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Nucellar embryogenesis and plantlet regeneration in monoembryonic and polyembryonic mango (*Mangifera indica* L.) cultivars

Manish Shukla, Khair Tuwair Al-Busaidi*, Ghaya Sultan Al-Blushi, Abdullah Hamed Al-Burashdi, Hamoud Darwish Al-Hasani, Mohammed Hamed Al-Jabri and Basem Saif AL-Kalbani

Plant Production Research Centre, Fruit Crop Research Section, Directorate General of Agriculture and Livestock Research, Ministry of Agriculture and Fisheries, P. O. Box 50, Seeb, Postal Code 121, Sultanate of Oman.

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Biotic and abiotic stress particularly fungal diseases and salinity are major challenges facing mango cultivations in Oman. Micropropagation technique for multiplying disease resistant and salinity tolerant elite cultivars could be utilized to replace dead and infected plants in mango orchards but standardize *in-vitro* regeneration protocol via somatic embryogenesis is prerequisite. Nucellar tissues from immature mango fruits of monoembryonic cultivars Alphonso, Amrapali, Dashehari and Zafran, and polyembryonic cultivars Carabao and Turpentine were used as explants to induce somatic embryogenesis plantlets. Gamborg's B5 macronutrients, Murashige and Skoog micronutrients, iron source, vitamins and organics were used as standard basal media for all types of media used at each stage of somatic embryo development and regeneration. Induction medium 2 containing 2 mg/l 2,4-Dichlorophenoxyacetic acid and 0.5 mg/l 6-Benzylaminopurine were induced highest percentage of primary somatic embryos for Alphonso (22.08%) while induction medium 3 having 1 mg/l 2,4-Dichlorophenoxyacetic acid with sucrose 60 gm/l and induction medium 1 containing 1 mg/l 2,4-Dichlorophenoxyacetic acid and 0.25 mg/l 6-Benzylaminopurine induced highest percentage of primary somatic embryos in Carabao (29.17%) and Turpentine (42.71%) respectively. Maximum somatic embryo germination were achieved in germination medium 2 containing 0.1 mg/l Indole-3-acetic acid and 0.5 mg/l Gibberellic acid for Alphonso (7.34%) and Turpentine (3.34%) while for Carabao (18.59%) in germination medium 1 which does not contain any plant growth regulators. Germinated plantlets are surviving well in ex-vitro conditions after 4 months of transfer to greenhouse and survival rate of 66.66% for Alphonso, 26.68% for Carabao and 49.16% for Turpentine was obtained.

Key words: Mango, nucellar embryogenesis, monoembryonic, polyembryonic, somatic embryo, germination, survival rate.

INTRODUCTION

Mango (*Mangifera indica* L.) a popular fruit of tropical and subtropical region belongs to the dicotyledonous family

Anacardiaceae. It is widely cultivated in this region due to its delicious taste, high nutritive value, varietal diversity and higher demand for food processing industry in many parts of the world. Mango was introduced to the Sultanate of Oman from the Indian subcontinent and East Africa over hundreds of years ago. Now, it is fourth most important fruit crop after date palm (*Phoenix dactylifera* L.), banana (*Musa* spp) and lime (*Citrus aurantifolia* Swingle) in terms of area (148,514 ha) and production (14257 ton) (MAF, 2015). Mango cultivars are either monoembryonic or Indian type, or polyembryonic or Southeast Asia type (Mukherjee and Litz, 1977). Seedlings of polyembryonic cultivars are true-to-type unlike monoembryonic cultivars which are genetically not homogenous. Evaluation of mango genotypes resulted into the recommendation of 16 good quality and high yielding cultivars for cultivation in Oman and majority of them are monoembryonic such as Alphonso, Amrapali, Dashehari, and Zafran of Indian cultivars (MAF, 1989; 1990, 1991, 1992). These cultivars are semi-vigorous with semi erect tree shape and early ripening (April to mid-may) with average yield ranged from 45 to 100 kg /tree and fruit quality of 17% total soluble solids (MAF, 1989, 1990). Monoembryonic cultivars are mainly propagated by vegetative method as in Ambalavi, where de-novo adventives embryony is lacking in the nucellus of ovules to get true-to-type plant (Chaturvedi et al., 2004). Grafting and air layering are still a popular practice in propagation of mango monoembryonic cultivars worldwide which is expensive and time-consuming. In Oman, heterozygous rootstock seedlings of local strain named "Omani mango" have been used to propagate recommended monoembryonic mango cultivars resulting in non-uniform trees.

The most important challenge for mango growers in Oman is mango sudden decline disease, caused by the fungus *Ceratocystis fimbriata*, introduced recently and destroyed about 60% of the mango orchards (Al Adawi et al., 2003, 2006; Al Subhi et al., 2006; Galdino et al., 2016). Ministry of Agriculture and Fisheries has also introduced polyembryonic cultivars considering resistant to mango sudden decline disease but the problem was not resolved effectively. Therefore, there is an imperative to find an effective and expeditious method of mango propagation such as *in vitro* regeneration via nucellar organogenesis (Hartmann et al., 1997). Nucellar tissues from ovules of immature mango fruits were used to induce nucellar embryogenesis and this is a widely accepted micropropagation technique for mango tissue culture to obtain true-to-type plants. Somatic embryo formation and plantlet regeneration can be achieved from induced callus of nucellar explants of mango fruits (Chaturvedi et al., 2004; Laxmi et al., 1999). This technique has been used successfully by many researchers (Al-Busaidi et al., 2016; Chaturvedi et al., 2004; Lad et al., 1997; Litz et al., 1984; Malabadi et al., 2011; Nower, 2013; Pateña and Barba, 2011) to multiply monoembryonic and polyembryonic mango cultivars but the response is genotype dependent (Ara et al., 2000; Litz, 1984; Litz et al., 1982). Standardize protocol for somatic embryogenesis and plantlet regeneration is foremost required to obtain true-to-type and disease free mango plantlets in large numbers. Therefore, the present study aims to develop an efficient and reliable protocol for nucellar embryogenesis and plantlet regeneration for *M. indica* cultivars Alphonso, Amrapali, Dashehari, Zafran, Carabao and Turpentine.

MATERIALS AND METHODS

Explants source and culture medium

Nucellar tissues from ovular halves of immature mango fruits are used as explants for callus initiation and primary somatic embryo induction for developing tissue culture protocol of Mango (*M. indica* L.) monoembryonic cultivars of Alphonso, Amrapali, Dashehari and Zafran and polyembryonic cultivar of Carabao and Turpentine. Immature mango fruits of size 2.0 to 3.5 cm (small size) and 3.5 to 5.0 cm (medium size) in length (approx. 30 to 40 days of pollination) were collected from *Mangifera indica* L. cv Alphonso, Amrapali, Dashehari, Zafran, Carabao and Turpentine (but only small size fruits for Turpentine) trees from mango gene bank at Wadi Hebi Research Farm, Ministry of Agriculture and Fisheries, Sultanate of Oman. Processing of fruits and all the subsequent experiments were conducted at the Tissue Culture Unit, Directorate General of Agriculture and Livestock Research, Ar Rumais, Oman during the year 2014 to 2016. Mango fruits of two sizes were processed separately and cultured on same day after collection. Fruits were washed thoroughly with soap and running tap water for few minutes. Then surface sterilization was done by sequential treatment with 70% ethanol (v/v) (10 min), 0.1% $HgCl_2$ (w/v) (5 min) and finally rinsed with sterile double distilled water 3 to 4 times under laminar flow hood (Al-Busaidi et al., 2016). Culture media with different compositions of salts, organics and plant growth regulators (Table 1) were evaluated for each stage of nucellar embryogenesis and plantlets regeneration to achieve tissue culture raised mango plantlets in 6 mango cultivars. Modified basal media composition containing Gamborg's B5 macronutrients (Gamborg, 1970), MS micronutrients, Fe-EDTA, vitamins and organics (Murashige and Skoog, 1962) were used in all different media types, that is, induction media (IND), proliferation media (P), maturation media (M) and germination media (GM) for somatic embryo induction, proliferation, maturation and germination stages respectively. Various concentrations of plant growth regulators, L-glutamine, malt extract, L-ascorbic acid, PVP (Polyvinylpyrrolidone), sucrose, phytagel and activated charcoal were also added along with basal media in different media types (IND1, IND2, IND3, P, M, GM1, GM2, GM3, GM4 and GM5) based on their requirements at different developmental stages of somatic embryogenesis (Table 1). pH was adjusted to 5.8 and solidifying agent phytagel was added at concentration 2.5 gm/l in all media types just before sterilization of media.

*Corresponding author. E-mail: abouanfalalbusaidi@yahoo.com. Tel: +968 26893131.

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Table 1. Media compositions for nucellar embryogenesis and plant regeneration in 6 Mango cultivars viz. Alphonso, Amrapali, Dashehari, Zafran, Carabao and Turpentine.

Components	Induction media			Proliferation medium	Maturation medium	Germination media				
	IND1	IND2	IND3	P	M	GM1	GM2	GM3	GM4	GM5
B5 Macronutrients	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	Half strength
MS Micronutrients	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated
MS Fe-EDTA	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated
MS Vitamins	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated	As formulated
2,4-D (mg/l)	1	2	1	-	-	-	-	-	-	-
BAP (mg/l)	0.25 l	0.50	-	-	-	-	-	0.5 l	-	-
IAA	-	-	-	-	100 µg/l	-	0.1 mg/l	0.1 mg/l	0.1 mg/l	-
NAA	-	-	-	-	-	-	-	-	-	-
Kinetin	-	-	-	-	-	-	-	-	-	-
GA3 (mg/l)	-	-	-	-	-	-	0.5	0.5	-	-
ABA	-	-	-	-	100 µg/l	-	-	-	-	-
PEG (mg/l)	-	-	-	-	100	-	-	-	-	-
L-Glutamine (mg/l)	400	400	400	400	-	400	400	400	-	400
Malt extract (mg/l)	500	500	500	500	-	500	500	500	500	500
L-Ascorbic acid (mg/l)	100	150	100	100	-	-	-	-	-	-
PVP (mg/l)	100	50	100	100	-	-	-	-	-	-
Sucrose (g/l)	30	30	60	30	30	20	20	20	20	40
Phytagel (mg/l)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Activated charcoal (mg/l)	-	-	-	-	-	50	50	50	50	-
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8

2,4-D, 2,4-Dichlorophenoxyacetic acid; ABA, Abscisic acid; BAP, 6-Benzylaminopurine; GA3, Gibberellic acid; IAA, Indole-3-acetic acid; NAA, 1-Naphthaleneacetic acid; PEG, *Polyethylene glycol;* PVP, Polyvinylpyrrolidone; MS, Murashige and Skoog media; B5, Gamborg's media; IND, Induction media; P, Proliferation media; M, Maturation media; GM, Germination media.

Callus induction, somatic embryo production and proliferation

The sterilized immature mango fruits of both small and medium size from all 6 cultivars were opened under laminar flow hood and intact ovule cut longitudinally into two halves. After removing the zygotic embryo part carefully, the ovular halves having nucellar tissues were cultured in GA-7 Magenta plant culture vessels containing induction media IND1, IND2 and IND3 under the aseptic conditions and incubated at 25 ± 2°C, 60% relative humidity (RH) in dark conditions for induction of somatic embryogenesis. Nucellar tissues from ovules of immature mango fruits are preferred explants source to induce nucellar embryogenesis and *in-vitro* regeneration for monoembryonic and polyembryonic mango cultivars (Chaturvedi et al., 2004; Laxmi et al., 1999; Malabadi et al., 2011). Based on previous reports and our preliminary experiments (Al-Busaidi et al., 2016) three different media compositions were used in this study to evaluate the response of mango cultivars and size of explants to different media compositions. Frequent sub-culturing was performed initially to reduce the explants browning due to phenolics exudation. Explants browning in post culture stages are one of the major hurdles in mango tissue culture experiments and several strategies were developed for prevention of explants browning effectively. L-Ascorbic

acid (100 mg/l) and 0.3% PVP were added in the induction (IND1, IND2 and IND3) and proliferation (P) media to control explants browning, along with frequent sub-culturing of explants initially and incubation of nucellar cultures in dark conditions to minimize the effects of phenolic exudations (Ara et al., 2000; Litz, 2003).

The numbers of explants showing callus initiation, pro-embryonic calli (PEC) and primary somatic embryos (PSEs) production were recorded after 60, 90, 120 and 150 days of culture from both size fruit explants of each cultivar and percentage callusing and PSE induction was calculated. PEC and initial globular shaped PSEs form all 6 cultivars were transferred to proliferation media (P) for proliferation. Primary somatic embryos (PSEs) induced in different induction media were transferred to proliferation media (P) for further proliferation and multiplication to produce large number of somatic embryos for maturation and germination. Production of large number of somatic embryos (SEs) from primary somatic embryo explants is called proliferation of somatic embryos. PSEs were sub-cultured onto proliferation media (P) to induce and production of secondary somatic embryos.

Maturation and germination of somatic embryos

Proliferated somatic embryos of heart stage, torpedo stage and cotyledonary stage were transferred to maturation media (M) (Table 1) for maturation. Maturation of somatic embryos is an important step to establish bipolarity in globular somatic embryos, minimize the fasciation and synchronization in the development of somatic embryos (Krishna and Singh, 2007; Singh et al., 2001; Thomas, 1999). The cultures at maturation stage were incubated for 4 weeks at 25 ± 2°C, 55 to 0 % RH in dark. Matured cotyledonary stage somatic embryos were transferred to different germination media GM1, GM2, GM3, GM4 and GM5 (Table 1) for germination of somatic embryos and plantlets formation. The cultures in germination media were incubated at 25±2°C temperature, 55 to 60% RH and 16 h photoperiod with 40 $mmolm^2S^{-1}$ light intensity (Al-Busaidi et al., 2016). Numbers of germinated somatic embryos (visible leaves, shoots and roots) were counted after 6 weeks of transfer to germination media and percentage germination was calculated for each germination media. Somatic embryos were considered to be germinated when plantlets have been formed and well developed shoots, roots and green leaves are visible and it is also important to mention that germinated embryos are considered as plantlets in this study.

Acclimatization and hardening

Germinated mango plantlets of different cultivars were transplanted into plastic plant trays containing mixture of sand, peatmoss and perlite in 1:1:0.5 ratios and transferred to the green house for acclimatization and hardening. These plantlets were irrigated on alternate days and covered with transparent polyethylene sheets initially for one month to maintain high humidity and low transpiration. Polythene covers were removed after one month and plants were transferred to medium size black plastic pots (30 × 15 cm dimensions) individually in the same sand mixture and nutrients and water were added by fertigation. Total number of mango plantlets transferred to the green house, their growth conditions and number of plants survived after each month of transfer were recorded for each cultivar to calculate the survival percentage in green house conditions.

Statistical analysis

All the experiments were performed as a completely randomized design (CRD). Each experiment was conducted minimum three times with four replicates and minimum 20 explants or 40 somatic embryos in each replicate. All the collected data were tabulated using Microsoft Excel software 2010 and statistically analyzed by the software GenStat Release 11.1 (VSN International, Hemel Hempstead, UK). Analysis of variance (ANOVA) was done for data analysis using GenStat Release 11.1 and significant differences between treatments were determined on the basis of Duncan's multiple range test at $p<0.05$.

RESULTS AND DISCUSSION

Effect of explants size on callus and primary somatic embryo induction in mango cultivars

Nucellar tissues from all 6 mango cultivars were callused and produced primary somatic embryos variously in different induction media. Although data for number of explants callused and number of explants produced primary somatic embryos (PSEs) were recorded for 60, 90, 120 and 150 days old cultures in induction media but highest callusing was observed in 90 days old culture and highest PSE induction was observed in 120 days old culture in both fruit size explants of all 6 mango cultivars studied (data not shown). Data from Tables 2 and 3 clearly indicates that maximum callusing and PSE induction was observed mainly in small size fruit (2.0 to 3.5 cm) explants in all 6 cultivars. Higher percentage of callus initiation was observed in small size fruit explants of Alphonso (43.4%), Dashehari (32.64%), Carabao (40.97%) and Turpentine (42.36%) while Amrapali (70.83%) and Zafran (36.81%) recorded maximum callusing percentage in medium size fruit (3.5 to 5.0 cm) explants after 90 days of culture in induction media (Table 2). Although higher callus initiation was observed in medium size explants of Amrapali and Zafran but these calluses were non-embryogenic in nature which does not form any primary somatic embryos (Tables 2 and 3).

Nucellar explants from small size fruits were performed extremely well in induction of PSEs in almost all cultivars except Dashehari, while medium size fruit explants of Carabao also induced the formation of PSEs in less numbers. Highest percentage of PSE induction was observed in Turpentine small size fruits explant (28.82%) while Carabao and Alphonso small size fruits explant induced 25 and 15.35% PSEs respectively (Table 3). Very less PSE induction was recorded in Amrapali and Zafran small size fruit explants while almost none of the explants induced PSE formation in Dashehari.

Induction of callusing was recorded in both small and medium size fruits nucellar explants of all 6 cultivars of mango but significantly ($P\geq0.05$) higher number of small size explants produced PSEs only in monoembryonic cultivar Alphonso and polyembryonic cultivars Carabao and Turpentine. Chaturvedi et al. (2004) reported 50% of explants showed induction of nucellar embryogenesis from nucellus tissue of young fruits of size 2.5 cm of monoembryonic cv Ambalavi and Malabadi et al. (2011) achieved induction of somatic embryos from nucellar tissues of 3.0 to 4.0 cm long immature mango fruits of cultivar Ratnagiri. Higher callusing percentage does not

Table 2. Effect of mango cultivars, fruit size and media composition on callus induction.

% Callus induction								
Fruit size (B)	**Small size fruit (2.0-3.5 cm**				**Medium size fruit (3.5-5.0 cm)**			
Media (A) Cultivar (C)	**IND1**	**IND2**	**IND3**	**Mean**	**IND1**	**IND2**	**IND3**	**Mean**
Alphonso	59.37^{abcd}	45.83^{abcdefgh}	25.00^{defgh}	43.4^{NS}	12.50^{h}	37.50^{bcdefgh}	25.00^{defgh}	25.00^{b}
Amrapali	31.25^{cdefgh}	46.87^{abcdefgh}	50.00^{abcdefg}	42.71	79.17^{a}	62.50^{abc}	70.83^{ab}	70.83^{a}
Dashehari	33.33^{cdefgh}	39.58^{bcdefgh}	25.00^{defgh}	32.64	32.50^{cdefgh}	15.62^{gh}	43.75 ^{bcdefgh}	30.62^{b}
Zafran	32.29^{cdefgh}	16.67fgh	44.37^{bcdefgh}	31.11	29.17 ^{cdefgh}	43.73 ^{bcdefgh}	37.50^{bcdefgh}	36.81^{b}
Carabao	39.58^{bcdefgh}	31.25^{cdefgh}	52.08^{abcdef}	40.97	21.87^{efgh}	15.62^{gh}	16.67^{fgh}	18.05^{b}
Turpentine	54.17^{abcde}	31.25 ^{cdefgh}	41.67^{bcdefgh}	42.36	-	-	-	-

Means within column followed by the same letter(s) were not significantly different according to Duncan's multiple range test at 5% level.

Table 3. Effect of mango cultivars, fruit size and media composition on primary somatic embryos (PSE) induction.

% Primary somatic embryo (PSE) induction								
Fruit size (B)	**% Primary somatic embryo (PSE)**				**Medium size fruit (3.5-5.0 cm)**			
Media (A) cultivar (C)	**IND1**	**IND2**	**IND3**	**Mean**	**IND1**	**IND2**	**IND3**	**Mean**
Alphonso	15.63^{cdefg}	22.08^{bcd}	8.33^{defg}	15.35^{b}	12.50^{cdefg}	0.00^{g}	0.00^{g}	4.17^{b}
Amrapali	0.00^{g}	6.25^{defg}	8.33^{defg}	4.86^{c}	0.00^{g}	0.00^{g}	3.13^{fg}	1.04^{b}
Dashehari	0.00^{g}	0.00^{g}	0.00^{g}	0.00^{c}	3.13^{fg}	0.00^{g}	0.00^{g}	1.04^{b}
Zafran	7.29^{defg}	2.08^{fg}	0.00^{g}	3.13^{c}	0.00^{g}	4.17^{efg}	0.00^{g}	1.39^{b}
Carabao	27.08^{bc}	18.75^{cdef}	29.17^{abc}	25.00^{a}	6.25^{defg}	9.38^{defg}	16.67^{cdefg}	10.76^{a}
Turpentine	42.71^{a}	22.92^{bcd}	20.83^{cde}	28.82^{a}	-	-	-	-

Means within column followed by the same letter(s) were not significantly different according to Duncan's multiple range test at 5% level.

correspond with higher PEC initiation or PSE formation also, because they may be non-embryogenic callus. Significantly higher percentage of PSE formation was observed only in 3 cultivars, that is, Turpentine, Carabao and Alphonso.

The results of the present study indicate that explants of small size fruits gave significantly the highest number of callused explants and primary somatic embryos compared to medium size (Table 2). In another study, nucellar tissues from immature mango fruit of size 2.5 to 4.5 cm long were used to induce somatic embryogenesis in cultivar Alphonso (Deore et al., 2000) while rapid production of somatic embryos with normal morphology and germination were achieved in monoembryonic mango cultivars Alphonso, Mundan and Baneshan from the nucellar explants (Jana et al., 1994). An interaction between explants size and cultivars significantly affected on the number of explants callused. It is distinct from our results that small size explants was more favorable for callusing than medium size and also revealed that % PSE induction significantly affected with size of explants as well as genotype.

Effect of media composition on callus and primary somatic embryo induction from nucellar explants in mango cultivars

Percent callus and primary somatic embryo (PSE) induction data from Tables 2 and 3 distinctively indicates that induction media 1 (IND1) and 3 (IND3) worked well for callus initiation and PSE induction in both mono and polyembryonic cultivars. Maximum callusing was observed either in media IND1 or IND3 in most of mango cultivars studied in both small and medium size fruit explants (Table 2). Highest callus initiation in Alphonso (59.37%) and Turpentine (54.17%) was recorded in media IND1 for small size fruit explants while highest callus initiation in Zafran (44.37%) and Carabao (52.08%) was observed in media IND3 (Table 2). Maximum callus initiation for Amrapali (79.17%) and Dashehari (43.75%) was observed in media IND1 and IND3 respectively for medium size fruit explants but this callus were non-embryogenic in nature. Induction of PSE formation was ascertained mostly in media IND1 as in case of Alphonso, Zafran, Carabao and Turpentine and IND3 as in case of Amrapali and Carabao. Maximum PSE induction for Alphonso (22.08%) was recorded in media IND2 while maximum PSE induction for Carabao (29.17%) and Turpentine (42.71%) was recorded in media IND3 and IND1 respectively (Table 3 and Figure 1). None or less PSE induction was recorded for Dashehari and Amrapali cultivar in medium size fruit explants and Carabao only induced PSE in media IND3.

Either 1 mg/l 2,4-D alone (IND3) or in combination with 0.25 mg/l BAP (IND1) was sufficient for producing embryogenic callus and induction of PSEs in most of the

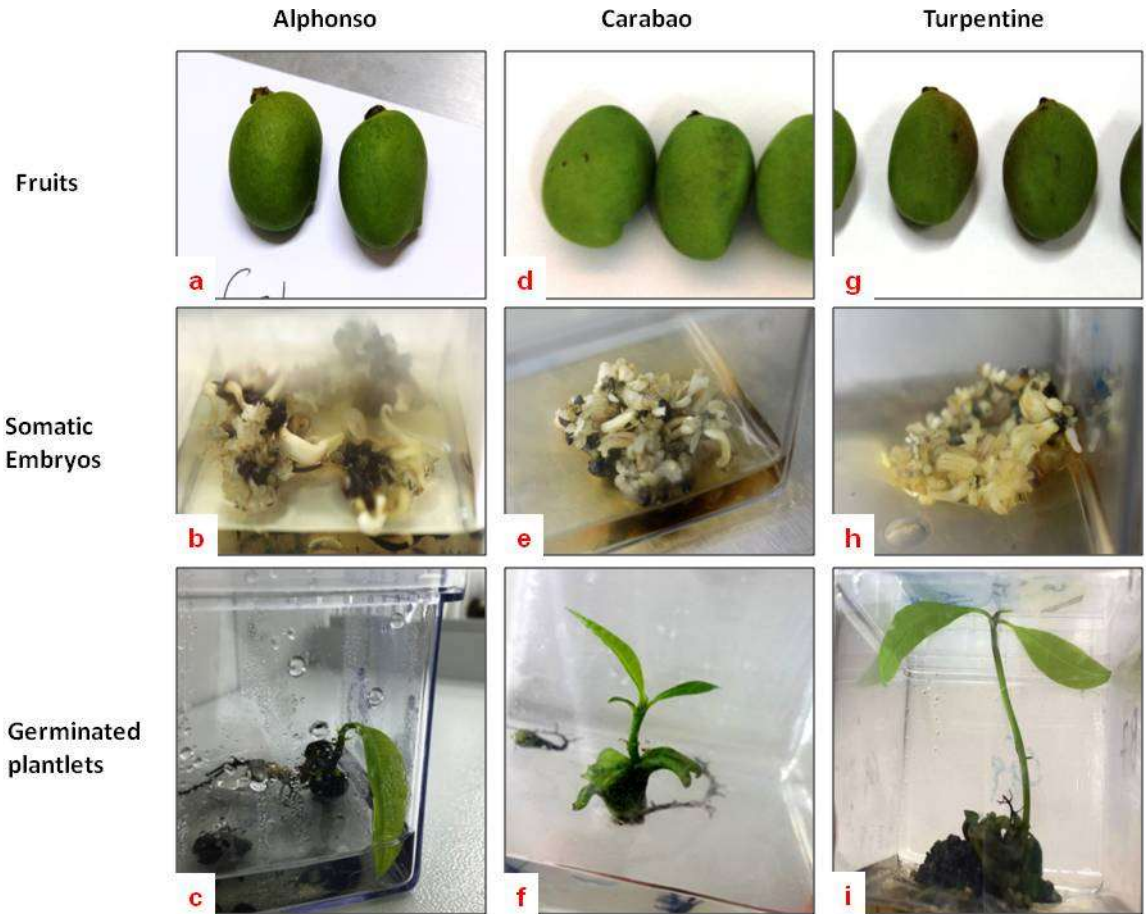

Figure 1. Somatic embryogenesis in Alphonso (a, b, c), Carabao (d, e, f,) and Turpentine (g,h,i).

cultivars studied here. The effect of an interaction between media and cultivar has significantly (P≥0.05) affected on number of explants callused and primary somatic embryo induction (Tables 2 and 3).

Induction media 1 (IND1) having 1 mg/l 2,4-D, 0.25 mg/l BAP, 400 mg/l L-Glutamine and 500 mg/l malt extract along with basal media components induced higher callusing and PSE induction for polyembryonic cultivar Carabao in our studies, but Pateña and Barba (2011) reported PSE induction ranged 16 to 86% in their experiments on Carabao when 0.5 mg/l 2,4-D, 100 ml/l coconut water, 6% sucrose with basal media components used for induction and proliferation of primary somatic embryos which is also dependent on the strain, collection time and tree source.

The data in the present study indicates that primary somatic embryo induction (% PSE) significantly affected by media compositions and mango genotypes as an interaction effect for these two factors (Table 3). Aside of media compositions, size of explants used affected significantly on number of callused explants and PSE induction in different mango cultivars. In another study, on monoembryonic cultivars of Amrapali and Chausa, Ara et al. (2000) observed that 2, 4-D 1 mg/l with basal media components and 6% sucrose was sufficient to stimulate the induction of callus and pro-embryonic calli (PEC) from nucellar tissues explants, while 4.52 µM 2,4-D and 2.27 µM thidiazuron (TDZ) with MS as the basal medium were able to induce somatic embryo formation in Ratnagiri cultivar (Malabadi et al., 2011). This has been well established that plant growth regulator 2,4-D is an essential requirement for primary somatic embryo induction in monoembryonic and polyembryonic mango cultivars, but prolonged presence of 2,4-D in induction media inhibits the proliferation and further growth of somatic embryos beyond globular stage (Krishna and Singh, 2007). Optimum 2,4-D and BAP concentration, malt extract and L-glutamine along with basal media are essential components in improving the embryogenic response and primary somatic embryo induction from nucellar explants. In another study, embryogenic callus induction was obtained from nucellar explants of Alphonso in modified MS medium containing 1 mg/l 2,4-D, 400 mg/l glutamine, 100 mg/l ascorbic acid, 500 mg/l PVP, 60 g/l sucrose and 2.5 g/l Phytagel and the somatic embryos were developed in same medium without 2,4-D

Table 4. Effect of media composition on somatic embryo (SE) germination in different mango cultivars from small size fruit explants.

Media (A) cultivar (C)	Number of SEs germinated						% SEs germination					
	GM1	GM2	GM3	GM4	GM5	Mean	GM1	GM2	GM3	GM4	GM5	Mean
Alphonso	2.67^{cd}	1.67^{cd}	0.00^{d}	2.00^{cd}	2.67^{cd}	1.80^{b}	2.23^{d}	7.34^{bc}	0.00^{d}	2.03^{d}	3.94^{cd}	3.11^{b}
Carabao	19.67^{a}	1.33^{cd}	0.33^{d}	9.67^{b}	8.00^{bc}	7.8^{a}	18.59^{a}	10.85^{b}	1.39^{d}	10.34^{b}	15.83^{a}	11.40^{a}
Turpentine	1.33^{cd}	2.67^{cd}	0.67^{d}	3.67^{bcd}	2.33^{cd}	2.13^{b}	0.87^{d}	3.34^{cd}	2.08^{d}	2.04^{d}	1.66^{d}	2.00^{b}
Mean	7.89^{a}	1.89^{bc}	0.33^{c}	5.11^{ab}	4.33^{ab}	-	7.23^{a}	7.18^{a}	1.16^{c}	4.80^{a}	7.14^{ab}	-

Means within column followed by the same letter(s) were not significantly different according to Duncan's multiple range test at 5% level.

(Deore et al., 2000) while embryogenic nucellar cultures were established successfully for Carabao in induction media containing 4.5 µM 2,4-D (minimum 28 days of treatment), 400 mg/l glutamine, 60 g/l sucrose, 2 g/l gellan gum and basal media B5 major salts, MS minor salts and organics (Lad et al., 1997). Efficient somatic embryogenesis and different stages of somatic embryos has also been reported in five polyembryonic cultivars, that is, Chino, Sabre, Omo, Heart and Turpentine N2-17-2 from nucellar cultures in a liquid media containing 20% coconut water by Litz et al. (1982). Modified basal media with other essential components was used by Ara et al. (2000) and Pateña et al. (2002) for induction of somatic embryogenesis and *in-vitro* regeneration for Amraplai and Carabao cultivar respectively. Varying concentration of different plant growth regulators along with modified basal media and other essential components such as sucrose and phytagel are required at each stage of somatic embryogenesis and plant regeneration as also reported by Chaturvedi et al. (2004) and Al-Busaidi et al. (2016).

It was also observed from our results that low concentration of BAP (0.5 mg/l) and withdrawal of 2,4-D from proliferation media was an important step for getting large numbers of somatic embryos in lesser time and also conversion of PEC and globular somatic embryos into heart shaped embryos and early cotyledonary stage somatic embryos, and similarly. Ara et al. (2000) also found in their studies on monoembryonic cultivars Amrapali and Chausa that the presence of 2,4-D in the medium inhibited the further progression of somatic embryo development. It is obvious by the results that the effectiveness and the positive role of the media compositions on mango callus initiation and PSE formation largely dependent on the size of the explants used, which confirms the effect of the explants size on *in-vitro* regeneration of mango genotypes.

Effect of media composition on somatic embryo germination in mango cultivars

Data in Table 4 present the effect of media composition on germination of somatic embryos in 3 different mango cultivars. Germination of matured cotyledonary stage SEs was observed only in cultures from small size fruit explants in 3 cultivars, that is, Alphonso, Carabao and Turpentine (Figure 1). SE induction and proliferation was also recorded in few cultures containing explants of medium size fruits of Alphonso, Amrapali, Dashehari, Zafran and Carabao, but these SEs were not germinated in any of the germination media except Carabao which was germinated in GM1 (data not shown). Higher percentage germination of SEs was recorded in germination media 2 (GM2) for Alphonso (7.34%), GM1 for Carabao (18.59%) and GM2 for Turpentine (3.34%) (Table 4). Germination media 5 (GM5) also stimulated the germination of SEs of Alphonso and Carabao in higher number. Germination media 2 (GM2) containing 0.1 mg/l IAA and 0.5 mg/l GA3 along with basal media has shown significantly higher percent germination of SEs of Alphonso and Turpentine while Carabao recorded significantly higher germination percentage in GM1 (18.59%) and GM5 (15.83%) media (Table 4), where GM1 did not contain any plant growth regulator and GM5 contains 0.5 mg/l NAA, 2.5 mg/l kinetin and 1 mg/l GA3 along with half strength B5 in basal media and other components (Table 1). In another study, 1 mg/l GA3 in a liquid medium containing half strength B5 macrosalts was used for germination of matured somatic embryos in Amrapali and Chausa by Ara et al. (2000). In general, germination media GM2 and GM5 had shown a good response for germination of SEs in monoembryonic cultivar Alphonso and polyembryonic cultivar Turpentine but germination of SEs for Carabao was the best in a germination media GM1 which did not contain any plant growth regulator.

Sucrose concentration has been reduced to 20 g/l in germination media GM1 to GM4 as reported by Litz (2003) and Laxmi et al. (1999), who suggested that reduced sugar concentration is important for germination and plantlet formation in mango tissue culture. In our study, significantly ($P \geq 0.05$) higher germination rate (18.59%) for Carabao was recorded in GM1 media without any plant growth regulators (PGR) which indicates that somatic embryo germination and plantlet formation might be achieved without any PGR, only in presence of basal media components with L-glutamine and malt extract. Similarly, Pateña and Barba (2011) were able to regenerate plantlets of Carabao in mango

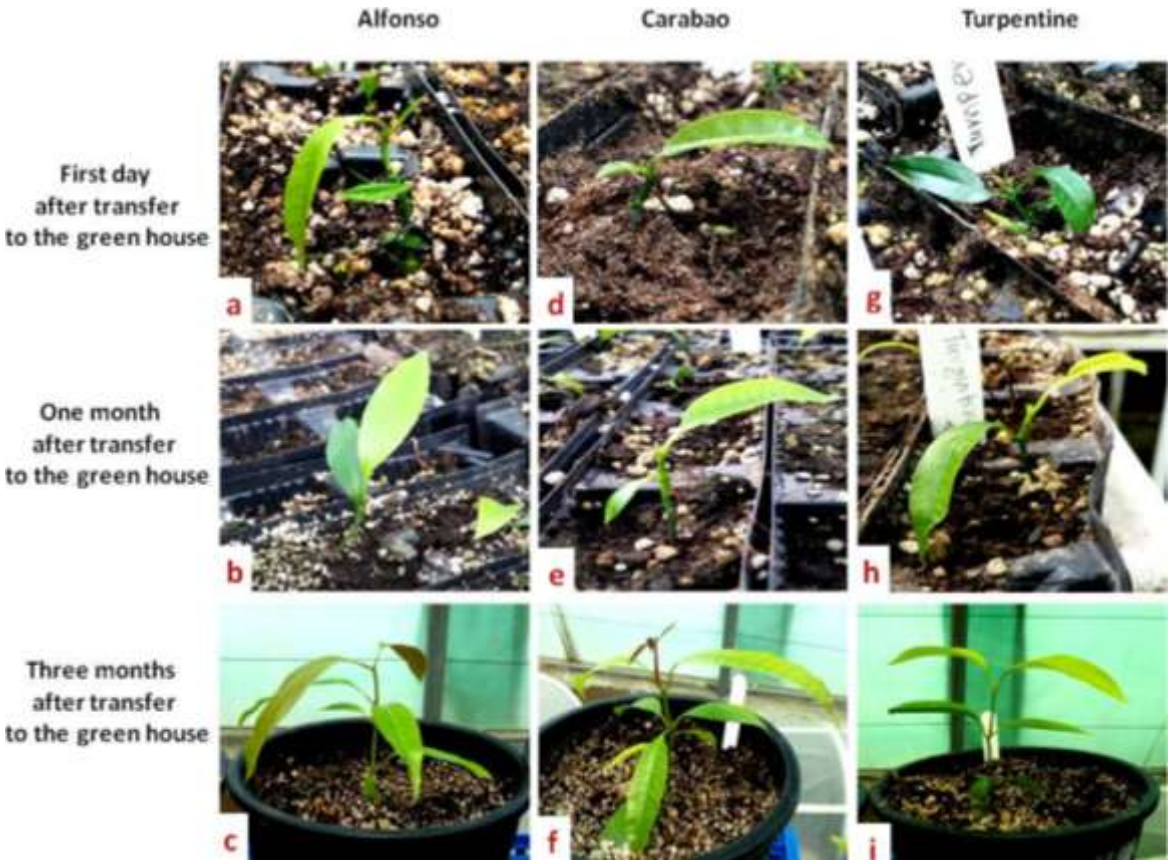

Figure 2. Hardening of tissue culture raised mango plantlets in green house Alphonso (a, b, c), Carabao (d, e, f,) and Turpentine (g,h,i).

medium for plantlet regeneration (MMPR) without any PGR as well, while Malabadi et al. (2011) succeeded in germination of somatic embryo in Ratnagiri cultivar in half strength MS basal media without any PGRs.

The results in this study revealed that there is a significant interaction (P≥0.05) effect between germination media and mango cultivars on the number of somatic embryos (SEs) germinated (Table 4). Matured cotyledonary stage somatic embryos have taken about 6 weeks for germination and first true mango plantlet (having well developed shoot, leaves and roots) was formed after approximately 8 months of explants culture.

Hardening and *ex-vitro* survival of germinated mango plantlets

Germinated plantlets of Alphonso, Carabao and Turpentine performed steady growth and better survival rate after one month of transfer to the greenhouse (Figure 2 and Table 5). Few of the plants of all 3 cultivars did not survive more than one month of transfer to the green house due to robust environmental conditions but after first one month, all plants were performed better in growth parameters and survived largely showing acclimatized to the new environmental conditions (Figure 2). About 67% plantlets of Alphonso, 27% of Carabao and 50% of Turpentine were survived more than 3 months after transfer to the greenhouse conditions (Table 5). All these plants are still surviving and growing well even after 6 months of transfer to the greenhouse. High survival rate for Alphonso cultivar in this study is promising compared to the previous reports about survival rate of other tissue culture raised mango plants in *ex-vitro* conditions such as 50% survival rate for Amrapali and no survival for Chausa was reported by Ara et al. (2000). About 70% transplant success was achieved by Chaturvedi et al. (2004) for a monoembryonic cv Ambalavi and these plantlets were survived more than 4 months. About 50% survival rate for Turpentine is still good but survival rate for Carabao (27%) is required to be improved and even better acclimatization strategy to be explored for Carabao. Pateña and Barba (2011) successfully transplanted tissue culture raised *ex-vitro* grafted plantlets of Carabao in field after 1.5 years of acclimatization in greenhouse conditions and only 14.3 to 50% survival rate achieved for these *ex-vitro* grafted plantlets in green house.

Table 5. Hardening and *ex-vitro* survival of tissue culture raised mango plants.

Cultivars	Batch No.	No. of plants transferred to green house for hardening	No. of plants survived after 1 month of transfer	No. of plants survived after 3 months of transfer	% plants survived after 3 months of transfer	Mean % survival after 3 months of transfer
Alphonso	Batch 1	12	7	7	58.33	66.66
	Batch 2	6	5	4	66.66	
	Batch 3	8	6	6	75.00	
Carabao	Batch 1	28	2	2	7.14	26.68
	Batch 2	16	7	5	31.25	
	Batch 3	12	6	5	41.66	
Turpentine	Batch 1	8	5	3	37.50	49.16
	Batch 2	6	5	3	50.00	
	Batch 3	5	4	3	60.00	

In this study, tissue culture raised mango plants were produced successfully for 3 cultivars Alphonso, Carabao and Turpentine from nucellar explants of small size fruits within 8 months of time and this is the first report where tissue culture mango plantlets were developed for cultivars Alphonso and Turpentine through nucellar embryogenesis.

Conclusions

Tissue culture raised mango plants were successfully developed for Alphonso, Carabao and Turpentine cultivars through nucellar embryogenesis. Primary somatic embryos were produced and proliferated largely in Alphonso, Carabao and Turpentine small fruit size explants. Some primary somatic embryo induction was also noticed in Amrapali and Zafran but these somatic embryos were unable to germinate. Gamborg's B5 macronutrients, MS micronutrients, iron source and vitamins, and organics were used as basal media at each stage of development. Induction media containing 1 mg/l 2, 4-D alone or in combination with 0.25 mg/l BAP along with 400 mg/l L-glutamine, 500 mg/l malt extract and essential basal media components are the best for obtaining embryogenic callus and somatic embryos in most of the cultivars while 2 mg/l 2,4-D and 0.50 mg/l BAP worked well for inducing somatic embryo formation in Alphonso. Germination of matured somatic embryos were achieved in germination media without any plant growth regulators or only 0.1 mg/l IAA and 0.5 mg/l GA3 along with basal media in Alphonso, Carabao and Turpentine. Better acclimatization strategy and higher survival rate has also been accomplished in Alphonso, Carabao and Turpentine cultivars. An efficient somatic embryogenesis and plantlet regeneration system was developed from nucellar explants by using modified basal media and different concentration of plant growth regulators at each stage in 3 mango cultivars i.e. Alphonso, Carabao and Turpentine. Further studies to increase germination percentage and a strategy for better acclimatization are required to use this protocol for mass propagation of these 3 mango cultivars.

Conflicts of Interests

The authors have not declared any conflict of interests.

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