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A N I N T E R N A T I O N A L J O U R N A L

Recurrent Somatic Embryogenesis and Plantlet Regeneration in *Psidium guajava* L.

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ABSTRACT

A simple and efficient protocol for recurrent somatic embryogenesis and plant regeneration is one of the prerequisites for genetic improvement of guava. An efficient reproducible regeneration somatic embryogenesis protocol was developed in four genotypes of Psidium guajava L. using immature zygotic embryo as starter explant. Somatic embryogenesis induction was obtained on MS basal medium supplemented with 2.0 mgL^{-1} 2, 4-D, 400 mgL^{-1} L-glutamine, 6% sucrose and 500 mgL^{-1} Malt extract. Following SE induction different developmental stages of somatic embryos (Globular, heart-shaped, torpedo, cotyledonary) was directly obtained and further recurrent embryogenesis also obtained upon prolonged incubation in induction media. Addition of polyethylene glycol (50 mgL^{-1}) significantly improved the embryos maturation in MS supplemented with and 3% sucrose. The regeneration in MS medium supplemented with BAP (0.5 mgL^{-1}), NAA (0.2 mgL^{-1}), casein hydrolysate (100 mgL^{-1}) and 3% sucrose. High plant regeneration frequency and intensity of somatic embryos (58.5%) obtained. Plant maturation on MS medium supplemented with BAP 2.0 mgL^{-1} with 2% sucrose. The rooted plants was successfully acclimatize in the greenhouse with a survival rate of 85%. This somatic embryogenesis protocol developed would be helpful in establishment of genetically modified guava aimed at seedlessness, increased shelf life and wilt disease.

Key words: Somatic embryogenesis, zygotic embryo, recurrent, maturation, acclimatization, induction, regeneration

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INTRODUCTION

Psidium guajava L. (Myrtaceae) generally known as guava 'the apple of tropics' consider one of the most important fruit of high nutraceutical value in international trade and domestic economy of several tropical and subtropical countries (Chandra et al., 2010; Kamle et al., 2013). Guava contains four times higher vitamin C than any other fruits. High concentration of pectin in guava plays noteworthy role in cholesterol drop and thus losses the cardiovascular diseases (Singh et al., 2005). Guava is low in calories and fats but carry several antioxidant poly-phenolic and flavonoid compounds that play a crucial role in prevention of many important diseases like cancer, aging, infections, etc. Scientific studies suggest that regular intake of vitamin C helps human body develop resistance against infectious agents and scavenge cancer (Yadava, 1994). Guava has a vast gene pool and genetically diverse in nature. In general, guava propagates through seeds but seedlings are variable in both plant and fruit characteristics due to heterozygous nature of crop (Saw *et al.,* 1991). Conventional guava propagation employed through cutting, grafting, stooling or air layering met with limited success in development of disease resistant cultivars due to the narrow genetic variability present in the guava germplasm (Chandra *et al.,* 2004). Therefore, the best alternative on the road to guava genetic improvement is to resort somatic embryogenesis that help in mass propagation of elite and new genotypes in shorter periods.

Somatic embryogenesis (SE) is a perfect confrontational system in which every somatic cells under special circumstances build up into embryos and passing through various developmental stages ultimately give rise to a complete plantlet formation (Arnold et al., 2002). SE is one form of asexual reproduction by which somatic cells, under suitable induction conditions, undergo a complete genome shift and embark into a new developmental pathway ending in the formation of asexual embryos morphologically identical to their zygotic counterparts (Zimmerman, 1993; Schmidt et al., 1997; Arnold et al., 2002; Feher et al., 2003). During this unique developmental process, cells have to dedifferentiate, activate cell division, and reprogram their physiology, metabolism, and gene expression patterns (Yang and Zhang, 2010). Thus, SE is an ideal demonstration of plant totipotency, which represents how a somatic cells contain the essential genetic draft for complete plant development without fertilization (Zimmerman, 1993). In SE, direct root and shoot induction occurs, thus the plantlet could be multiplied and acclimatize fast. 2, 4 –D is a master regulator of somatic embryogenesis induction in an array of crops (Arnold et al., 2002; Van Winkle et al., 2003; Feng et al., 2009) and a stress-inducing herbicide which invokes embryogenic efficiency as an adaptation mechanism (Feher et al., 2003; Feher et al., 2005). The formation of an embryogenic cell is related to nuclear DNA methylation in the presence of 2, 4-D (Leljak– Levanic et al., 2004). Thus, the dynamic changes occurs in chromatin structure induced by DNA methylation under the influence of 2, 4-D lead to genomic reprogramming in somatic cells. This modification might affect hormone responsive genes required for acquisition of embryogenic competence. The somatic cells within a plant contain the genetic information necessary to form a complete and functional plant (Feher et al., 2003). Even though they are not exactly the same as zygotic embryos (Dodeman et al., 1997). There are several reports on regeneration of normal plants from somatic embryos in different species. In SE chances of the occurrence of chimera is less thus a more efficient way of regeneration of the transformed plants. Besides, plants in which somatic embryogenesis was obtained, secondary or recurrent embryogenesis was often present, a phenomenon in which new embryos emerges from somatic embryos (Raemarkers et al., 1995). Secondary somatic embryogenesis has many uses in biotechnology, such as the mass propagation of new plants, cryopreservation, genetic transformation and the induction of mutation (Litz and Gray, 1995; Raemarkers et al., 1995). Somatic embryogenesis in guava for biotechnological improvement programme has been reported by a various researchers (Akhtar et al., 2000; Chandra et al., 2004; Kosky *et al*. 2005; Rai et al., 2007; 2008; Saxena et al., 2007; Singh et al., 2007; Rai et al., 2009; Moura and Motoike, 2009; Akhtar, 2010; Kamle et al., 2013). Numerous problems encountered during the optimization of a high-efficiency protocol for induction of somatic embryogenesis from zygotic embryo explants of various cultivars of guava. Immature zygotic embryos utilized as the primary explants (Jaiswal and Litz, 1993) for the induction of somatic embryogenesis. The induction of somatic embryogenesis starting from mature and immature zygotic embryos has shown good results and efficiency in several plants of family Myrtaceae (Cruz *et al.,* 1990; Canhoto and Cruz, 1996; Canhoto *et al.,* 1999, Guerra *et al.,* 1997). The advancement of efficient protocols for plant regeneration is one of the

significant essentials for starting genetic transformation system in guava. In this research paper, we report an efficient recurrent regeneration via somatic embryogenesis in guava that can potentially pave the way in development of genetically modified guava with improved traits like seedlessness, increased shelf life and *Fusarium* wilt disease.

MATERIAL AND METHODS

Preparation Of Explant

Four commercial cultivars of guava (Allahabad Safeda, Lalit, Sardar (L-49) and Shweta) were sampled from the Orchard Block of Central Institute for Subtropical Horticulture, Lucknow, India. To ensure self-fertilization, un-opened flower buds were bagged and the 70 days post-anthesis immature fruits were collected. Immature fruits were brought to the laboratory and surface sterilized with water and then soaked in $HgCl_2$ (0.1%) for 2-3 minutes containing one drop of Tween-20 for surface sterilization, washed thoroughly the fruits with sterile distilled water in order to remove traces of mercuric chloride. Flame sterilized the guava fruit using 90% ethanol for few seconds under laminar airflow. The sterilized fruit was bisected followed by the removal of immature seeds for zygotic embryos excision under aseptic condition. Immature zygotic embryos were chose as starter explants for inducing somatic embryogenesis. Dissection of zygotic embryos was isolated as described by Akhtar (2010).

Induction Of Somatic Embryogenesis

Excised immature zygotic embryos isolated from fruits of guava 70 (Days after Pollination) days was inoculated on petridish containing Murashigue and Skoog (1962) medium supplemented with 2, 4-D ($2mgL^{-1}$), L-glutamine ($400\ mgL^{-1}$), malt-extract ($500\ mgL^{-1}$) and 6% sucrose. The pH of the medium adjusts to 5.8±0.2. Each petridish contain ten zygotic embryos and incubate at 25 ± 2 °C in dark for 4-6 weeks in induction medium. Globular embryos appeared after 6-8 weeks of inoculation, which formed bipolar-shape (Fig1). Somatic embryos subculture at every 4 weeks up to 12 weeks incubation in dark. Subculturing on induction medium devoid of hormone resulted in repetitive embryogenesis.

Figure 1 - Different developmental stages of somatic embryo induction in *Psidium guajava* L. a) 70 day zygotic embryo as explant source, b) Pre embryogenic mass of cells (PEMCs), c) protuberance of transparent PEMC in clump, d) globular & heart shaped embryo, e) elongated embryo, f) torpedo-shaped embryos, g) cordiform embryo, h) embryo proliferation, i) recurrent embryogenesis.

Evaluation Of Somatic Embryogenesis

1). Frequency of Embryogenesis (FE): The proportion of explants that showed at least one somatic embryo at any stage of development, out of the total number of explants inoculated in a particular treatment. FE (as a percentage) were calculate as follows:

$$FE\ (\%) = \frac{\textit{Sum of the number of explants showing at least one Somatic embryo per treatment}}{\textit{Total number of explants inoculated per treatment}} \times 100$$

2). Intensity of Embryogenesis (IE): The mean number of somatic embryos produced per responsive explants in a particular treatment. The average number of somatic embryos produced per culture (ANEPC) represents IE and calculate as follows:

$$IE\ (ANEPC) = \frac{\textit{Sum of total number of somatic embryos produced in each explants in a particular treatment}}{\textit{Total number of explants showing at least one Somatic embryo per treatment}} \times 100$$

Histological Analysis

Samples examine after 21 days of culture for the histo-chemical analysis of somatic embryos. The tissue samples were fix in FAA in ratio of 5: 5: 90 (5ml 40% formaldehyde: 5 ml Glacial Acetic acid: 90 ml 70% ethanol). Ethanol dilution varies according to the soft tissue. After dehydration in an alcohol series (70% for 60 min, 95% for 30 min and 100% for 15 min') the samples were subsequently embedded in paraffin wax at 58^0C. Serial 8 µm thick sections were cut using a rotatory microtome (Leica, RM2125) using standard procedure and stained with deal field hematoxylin, fushin-astra blue combination and mounted in Canada balsam oil and photograph under a light microscope Leitz DMRB photomicroscope (Leica, Wetzler, Germany).

Maturation, Plantlet Regeneration and Acclimatization

For maturation, somatic embryos were placed in a MS basal medium containing PEG (50 mgL-1) and 3% sucrose.

Somatic embryos isolated from the clumps were placed onto the ½ MS medium supplemented with BAP (0.5 mgL^{-1}) and NAA (0.2 mgL^{-1}), casein hydrolysate (100 mgL^{-1}) containing 3% sucrose for plantlet regeneration for 2-3 weeks. Later, plantlets transfer to ½ MS medium supplemented with BAP (2.0 mgL^{-1}) and 3% sucrose for elongation and shoot proliferation. Four weeks after cultured into proliferation medium plantlets reached size of 5-8cm along with developed root and shoot system, transfer plantlets to glass-bottles containing coco-peat moistened with ½ MS plant salt mixture and covered with a plastic sheet for acclimatization. After a week made few perforations in the plastic sheet for aeration to plantlets and then shifted to green house at 25 ± 2°C. In greenhouse, transfer plants into pots filled with sand: soil: FYM (1:1:1) and remove the polybags and let plants acclimatize completely too external environment. The plants grown in growth chambers (24°C±2 °C and 16 h photoperiod with 80% relative humidity). Now, transfer plants into the field.

Statistical Analyses

All experiments was repeat at least three-times. All results were subject to two way analysis of variance (ANOVA) to detect significant variation at CD ± 0.05. All cultures were observed daily and changes in the zygotic embryo explants were noted after each week. Embryogenic responses were usually record 8–10 days after culture initiation. The efficacy of the various treatments calculated using two different embryogenic parameters as described by Akhtar (2010). Data were analyzed for two-way analysis of variance (SPSS version 16). Significance of difference ($P<0.05$) was detected.

RESULTS

In the present study, an efficient and highly reproducible system for *Psidium guajava* L. somatic embryogenesis was developed (Fig; 1a-i). **Somatic embryogenesis induction:** Somatic embryogenesis induction achieved using the immature zygotic embryo (70 DAP) as explant on MS medium, containing 2, 4-D (2.0 mgL^{-1}) and L-glutamine (400 mgL^{-1}) and 6% (w/v) sucrose. Direct embryogenesis observed after 4 weeks post culture induction and later pre-embryogenic masses of cells (PEMC) developed directly from the immature zygotic embryo explants in all treatment except in the control after 4 weeks (Fig1). The addition of 2, 4-D (2 mgL^{-1}) with L-glutamine (400 mgL^{-1}) significantly increased formation of somatic embryos. Removal of auxin or decreasing concentration of auxin led to formation of repetitive embryogenesis (Chandra et al., 2004). After 6 weeks of incubation in induction, medium, granular

and shiny masses of cellular embryos observed (Fig1). This process continues in subsequent sub culturing leading to the successive formation of somatic embryos that emerged from the peripheral areas of the callus mass after 8-weeks of culture incubation. Various developmental stages observed of somatic embryos first appeared as clusters of globular embryos, further into heart-shaped or cordiform shape then torpedo-shaped and finally into cotyledonary stage to form a complete plantlet.

Maturation, Proliferation and Regeneration of Somatic embryos: Maturation is a key phase between embryo development and germination. To achieve maturation somatic embryos transferred to MS medium supplemented with polyethylene glycol (50 mgL^{-1}) and 3% sucrose produced 83.64% cotyledonary embryos into maximum 74.28% matured embryos. For regeneration, transfer to 1/2 strength MS medium fortified with BAP (0.5 mgL^{-1}), NAA (0.2 mgL^{-1}) and casein hydrolysate (100 mgL^{-1}) containing 3% sucrose for 2-3 weeks (Fig2). For conversion of somatic embryos into plantlets, the somatic embryos now exposed to 16/8 hr. photoperiod. This is the most critical stage for establishment of any successful regeneration protocol depends on conversion efficiency. Percentage of conversion record after 3 weeks (Fig4). High plant regeneration frequency and intensity of somatic embryos (58.5%) obtained.

Figure 2 - a) Different stages of plantlet regeneration in guava through somatic embryogenesis pathway a) Maturation of somatic embryo b) plantlet germination c) rooting in plant d) elongation of plantlets e) shoot proliferation f) plant maturation g) plant in coco-peat h) hardening in poly-house i) Plants in soil-pots.

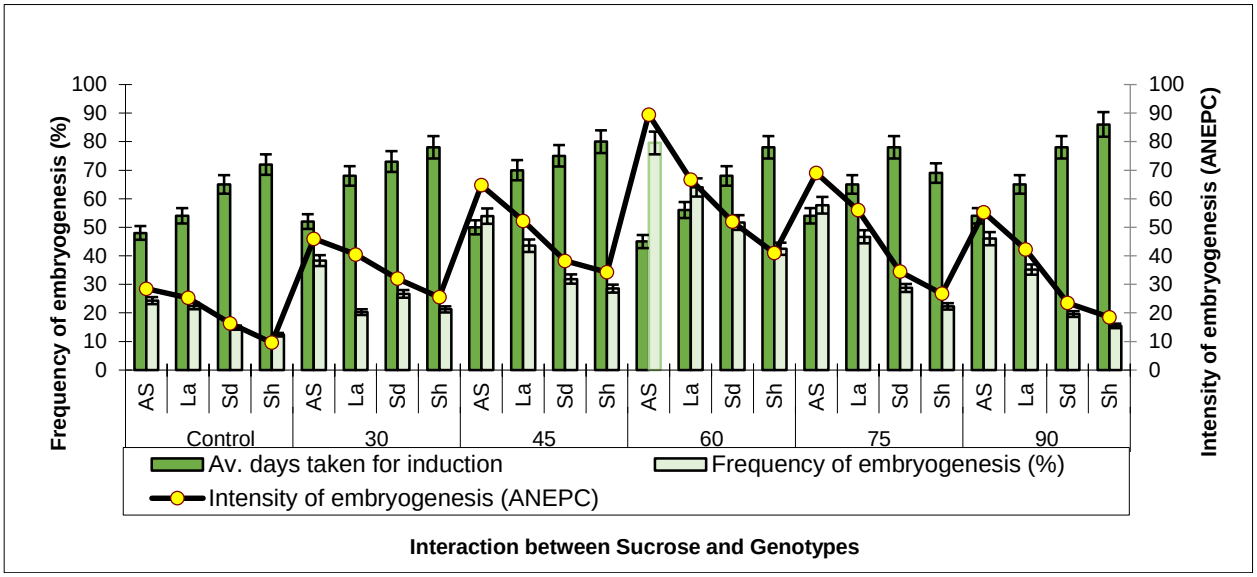

Figure 3 - Effect of sucrose and its interactive effect on the four genotypes of guava. Represents maximum embryogenesis induction in terms of frequency and intensity with reference to the four genotypes

Embryogenic Potential of Genotypes:

Embryogenic potential of four guava genotypes viz. Allahabad Safeda, Lalit, Sardar (L-49) and Shweta was estimated on the maximum embryogenesis induction, maturation, plantlet regeneration was recorded in Allahabad Safeda which accounts for 32.5% plantlet regenerated followed by Lalit in which 23.3% regeneration was recorded. Least plantlet regeneration was recorded in Sardar (L-49) and Shweta which accounts for only 15.3%. Comparative overall embryogenic potential we reported Allahabad Safeda as the most promising genotype of guava in terms of induction, maturation, proliferation via somatic embryogenesis through tissue culture as thus, could potentially use further in establishment of genetic transformation protocol.

Effect of carrier substrate in acclimatization of Plantlets:

Four different carrier substrates blend with bioagent *Trichoderma harzianum* was used *viz*., perlite and *T. harzianum*, Vermiculite + *T. harzianum* and cocopeat + *T. harzianum* and Sand + Soil + FYM for acclimatization of guava plantlets. Maximum survival of plants (data not shown) in substrate Coco-peat + *T. harzianum* (85.40 cm) was obtained and followed by Sand + Soil + FYM (75.20 cm). Later the genetic fidelity analysis of all plants was confirm using Molecular markers.

DISCUSSION

Advancement of efficient protocol for plant regeneration is crucial for establishment of genetic improvement. An orderly series of characteristic embryogenesis system have developed in guava under present investigation. Immature zygotic embryos have proved to be an efficient regenerative tissue for the many recalcitrant tropical fruit species. Zygotic embryos are made up of PEDC's (pre-embryogenic determined cells) in which, cells have the embryogenic competence and could easily induced to follow the embryogenic pathways (Sharp et al., 1980). Our results revealed that immature zygotic embryo (70 days post-anthesis) undergo rapid induction of somatic embryogenesis in terms of highest frequency and intensity over immature mesocarp. Our results are also in accordance with the results of Vilchez et al., 2000; Rai et al., 2007, Kosky et al., 2005 and Akhtar, 2010. Immature zygotic embryos have proved to be a potent regenerative tissue for the many recalcitrant tropical fruit species. 2, 4-D is consider as one of the main inductive factors for somatic embryogenesis (Ammirato, 1993) and is well established for many species (Dunstan *et al.*, 1995) including several members of the Myrtaceae family (Canhoto *et al.*, 1998). Among other Myrtaceae plants in which somatic embryogenesis has been induced *Feijoa sellowiana* (pineapple guava) and *Myrtle communis* where somatic embryos differentiate mostly from mature zygotic embryo (Canhoto and

Cruz, 1996; Canhoto *et al.*, 1999). Our results revealed that 2 mgL^{-1} 2, 4 - D was the best concentration inducing somatic embryogenesis in *Psidium guajava* L. among all the four genotypes tested in terms of highest frequency and intensity of embryogenesis (Fig3). Conversely, Akhtar, (2010) and Rai *et al.* (2007) specified that 1.0 mg/l 2, 4-D concentration found best for somatic embryogenesis in guava. The lower doses of 2, 4-D (1.0 mg/l) was recommended to induce maximum embryogenesis with increase in concentration of 2, 4-D (>1.0 mg/l) was increased the frequency and intensity of somatic embryo diminution. In case of guava (*Psidium guajava* L.) genotypes exposure of 2, 4-D in continuous treatment up to 4-6 weeks can lead to sufficient embryogenesis induction and with less morphogenic abnormality. Similarly, Moura and Motoike, (2009) reported that 2, 4-D alone cannot induce somatic embryogenesis in guava cv. Paluma. The addition of 2, 4-D (2.0 mgL^{-1}) with L-glutamine (400 mgL^{-1}) significantly increased formation of somatic embryos. Furthermore, combination of 2, 4-D and glutamine induced high production of SEs (Chandra et al., 2004). Fortification of an organic nitrogen form has a positive influence on somatic embryogenesis (Khlifi and Tremblay, 1995; Carman *et al*., 1996; Garin *et al*., 2000; Robichaud *et al*., 2004; Chandra *et al.*, 2004; Kosky *et al.*, 2005; Biswas *et al.*, 2005; Rai et *al*., 2007; Zouine and Hadrami, 2007; Gerdakaneh et al., 2011). L-Glutamine had shown improved somatic embryogenesis in both monocotyledons and dicotyledons (Dhir et al., 1991; Yin et al., 1993; Ke et al., 1996; Vani and Reddy et al., 1996; Srivastava and Chawla 2001). The addition of L-glutamine (400 mgL^{-1}) in media influenced efficient somatic embryogenesis formation in *P. guajava*. Similarly, several reports confirmed that formation of direct SEs was enhanced by glutamine (Baskaran and Jayabalan 2009; Deo et al., 2010; Gerdakaneh et al., 2011). After 4-5 weeks in induction medium small clusters of pre-embryogenic mass cells (PEMC) of cytoplasm rich cells were visible (Fig.1).

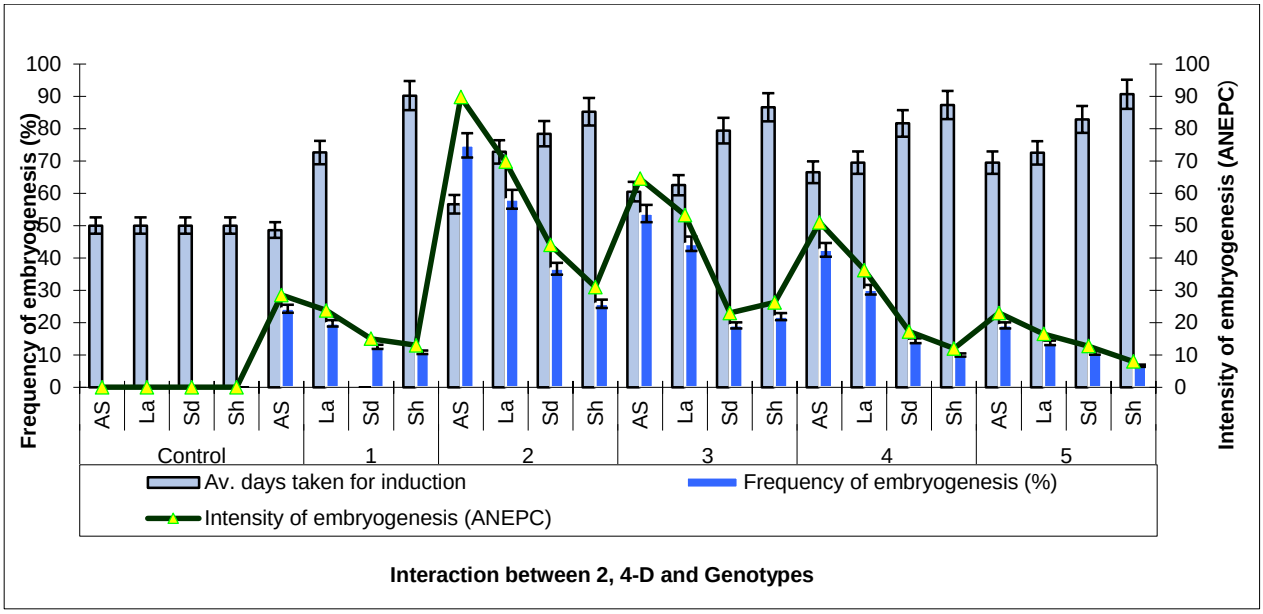

Figure 4 - Effect of 2, 4-D and its interactive effect on the four genotypes of guava. Represents maximum embryogenesis induction in terms of frequency and intensity with reference to the four genotypes

Since direct somatic embryogenesis had often seen in hypocotyls region in several species including Myrtaceae, it is likely that PEMC originate from hypocotyls region. *In vitro* growth and development of tissues requires a carbon source because of heterotrophic mode of nutrition. Sucrose is the most conjoint carbohydrate source used in the plant tissue culture and present dominantly in the phloem (Zimmerman and Zeigler, 1975). Research claimed that carbohydrate concentration affects greatly in the formation of somatic embryos (Lou and Kako, 1995; Luo et al., 1996; Nakagawa et al., 2001). Similarly, enhanced induction of somatic embryos achieve by adding high concentration of sucrose to the culture medium in cucumber (Luo et al., 1996), iris (Jehan et al., 1994) and melon (Nakagawa et al., 2001). Previously, 5% sucrose in guava (Kosky et al., 2005; Rai et al., 2007) found best for induction of somatic embryogenesis. Our results revealed that 6% sucrose found best for inducing maximum embryogenesis in

guava with less time supports our previous report by Chandra et al. (2004) in mesocarp tissue of guava. However, at lower concentration (2%, 3%, and 4%) of sucrose not efficiently induce somatic embryogenesis (Rai et al., 2007). High carbohydrate content in culture medium found increased somatic embryogenesis in many plant species (Luo et al., 1996; Rai et. al., 2009). However, it is a common fact that the role of high sucrose concentration in somatic embryogenesis may influence the cell osmolarity (May and Trigiano, 1991). Therefore, the role of sucrose in the present study interpreted as both nutritional and osmotic regulatory functions of the carbohydrate which creates a stress that can lead to embryogenesis induction. Therefore, it is recommended that, osmotic effect of sucrose may cause normal development of somatic embryos. Genotype is the most important factor influencing embryogenic response and SE is highly genotype dependent process. Some genotypes are highly regenerative where as others are recalcitrant especially in case woody tree species. Variability in the induction and frequency of the obtained embryos observed among different species of genera and within the cultivars. Considerable variations in embryogenic capacity observed between individuals of one cultivar or species. Genotype-dependent embryogenic capability was widely reported. In some genotypes, genes involved in plant regeneration may be suppressed due to inappropriate culture medium and culture condition. According to the present findings, we claimed Allahabad Safeda as found best genotype for establishing successful somatic embryogenesis. Individual genotypes may also have unique requirements for optimal regeneration capacity. Such genotypic differences in embryogenic capacity might reflect current differences in the ability to activate key elements in the embryogenic pathway (Merkle et al., 1995).

The use of PEG as an osmoticum in maturation media for somatic embryos has proven effective in increasing germination and conversion (Capuana and Debergh, 1997). Polyethylene glycol reported to improve the quality of somatic embryos by promoting normal differentiation of the embryonic shoot and root (Stasolla et al., 2003). PEG molecules are too large to move through the cell wall and do not cause plasmolysis. Non-plasmolyzing osmotic are more effective in promoting somatic embryo maturation (Linossier et al., 1997; Walker and Parrott, 2001). In our findings conversion of guava somatic embryos into plantlets achieve successfully on 1/2 MS medium fortified with BAP (0.5 mgL^{-1}) and NAA (0.2 mgL^{-1}), casein hydrolysate (100 mgL^{-1}) containing 3% sucrose results in 52.36% plantlet regeneration. Similarly, Rai et al. (2009) also reported the use of PEG in guava embryo maturation. Carrier substrate plays a major role in hardening and acclimatization of tissue culture raised plantlets. This is the most critical stage to look after the tissue-cultured plantlets before planting into field as the maximum plants dies during hardening stage. Coco peat as carrier substrates have marked effect on the plant height, number of leaves, number of roots, length of roots, number of shoots/explant and their survival per cent. This indicated that coco peat was effective carrier substrate for acclimatization. Coco peat absorb water and release gradually. It provides sufficient aeration for development of healthy roots. However, in case of guava successful acclimatization of rooted shoots reported in peat-based compost and autoclaved FYM + Sand (1:1) by various workers (Amin and Jaiswal, 1988; Prakash 1992; Prakash and Tiwari, 1993; 1996). Hardened plantlets shifted in field where they are growing vigorously. Mishra et al. (2004) have found that micro propagated plantlets of Bael can be acclimatized under 50 % shade and high relative humidity (70%). Our results also corroborated with Mishra et al. (2007) that the survival of rooted plantlets of *Psidium guajava* L. was high in coco peat substrate. Maximum survival of plants (84%) noticed on autoclaved coconut husk fortified with ½ MS plant salt mixture. The transfer of tissue culture raised plantlets to field conditions is one of the most critical factors that cause higher production costs. High mortality rate observed when plants transfer to field conditions as cultured plants had non-functional stomata, weak root system and poorly developed cuticle. In order to increase growth and reduce mortality in plantlets at the acclimatization stage, research focused on the control of the environmental conditions (both physical and chemical). Addition of bio agents such as *Trichoderma harzianum* in the carrier substrate not only enhances survival of the plant but it also augmented development of fibrous root and vigor of the plant. Fortification of *T. harzianum* in coco peat found best results for increasing survival and growth of guava plants. Thus, bio hardening by employing bioagents promote growth and encourage mutual association that the plantlets may oppose upon transplantation to natural conditions (Varma and Schuepp, 1996; Hernandez *et al*., 1999; Sahay and Varma 1999; Pandey *et al*., 2000). Bioagents such as *Glomus aggregatum*, *T. harzianum* and *Piriformospora indica* found very effective in improving the establishment frequency.

Bio hardening is an emerging dimension of tissue culture technique for implementation of successful hardening and acclimatization of plants (Lovato et al., 1996; Nowak, 1998; Sahay and Varma, 1999; Pandey *et al*., 2000; Rai, 2001; Srivastava *et al.*, 2002).

CONCLUSION

In this research paper, we established a highly efficient and regenerative protocol for recurrent somatic embryogenesis in guava genotypes Allahabad Safeda. This consistent repetitive embryogenesis system will provide a reliable source for genetic improvement of *Psidium guajava* L. and can potentially useful in further expansion of genetically modified guava with enhanced aroma, better shelf life, resistance to wilt and seedlessness.

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