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SHOOT MULTIPLICATION AND PLANT REGENERATION OF GUAVA (*Psidium guajava* L.) FROM NODAL EXPLANTS OF *IN VITRO* RAISED PLANTLETS

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A B S T R A C T

A micropropagation method is described for guava (*Psidium guajava* L.) using nodal explants from somatic embryo-derived young and aseptic plantlets. Multiple shoots were induced from axillary buds on MS medium containing different concentrations of N^6-benzylaminopurine (BAP), either alone or in combination with kinetin (Kn), indole-3-acetic acid (IAA) or α-naphthalene acetic acid (NAA). Medium containing 1 mg l^{-1} BAP was the most effective for shoot multiplication. *In vitro* regenerated shoots developed roots either on MS medium alone or on MS medium supplemented with indole-3-butyric acid (IBA). The rooted plantlets were successfully acclimatized.

Key words: acclimatization, micropropagation, microshoots, organogenesis, *Psidium guajava*

INTRODUCTION

Guava (*Psidium guajava* L., *Myrtaceae*) is an important fruit crop of tropical and sub-tropical regions of the world. It is one of the richest natural sources of vitamin C and a good source of calcium, phosphorus,

iron and pectin. Guava fruit, leaves, roots and bark are used in local medicines to treat gastroenteritis, diarrhoea and dysentery (Jaiswal and Amin, 1992; Rai et al., 2008a). The major agronomic and horticultural problems facing the guava industry are severity of wilt disease and susceptibility to many pathogens and stresses, low yield and short fruit shelf life, high seed content etc. (Rai et al., 2007). Conventional breeding techniques have limited scope of improvement of guava owing to long juvenile period, self incompatibility and heterozygous nature. Conventional propagation methods, i.e. grafting and stool layering, suitable for improvement of guava already exist, but due to the long juvenile period they are time consuming and cumbersome (Jaiswal and Amin, 1992). Propagation from seeds is not practical because they germinate poorly and unevenly and long time is required for seedling emergence (Doijode, 2001). Biotechnology could resolve some of the most serious problems of the guava industry. Transfer of useful traits such as resistance to diseases, insects and stresses between plant species has played an important role in crop improvement (Goodman et al., 1987). The crop improvement through genetic transformation requires an efficient *in vitro* regeneration system (Rai et al., 2008a).

In guava, organogenesis form shoot tips and nodal segments obtained from mature trees was reported for the first time by Amin and Jaiswal (1987, 1988) and Jaiswal and Amin (1987). However, several problems, such as exudation of phenolics and browning of media and explants, microbial contamination and recalcitrant *in vitro* tissues, are associated with *in vitro* culture of explants obtained from mature tree (Amin and Jaiswal, 1987). Acosta et al. (2002) have identified several microbial contaminants/pathogens in *in vitro* cultures of guava.

Regeneration of plants from seedling explants of guava has also been reported by several workers (Loh and Rao, 1989; Papadatau et al., 1990; Yasseen et al., 1995; Singh et al., 2002). However, about 67-95% seeds of guava are contaminated by bacteria and fungi, which impedes the use of standard tissue culture methods (Ali et al., 2007). Micropropagation of guava through direct somatic embryogenesis has been published earlier by Rai et al. (2007). Production of synthetic seeds using shoot tips and nodal segments, which may be applied in short-term storage and germplasm exchange of elite genotype of guava, has also been reported (Rai et al., 2008ab). However, for large-scale application in development of transgenic plants, there is a need for developing efficient regeneration system in guava, especially in terms of establishment of contamination-free culture and large-scale propagation.

In the work presented, investigation was undertaken to establish an *in vitro* micropropagation method of *Psidium guajava* L. cv. 'Banarasi' local via direct organogenesis from nodal explants obtained from *in vitro*-derived, uncontaminated material.

MATERIAL AND METHODS

Establishment of nodal explants cultures

Cultures were established using immature zygotic embryos following the procedure described by Rai et al. (2007). Zygotic embryos were excised from ten-week-old fruits and cultured on full-strength MS (Murashige and Skoog, 1962) medium supplemented with 1 mg l^{-1} 2,4-D and 5% sucrose and solidified with 0.8% (w/v) of agar. After eight days, the embryos were transferred to 2,4-D free MS medium with 5% sucrose. Globular stage somatic embryos were formed on the surface of zygotic embryo after 2-3 weeks of culturing. At the end of the fourth week, most of the cultures showed somatic embryos at globular, heart and cotyledonary stages of development on the whole surface of zygotic embryos. Earlier formed embryos proceeded towards torpedo stages. After 6-8 weeks, somatic embryos at torpedo stage were transferred to germination medium (half strength MS medium + 3% sucrose) at which they germinated and developed into plantlets within 2 weeks. Nodal explants (8-10 mm long; Fig. 1A) excised from 6-8 week old plantlets were used for shoot organogenesis and multiplication.

Shoot proliferation and rooting

For shoot organogenesis and proliferation, nodal explants were cultured on MS medium containing 3% (w/v) sucrose (Hi-Media, Mumbai, India), supplemented with different concentrations of BAP or Kn alone or in combination with 1.0 mg l^{-1} BAP and Kn or IAA or NAA at different concentrations (0, 0.1, 0.5 or 1.0 mg l^{-1}) (Sigma Chemical Co., St Louis, MO, USA). For rooting, 2-4 cm in length shoots regenerated on different media were excised from nodal segments and subcultured on MS medium containing different concentrations (0, 0.1, 0.5 or 1.0 mg l^{-1}) of IBA or NAA. The pH of the media was adjusted to 5.8 with 0.1 N HCl or 1 N NaOH, before addition of agar. Media were autoclaved at 121°C for 15 min. Cultures were maintained in a growth chamber at 25°C (±2°C) and in artificial light (16 h photoperiod and irradiance of 50 μmol m^{-2} s^{-1}) provided by white fluorescent tubes.

Acclimatization and field transfer of plantlets

Rooted shoots with 4-5 fully expanded leaves were planted in 7.5-cm in diameter plastic pots containing a mixture of sand and garden soil (3 : 1), covered with polyethylene bags for 15-20 days to prevent excessive water loss and watered twice a week. Plantlets were kept at 25°C (±2°C) in artificial light (16 h photoperiod and irradiance of 50 μmol m^{-2} s^{-1}) provided by white fluorescent tubes for 3-4 weeks and then the pots were transferred to direct sunlight. After 3-4 months, successfully established plantlets were subsequently transferred to field conditions.

Experimental design and statistical analysis

The culture responses were expressed in terms of percentage of

responding explants and number of shoots or roots per an explant. The observations and measurement were recorded after four weeks of culturing. For the above experiments, 24 replicates were used for each treatment and each experiment was repeated thrice. One replicate means one explant. The results are expressed as a mean ± SE of three independent experiments. The data were analyzed statistically using one-factorial analysis of variance and the significant differences between means were assessed by Duncan's multiple range test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Shoot primordia on nodal segments (Fig. 1B) were induced on MS medium with or without any plant growth regulators within 1-2 weeks. However, when growth regulators were absent, only one shoot per an explant developed. Multiple shoots were induced on nodal segments cultured on media supplemented with various plant growth regulators. Browning of explants due to phenolic`s exudation has been reported as a serious problem in establishing axenic cultures of tree species, including guava (Amin and Jaiswal, 1987, 1988; Jaiswal and Amin, 1987). However, this problem was not observed in this study where cultures were initiated from *in vitro* raised plants. This is probably because young seedlings do not synthesize phenolic substances (Chandra et al., 2005). Of the two cytokinins tested, BAP was more effective than Kn in inducing shoot proliferation. On medium containing Kn, only one shoot developed per an explant or there was no shoot proliferation at all (data not presented). Among various concentrations of BAP tested, the highest number of shoots per an explant was observed on medium containing 1 mg l^{-1} BAP (Tab. 1). BAP is reported to have stimulated shoot multiplication from different explants of several fruit tree species including *Syzygium cuminii* (Yadav et al., 1990), *Artocarpus heterophyllus* (Amin and Jaiswal, 1993), *Annona squamosa* (Nagori and Purohit, 2004), *Litchi chinensis* (Das et al., 1999), *Punica granatum* (Naik et al., 2000) and *Carissa carandas* (Rai and Mishra, 2005). Effectiveness of BAP in stimulating shoot multiplication in guava has also been reported previously by several workers (Amin and Jaiswal, 1987, 1988; Loh and Rao, 1989; Papadatau et al., 1990; Yasseen et al., 1995). 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 (Malik et al., 2005). The shoots raised *in vitro* and developed on 1.0 mg l^{-1} BAP, with length of 1.0-1.5 cm, did not elongate further. Therefore, they were transferred to the medium with lower concentration (0.5 mg l^{-1}) of BAP. After 4 weeks from the transfer, the average length of shoots reached 3.0-3.5 cm. Exposure of

Table 1. Effect of BAP on shoot multiplication from nodal segments of guava

Concentration of BAP [mgl^{-1}]	Percentage of responding explants	Number of shoots per explant
0.0	83.3 ± 4.2a*	1.00 ± 0.00d
0.1	41.6 ± 8.4bc	1.58 ± 0.10bc
0.5	58.3 ± 8.3b	1.87 ± 0.10b
1.0	91.6 ± 8.3a	2.45 ± 0.10a
2.0	33.3 ± 4.2c	1.25 ± 0.30cd

*Means in a column followed by the same letter are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test

Table 2. **A** – The effect of BAP in combination with Kn on shoot multiplication from nodal segments

Concentration of BAP + Kn [$mg\ l^{-1}$]	Percentage of responding explants	Number of shoots per explant
1.0 + 0.0	91.6 ± 8.3a*	2.45 ± 0.10a
1.0 + 0.1	83.3 ± 4.2a	1.50 ± 0.05c
1.0 + 0.5	91.6 ± 8.3a	1.91 ± 0.10b
1.0 + 1.0	58.3 ± 8.3b	1.58 ± 0.10c

*For explanations, see Table 1

B – The effect of BAP in combination with IAA on shoot multiplication from nodal segments

Concentration of BAP + IAA [$mg\ l^{-1}$]	Percentage of responding explants	Number of shoots per explant
1.0 + 0.0	91.6 ± 8.3ab*	2.45 ± 0.10a
1.0 + 0.1	95.8 ± 4.2a	1.69 ± 0.04b
1.0 + 0.5	79.2 ± 4.2bc	1.57 ± 0.05b
1.0 + 1.0	70.8 ± 4.2c	1.35 ± 0.02c

*For explanations, see Table 1

C – The effect of BAP in combination with NAA on shoot multiplication from nodal segments

Concentration of BAP + NAA [$mg\ l^{-1}$]	Percentage of responding explants	Number of shoots per explant
1.0 + 0.0	91.6 ± 8.3ab*	2.45 ± 0.10a
1.0 + 0.1	66.6 ± 4.2b	1.74 ± 0.07b
1.0 + 0.5	20.8 ± 4.2c	1.33 ± 0.16b
1.0 + 1.0	08.3 ± 4.2c	0.66 ± 0.30d

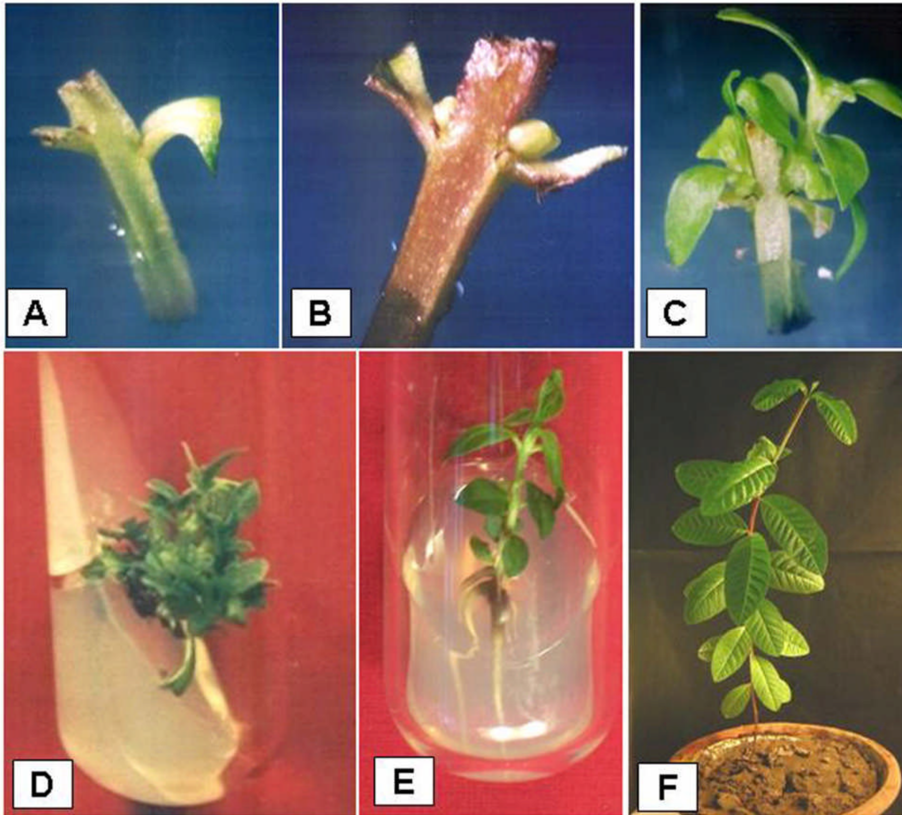
*For explanations, see Table 1

explants to higher BAP concentrations during induction phase may have led to accumulation of cytokinins, which inhibited further shoot growth (Malik et al., 2005). Combinations of BAP and Kn, IAA or NAA have also been used to induce shoot formation in numerous other species. However, in investigations presented combination of these plant growth regulators was less effective than of BAP alone (Tab. 2A, B, C). In contrary to our results, combinations of BAP and NAA have been shown effective in inducing shoot formation in *Annona muricata* (Bejoy and Hariharan, 1992) and lime (Al-Bahrany, 2002), whereas combinations of BAP and Kn have been beneficial for shoot multiplication in elephant apple, *Feronia limonia* (Hossain et al., 1994).

The shoots produced from nodal segments on different media were excised and transferred to MS medium containing auxins (IBA or NAA) for root initiation. Irrespective of media compositions, root initiation was observed after 1-2 weeks from the transfer. After four weeks of culturing, about 78.0% shoots developed roots on medium without IBA or NAA although usually only a single root per a shoot was observed. The occurrence of root formation on auxin-free medium may be due to the availability of endogenous auxin in *in vitro* shoots (Minocha, 1987). Among all the treatments tried, maximum number of roots per responsive explant was observed on medium containing 1.0 mg l^{-1} IBA (Fig. 1D, Fig. 2). When NAA was used to induce adventitious roots, a mass of callus was produced on the base of the shoots and callus formation increased with increasing concentration of NAA. Callus production not only decreased rooting frequency, but also resulted in formation of abnormal roots. Similarly as in our experiments, IBA has been shown to be very effective in root induction in various species of tropical trees including *Eucalyptus grandis* (Macrae and van Staden, 1990), *Syzygium cuminii* (Yadav et al., 1990), *Litchi chinensis* (Das et al., 1999), *Garcinia indica* (Malik et al., 2005) and *Terminalia arjuna* (Pandey et al., 2006).

Approximately 90.0% plants successfully acclimatized after 4-6 weeks from transplantation to the mixture of sand and soil (Fig. 1E). During adaptation in laboratory condition, shoot growth was slow and only 2-4 cm increase in shoot length was recorded after a month from transplantation. When these plants were kept in sunlight under natural condition, they grew faster and attained a height of 25-30 cm within a period of 2-4 months. About 3-4 month-old soil-established plants grown under laboratory conditions were successfully transferred to Botanical Garden of Banaras Hindu University. All plants transferred to Botanical Garden have survived and grew well in soil without any phenotypic aberrations.

The difficulties in establishing aseptic cultures from mature explants along with browning of explants have limited progress towards development of *in vitro* propagation of guava (Amin and Jaiswal, 1987, 1988; Jaiswal and



A. Freshly explanted nodal segment.
B. Shoot bud proliferation on MS medium containing 1 mg l^{-1} BAP after one week of culture.
C. Shoot development on MS medium containing 1 mg l^{-1} BAP after 2-3 weeks of culture.
D. Nodal explant with multiple shoots developed on MS medium supplemented with 1 mg l^{-1} BAP after 4 weeks of culture.
E. Rooted shoot on MS medium containing 1 mg l^{-1} IBA.
F. Well-hardened plant of guava in earthen pot.

Figure 1. Plantlets regeneration from nodal explants obtained from *in vitro* raised plantlets of guava

Amin, 1987) and several other plant species like *Zizyphus* sp. (Rathore et al., 1992) or *Terminalia arjuna* (Pandey et al., 2006). Since somatic embryo-derived young and aseptic plantlets were used as explant donors in the investigations presented, the problem of contamination and phenolic exudation was not observed. Although shoots regenerated directly from explants, the proposed methods may also be applicable in genetic transformation of guava. Elaborated propagation method would ensure a continuous supply of plants of guava cv. ‘Banarasi’ local produced in limited time and space.

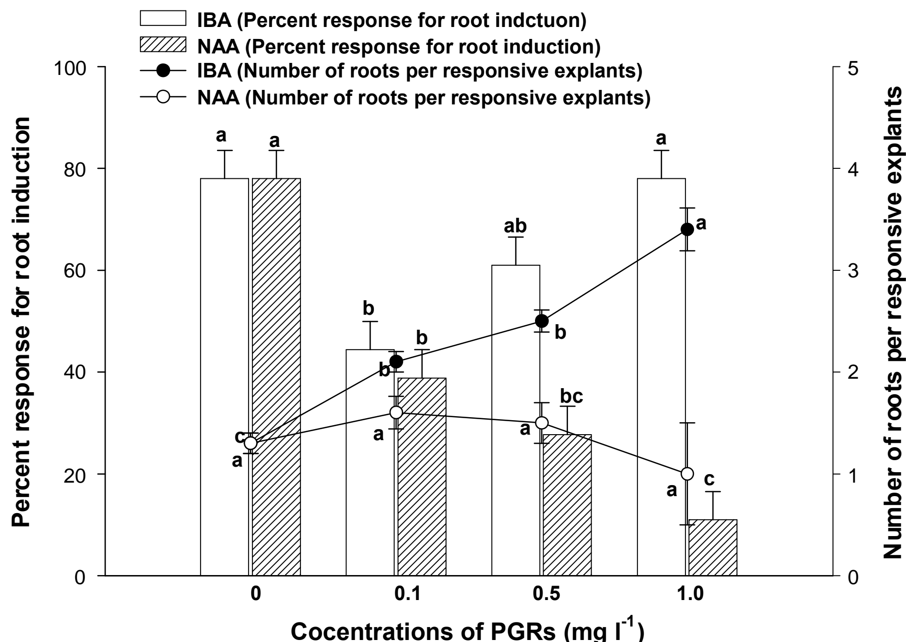


Figure 2. The influence of various concentrations of IBA or NAA on rooting of shoots of guava. Values are means ± S.E. of three independent experiments. Mean values followed with the same letter do not differ significantly at $p \leq 0.05$ according to Duncan's multiple range test

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ROZMNAŻANIE PĘDÓW I REGENERACJA GUAJAWY (*Psidium guajava* L.) Z EKSPLANTATÓW WĘZŁOWYCH ROŚLIN UPRAWIANYCH *IN VITRO*

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STRESZCZENIE

Opisano metodę mikrorozmnażania guajawy (*Psidium guajava* L.) używając eksplantatów węzłowych z młodych i aseptycznych roślin otrzymanych na drodze somatycznej embriogenezy. Wiele pędów było inokulowanych z pachwinowych pąków na pożywce MS zawierającej różne stężenia benzyloaminopuryny (BAP), zastosowanej pojedynczo lub w kombinacji z kinetyną (Kn), kwasem indolilooctowym (IAA) albo kwasem α-naftalenooctowym (NAA). Pożywka zawierająca BAP w stężeniu 1 mg l^{-1} była najbardziej efektywna. Ze zregenerowanych pędów *in vitro* rozwijały się korzenie zarówno na pożywce MS, jak i na pożywce MS uzupełnionej kwasem indolilomasłowym (IBA). Ukorzenione rośliny były pomyślnie aklimatyzowane.

Słowa kluczowe: aklimatyzacja, mikrorozmnażanie, mikropędy, organogeneza, *Psidium guajava*