable for micro propagation of some guava genotypes. Starting material for establishment of guava *in vitro* culture consists mostly of actively growing shoot tips or nodal segments as explants (1.0-3.0 cm size) collected during in spring are best suited for *in vitro* propagation of guava. Establishment of *in vitro* cultures of woody plants is greatly hampered by the browning of the explant and culture medium. In guava, several strategies have been employed to overcome the harmful effects of browning either through neutralization or through the avoidance of toxic substances in the medium.BAP (0.5 -2.0 mg/l) is the most frequently used cytokinin for guava micropropagation. The success of transplanting and survival of plants greatly depends on the quality of roots. Rooting *Psidium* spp. *in vitro* has proven to be difficult; however, several workers were successful in rooting. IBA is mostly used for inducing rooting followed by NAA, however use of dual auxins (IBA & NAA) resulted higher frequency of rooting. In guava, acclimatization has been accomplished in various substrates by progressively decreasing the relative humidity.

Key words : Guava, tissue culture, micropropagation, BAP, IBA, NAA, genetic fidelity.

Introduction

Guava (*Psidium guajava* L.) is one of the well known edible fruit of the tropical and sub-tropical climates. It is often referred to as "apple of the tropics". Guava, member of the Myrtaceae family is native to tropical America, stretching from Mexico to Peru (Samson, 1986). There are 150 species of *Psidium,* most of which are fruit bearing trees. Guava plant is widely adopted and can tolerate frost, drought and salinity conditions (Samson, 1986). It is considered as excellent source of ascorbic acid (Rathore, 1976) and also abundant in dietary fibres, vitamin-A (about 250 IU/100 g), pectin, calcium, phosphorus and iron. Excellent salad and pudding are prepared from the shell of ripe fruit. Guava jelly is well known to all and the common sour wild guava makes the best jelly, sherbets, ice-creams and guava powder. Two types of wines *viz.,* guava juice and guava pulp wines are also prepared from guava fruit (Bardiya *et al*., 1974). The roots, bark leaves and green fruits are used as medicine in the tropical world to halt gastroentistis, diarrhoea and dysentery (Rathore, 1976). It also contains many high-grade antioxidants such as, lycopene, carotenoids and polyphenols. (Jiménez *et al*., 2001). 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. (Feskanich *et al*., 2000; Gordon, 1996 and Halliwell, 1996). Furthermore, high concentrations of pectin content in guava fruit may play a significant role in reducing cholesterol. Guava production is facing major agronomic and horticultural problems including susceptibility to many

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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, 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, bacterial contamination and phenolics compounds exudation tend to limit *in vitro* cultures of the guava plant.

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. Micro propagation is now a well-established technology which has made significant contributions to the propagation and improvement of agricultural crops in general. It is applicable to various crops like ashwagandha (Bhuria *et al*., 2014) sugarcane (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

Theoretically, all plant parts are capable of regeneration *in vitro* except the bark which is dead tissue. In general, greater success has been reported with use of juvenile explants. Healthy, young and soft (actively growing) shoots are generally more amenable for culture than older woody tissue. New vegetative growths in guava have been reported to be reliable source of explant (Amin and Jaiswal, 1987; Kumar, 2001). The majority of workers have used actively growing shoot tips or nodal segments as explant (Loh and Rao, 1989; Papdatou *et al*., 1990; Parkash and Tiwari, 1996; Kumar and Tiwari, 2001; Singh *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). A propagation culture system from germinated seedlings has been established by Yasseen *et al*., 1995 and Shah *et al*., 2008).

Collection season and size of explant

In guava, generally it has been observed that explants collected from the base of the main stem during vigorous vegetative growth establish and proliferate well (Amin and Jaiswal, 1987). Nodal explants of guava collected during early spring season guava show less contamination and browning of media than explants collected during late autumn and summer (Parkash and Tiwari, 1996). Similarly, other workers have also reported best culture establishment and profuse sprouting of explants collected in spring (Kumar, 2001; Singh *et al.,* 2001, Meghwal *et al.,* 2003, Bisen, 2004). In general, explants of 1.0-3.0 cm size are best suited for *in vitro* propagation of guava (Amin and Jaiswal, 1987, 1988; Loh and Rao, 1989; Siddiqui and Farroa, 1996, Meghwal *et al.,* 2003; Bisen, 2004; Zamir *et al*., 2007, Xiaomei and Yang, 2011).

Sterilization of explant

In guava, effective surface sterilization was carried out with 0.05-0.50 per cent $HgCl_2$ (containing a few drops of Tween-20) for 2-5 min after a brief rinse in 70 per cent ethanol (Amin and Jaiswal, 1987;). Khattak *et al.* (1990) treated the seedling explants with 70 per cent ethanol for 1 min followed by 5 per cent NaOCl for 5 min and treatment of shoots which were plastic wrapped with 0.05 per cent $HgCl_2$ for 5 min. Parkash and Tiwari (1996) reported that apical shoots of 5-10 cm length were washed thoroughly under running tap water for 30-40 minutes, then washed with autoclaved 1 per cent KCl followed by 1 per cent NaOCl treatment for 5-7 min. The treated explants were then washed 3-4 times with sterile distilled water. Sidddiqui and Farrooq (1997) reported that shoot tip and nodal cuttings (3-5 cm long) from 3rd and 4th node segments were selected. These nodal cuttings were immediately transferred in a solution containing 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). Thereafter, 1-2 cm long nodal sections of guava seedlings were cut and then washed thoroughly under running tap water for 15 min. and then rinsed with 0.4 per cent mercuric chloride for 10 min. The maximum aseptic explants with shoot proliferation were obtained by a combination of surface sterilizing agents involving hydrogen peroxide (10%), silver nitrate (0.25%) and mercuric chloride (0.05%) one by one for 5, 6 and 3 min, respectively (Meghwal *et al.,* 2001; Bisen and Tiwari, 2006). Stem segments of guava were collected in a solution of 200 mg l^{-1} 8 hydroxy quinaline citrate + 0.1 per cent bavistin and brought to laboratory and washed in running tap water for 30 min. Further, the stem parts were surface sterilized by dipping them serially in multidisinfectant *viz.,* 70 per cent ethanol (15 sec.), 10 per cent (v/v) H_2O_2 (3 min) and 0.05 per cent $HgCl_2$ (3 min) (Singh *et al.,* 2001). But aseptic culture as well as maximum survival of explants was obtained when treated with ethyl alcohol (70%) for 30 seconds, mercuric chloride

(0.1%) for 5 min and sodium hypochloride (1%) for 6 min in sequential order (Kumar and Tiwari, 2001). For surface sterilization of nodal segments (2 cm) from aneuploid guava, multiple sterilants were employed in a series involving hydrogen peroxide (10%, v/v), silver nitrate (0.25%) and mercuric chloride (0.05%) for 5, 5 and 3 minutes, respectively followed by two to three rinses with sterile distilled water (Meghwal *et al.,* 2003). Zamir *et al*. (2007) briefly rinsed the explants with 70% ethanol and surface sterilization of these shoots was carried out with 0.05% Mercuric chloride ($HgCl_2$) and a drop of surfactant (Tween 80) was added and agitated at 100 rpm on a rotary shaker for 5 minutes. Then the shoot tips were rinsed three times with sterile distilled water under laminar flow bench. However, because of the danger of environmental pollution mercury compounds are not recommended. 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 (Xiaomei and Yang, 2011).

Phenolics

The tendency of guava to exude phenolic compounds into the media makes the regeneration process particularly difficult. Guava is a recalcitrant species with heavy 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 is generally considered to be the result of the oxidation of phenolic compound, released from the cut ends of the explants by polyphenoloxidases, feroxidases or air. *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 and Jaiswal, 1987; Fitchet, 1990; Siddiqui and Farooq, 1996 and 1997; Leon-de-Sierralta *et al*., 1997; Kumar and Tiwari, 2001; Meghwal *et al*., 2001; Meghwal *et al*., 2003; Bisen, 2004; Zamir *et al*., 2007 and Xiaomei and Yang, 2011).

Control of phenolics

In guava, several strategies have been employed to overcome the harmful effects of browning either through neutralization or through the avoidance of toxic substances in the medium. This includes (i) early spring growth which gave less contamination and browning (Kumar, 2001; Singh *et al*., 2001; Meghal *et al*., 2003), (ii) partial etiolation of stock plants of Allahabad Safeda and aneuploid No. 82 (Pusa Srijan) 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), (iii) transferring the explants in fresh medium at short intervals (Amin and Jaiswal, 1987, 1988; Singh and Tiwari, 1998; Siddiquai and Farooq, 1997; Kumar and Tiwari, 2001) (iv) 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 and Meghwal *et al*., 2003; Joshee *et al*., 2004 and Mangal *et al*., 2008) (v) use of absorbing agents such as activated charcoal, polyvinylpyrrolidone (PVP) (Amin and Jaiswal, 1988; Mohammed *et al*., 1995; Fougat *et al*., 1997; Siddiqui and Farooq, 1997; Kumar and Tiwari, 2001; Meghwal *et al*., 2003; Concepción *et al*., 2005*;* Xiaomei and Yang, 2011), (vi) agitation of explants in antioxidant solution (Meghwal *et al*., 2001; Meghwal *et al*., 2003; Zamir *et al*., 2007), (vi) air drying of explants in an air flow for 30-45 min prior to inoculation (Fitchet, 1989 and 1990) and (vii) 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). Regeneration of explants has been successfully achieved mainly in Murashige and Skoog's medium (Amin and Jaiswal, 1987; Fitchet, 1989; Loh and Rao, 1989; Khattak *et al*., 1990; Mohammed *et al*., 1995; Parkash and Tiwari, 1996; Ramirez and Salazar, 1997; Siddiqui and Farrooq, 1997; Meghwal *et al*., 2003; Bisen, 2004; Zamir *et al*., 2007; Shah *et al*., 2008; Xiaomei and Yang, 2011; Usman *et al*., 2012).

Plant growth regulators

For *in vitro* culture of guava, the concentration of plant growth regulator varied considerably from species to species 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) while, according to other workers (Loh and Rao, 1989 and Papadatau, 1990) 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) 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), Meghwal *et al.* (2001), Khattak *et al.*

(2002) and Zamir *et al*.(2007), 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) 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). 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). 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).

Plant growth regulators

Adventitious rooting in guava was obtained in half MS medium supplemented with 1 mm each of IBA and NAA (Amin and Jaiswal, 1987; Yasseen *et al*., 1995 and Shah *et al*., 2008). Shoots produced from guava explants produced roots in media containing 9.8 mM TBA (Mohammed *et al*., 1995). Shootlets 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 and Tiwari, 1996). 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). Best rooting (66.66%) was recorded with IBA + NAA (0.2 + 0.2 mg/l) in Chinese guava (Kumar, 2001). Singh *et al.* (2002) 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. Whereas, Meghwal *et al.* (2003) 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. However, Zamir *et al*. (2007) 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. Xiaomei and Yang, 2011 achieved maximum rooting (65%) when shoots were dipped in 4.9 mM Indole-3-butyric acid (IBA) solution for 1 min and then rooted.

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 somaclonal variation, which is often heritable as it represents induced genetic changes (Larkin & Scowcroft, 1981; Svabova & Lebeda, 2005). 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). Kamle *et al*. (2013) have reported various DNA based molecular markers for assessment of genetic fidelity in guava. Liu and Yang (2012) have reported ISSR markers for assessment of genetic fidelity in guava.

Conclusion

Efficient *in vitro* clonal propagation protocols have been developed all over the world for different guava spp. and varieties. For extending the guava cultivation in these areas a rapid and efficient method for clonal propagation of elite mature genotype is necessary. During two past decades, emerging biotechniques for tissue culture and micropropagation of superior guava cultivars have been discussed by several researchers are effective. 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 which needs 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 greatly advance our knowledge and provide information that will help address the issues of *in vitro* recalcitrance or *in vitro* plant growth and development.

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