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Full Length Research Paper

***In vitro* regeneration of Mango (*Mangifera indica* L.) cv. Baramasi through nucellar embryogenesis**

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

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Vegetative propagation via grafting is a common method to propagate mono-embryonic mango (*Mangifera indica* L.) varieties in Oman which is time consuming and expensive. Standardize *in-vitro* regeneration protocol in producing true-to-type, disease free and homogeneous high quality plants is prerequisite. Nucellar tissues from immature mango fruits of mono-embryonic cv. Baramasi were used as explants to induce somatic embryogenesis and plantlets regeneration. Several media compositions were evaluated for all the four stages that is, induction, conversion, maturation and germination. Modified Gamborg's B5 major salts, MS minor salts, iron source and organics were used as basal media with varying hormone concentrations. The highest number of callused explants (12.6) was observed in induction media 1 (IND1) containing 1 mg/l 2, 4-D, 0.25 mg/l BAP, 400 mg/l L-glutamine and 500 mg/l malt extract. About 49.6% callus produced somatic embryos (SEs) and maximum 69.7 SEs were proliferated from each embryogenic callus in conversion media 2 (CM2) having 0.5 mg/l BAP. The highest germination (35.9%) with well-developed shoot, leaves and roots was observed in germination media 5 (GM5) containing 0.1 mg/l IAA, 1 mg/l Kinetin and 0.5 mg/l GA3. About 65% of transplanted plants are still surviving in green house conditions even after 4 months of transfer.

Key words: Nucellar embryogenesis, mono-embryonic, genotype, callus, germination, maturation, induction, basal media.

INTRODUCTION

Mango (*Mangifera indica* L.) which belongs to the dicotyledonous family Anacardiaceae, is one of the most important tropical and subtropical fruit crops in the world. It is a widely cultivated fruit in the Sultanate of Oman and occupies fourth position in terms of area and production. (1,485 hectare and 14257 ton respectively, MAF 2015).

Mango is either mono-embryonic, Indian source or poly-embryonic from Southeast Asian race (Mukherjee, 1977). The seedlings of mono-embryonic varieties are not true to type unlike poly-embryonic varieties which are genetically homogenous. Worldwide, vegetative propagation is still a common method to propagate

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mono-embryonic mango varieties which is time consuming and expensive.

Baramasi is an Indian mono-embryonic cultivar and is well adapted under the Sultanate conditions. It is semi-dwarf in size and has a good yield (154 kg/ tree) with fruit quality of 18% total soluble solids, flowering and fruiting twice in a year, high temperature tolerant and disease resistance (Annual Research Report, 1989, 1990). However, being a mono-embryonic variety, nucellus of Baramasi cannot be used by conventional method of propagation to yield uniform planting material, since nucellus of mono-embryonic variety does not have a property of *de novo* adventive embryony as in the case of other mono-embryonic varieties such as Ambalavi (Chaturvedi et al., 2004). Under Omani conditions, heterozygous seedlings locally named "Omani mango" has been used as rootstock for propagation of such mango varieties resulting in non-uniform planting material. Therefore, in such cases the use of *in vitro* regeneration for the production of clones becomes prerequisite (Hartmann et al., 1997).

Somatic embryogenesis in mango is one of the micropagation techniques which depend on induction of nucellar tissue in ovular halves of fruits to produce callus, somatic embryo formation, proliferation of somatic embryos and their synchronized development into plantlets (Chaturvedi et al., 2004). The plants regenerated from nucellar tissues are true-to-the type and many researchers (Nower, 2013; Malabadi et al., 2011; Mishra et al., 2010; Krishna and Singh, 2007; Chaturvedi et al., 2004) have been succeeded in using this technique to multiply mono and poly-embryonic mango varieties. However, the response to this technique is genotype dependent (Litz et al., 1982 and Litz, 1986). No work has been reported on Baramasi so far and a standard reliable protocol of regeneration is the prime and foremost prerequisite to get true-to-type plants, free of diseases and homogeneous high quality planting material. Therefore, the present investigation aims to develop an effective and reliable protocol for nucellar embryogenesis and plantlet regeneration for *Mangifera indica* L. cv Baramasi.

MATERIALS AND METHODS

This investigation was conducted at the Tissue Culture Unit, Directorate General of Agriculture and Livestock Research, Rumais (23°41'15" N, 57°59'1" E) in the South Al Batinah Governorate, Oman during 2014-2016. Different compositions of media were evaluated for each stage of somatic embryogenesis and plantlets regeneration to obtain tissue culture raised mango plants from nucellar tissues of immature mango fruits. Modified Gamborg's B5 major salts (Gamborg, 1970), MS minor salts, MS Fe-EDTA and MS organics (Murashige and Skoog, 1962) were used as basal media in all four media types (Tables 1, 2 and 3) that is, induction media (IND), conversion media (CM), maturation media (M1) and germination media (GM) for induction, conversion and proliferation, maturation and germination stages respectively. Ara et al. (2000) and Patena et al. (2002) also used modified basal media to achieve

successful induction of somatic embryogenesis and regeneration. Different plant growth regulators and their varying concentration along with basal media are required at each stage of induction of embryogenesis and plant regeneration as reported by Chaturvedi et al. (2004) and Mishra et al. (2010).

Explant, surface sterilization and treatments

Immature mango fruits of about 3.5 to 5.0 cm in length were collected after approximately 30 to 40 days of pollination (Mishra et al., 2010) from *Mangifera indica* L. cv Baramasi trees from mango gene bank at Wadi Hebi Research Farm, Agricultural Research Department, and the Sultanate of Oman. Ovular halves of fruits containing nucellar tissues were used as explants to induce callusing, somatic embryo formation and germination to form complete mango plantlets of cv Baramasi. Nucellar tissue is an excellent choice as source of explants to induce nucellar embryogenesis and *in vitro* regeneration for mono-embryonic mango varieties as reported in several previous studies (Ara et al., 2000, Chaturvedi et al., 2004 and Laxmi et al., 1999). Collected immature mango fruits were washed intensively with running tap water and soap for few min. Thereafter, sequentially surface sterilized with 70 % (v/v) ethanol (10 min) and 0.1 % (w/v) HgCl₂ (5min) and finally rinse with sterile double distilled water 3 to 4 times under laminar airflow. Explants browning due to phenolic exudation in post culture stage are one of the major challenges in tissue culture of mango. Development of an effective pre-treatment methodology to control explants browning of mango is also a major hurdle in mango micropagation experiments. L-Ascorbic acid (100 mg/l) and 0.3 % PVP (polyvinylpyrrolidone) were added in the induction and conversion media to control explants browning (Mishra et al., 2010) along with frequent sub-culturing and incubation in dark conditions to minimize the effects of phenolic exudations (Ara et al., 2000). Pre-treatment of explants with antioxidants solutions (Hare Krishna et al., 2008), pre-culture in liquid induction media (Li et al., 2012), use of activated charcoal (0.5 gm/l) in basal media, incubation of nucellar cultures in dark with frequent sub culturing (Litz, 2003) are strategies which were found to be effective for prevention of browning of explants

Callus Induction and pro embryonic calli (PEC) initiation

For callus induction, the sterilized fruits were open and intact ovule containing nucellar tissues were cut longitudinally into two halves under the aseptic conditions of laminar air flow and cultured in magenta boxes contained induction media IND1, IND2, IND3 and IND4 (Table 1) and kept at 25 ± 2°C, 55 to 60% relative humidity (RH) in dark. Frequently Sub-culturing was performed at every third day for at least 3 times to reduce the explants browning due to phenolic exudation. The numbers of explants showing callus and pro-embryonic calli (PEC) initiation as well the number of days taken for callusing and PEC formation were recorded after 60, 90, 120 and 150 days of culture and percentage callusing was calculated at 90 and 120 days after first culture. Pro-embryonic calli (PEC) and initial globular shaped somatic embryos from Baramasi was further transferred in to conversion media and maturation media for complete somatic embryogenesis and regeneration of mango plantlets.

Conversion stage

After about 3 months of first culture, the callus and PEC were transferred into conversion medium CM1 and CM2 (Table 2). The callus and PECs were incubated at 25 ± 2°C temperature, 55 to

Table 1. Media compositions for callus induction via nucellar embryogenesis in mango var. Baramasi.

Media Compositions	IND 1	IND 2	IND 3	IND 4
Basal media	B5 Major salts and MS Minor salts	B5 Major salts and MS Minor salts	B5 Major salts and MS Minor salts	B5 Major salts and MS Minor salts
2,4-D	1 mg/l	-----	2 mg/l	1 mg/l
BAP	0.25 mg/l	0.25 mg/l	0.50 mg/l	0.25 mg/l
NAA	-----	1 mg/l	-----	-----
L-Glutamine	400 mg/l	400 mg/l	400 mg/l	400 mg/l
Malt Extract	500 mg/l	-----	500 mg/l	500 mg/l
L-Ascorbic acid	100 mg/l	100 mg/l	150 mg/l	100 mg/l
PVP	100 mg/l	-----	50 mg/l	100 mg/l
Sucrose	30 gm/l	30 gm/l	30 gm/l	30 gm/l
Phytigel	2.5 gm/l	2.5 gm/l	2.5 gm/l	2.5 gm/l
Activated charcoal	-----	-----	-----	500 mg/l
pH	5.8	5.8	5.8	5.8

Table 2. Media compositions for conversion of pro-embryonic calli into somatic embryos.

Media Compositions	CM 1	CM 2
Basal media	B5 Major salts and MS Minor salts	B5 Major salts and MS Minor salts
BAP	-----	0.5 mg/l
L-Glutamine	400 mg/l	400 mg/l
Malt Extract	500 mg/l	500 mg/l
L-Ascorbic acid	100 mg/l	100 mg/l
PVP	100 mg/l	100 mg/l
Sucrose	30 gm/l	30 gm/l
Phytigel	2.5 gm/l	2.5 gm/l
Activated charcoal	-----	-----
pH	5.8	5.8

60% RH in dark as reported by Mishra et al., 2010. The number of days taken to convert PEC into somatic embryos of different development stages (globular, heart, torpedo and cotyledonary) was recorded. Cotyledonary stage somatic embryos were transferred into maturation media for further maturation of embryos and germination while globular shaped and heart shaped somatic embryos were cultured again in CM 2 media containing 0.5 mg/l BAP for secondary and tertiary somatic embryo production.

Maturation and germination stage

Somatic embryos at torpedo and cotyledonary stage were transferred to maturation media (M1) containing B5 major salts, MS minor salts, iron-EDTA and organic supplements and vitamins as basal media with 100 µg/l each of ABA and IAA, 100 mg/l PEG, 30 gm/l sucrose, 2.5 gm/l phytigel and pH 5.8 for further maturation, synchronization in development and minimize the fasciation of developing somatic embryos. The cultures were incubated for 4 weeks at 25 ± 2°C, 55 to 60% RH in dark. About 10 weeks old mature cotyledonary stage somatic embryos were transferred into germination media GM 1, GM 2, GM 3, GM 4 and GM 5 (Table 3) containing BAP, IAA, Kinetin and GA3 plant growth regulators in

various concentrations to stimulate the germination of somatic embryos and plantlets formation. These cultures in germination media were maintained at 16 h photoperiod with 40 mmolm⁻²S⁻¹ light intensity, 55% RH at 25 ± 2°C (Mishra et al., 2010). Each germination media contains minimum 60 somatic embryos and germinated somatic embryos (visible shoots, roots and leaves) were counted after one month and transferred into liquid media of same composition without GA3 for further growth of leaves, roots and shoots. Somatic embryos were considered germinated when there is well developed shoots, roots and green leaves are visible and also, important to mention that here germinated embryos are counted as plantlets. Number of plantlets developed in each germination media was counted after 6 weeks and percentage germination was calculated in each germination media.

The *in vitro* raised plantlets of Baramasi have been transplanted in plastic pots containing sand and soil (3:1) mixture and wrapped with polythene bags to maintain high humidity inside and low transpiration. Initially, they maintained for one month in plant growth chamber with controlled light (16 h light cycle), temperature (25°C) and relative humidity (60%) rate as described by Ara et al. (2000) for Chausa. After one month, established mango plantlets were transferred into cooled greenhouse for further growth and acclimatization.

Table 3. Media compositions for germination of somatic embryos.

Media Compositions	GM 1	GM 2	GM 3	GM 4	GM 5
Basal media	B5 Major salts and MS minor salts	B5 Major salts and MS minor salts	B5 Major salts and MS minor salts	B5 Major salts and MS minor salts	B5 Major salts and MS minor salts
BAP				0.5 mg/l	
IAA			0.1 mg/l	0.1 mg/l	0.1 mg/l
Kinetin					1 mg/l
GA3		1 mg/l	0.5 mg/l	0.5 mg/l	0.5 mg/l
L-Glutamine	400 mg/l	400 mg/l	400 mg/l	400 mg/l	400 mg/l
Malt Extract	500 mg/l	500 mg/l	500 mg/l	500 mg/l	500 mg/l
Sucrose	20 g/l	20 g/l	20 g/l	20 g/l	20 g/l
Phytogel	2.5 g/l	2.5 g/l	2.5 g/l	2.5 g/l	2.5 g/l
Activated Charcoal	50 mg/l	---	50 mg/l	50 mg/l	50 mg/l
pH	5.8	5.8	5.8	5.8	5.8

Table 4. Effect of media formulation on callus induction of Baramasi variety via somatic embryogenesis after 120 days.

Media	Number of explants cultured	Number of callused explants	% callusing
IND 1	46.2	12.6 ^a	32.1 ^a
IND 2	9.0	0.67 ^b	7.9 ^b
IND 3	14.5	5.0 ^b	37.1 ^a
IND 4	43.75	6.3 ^b	14.5 ^c

Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range.

Statistical analysis

Randomized complete design (RCD) was used for all statistical analysis. All data were tabulated using Excel software 2010 and they were tested for normal distribution using the Shapiro-Wilk test. Analysis of variance (ANOVA) was done using GenStat Release 11.1 (VSN International, Hemel Hempstead, UK). The significance of media compositions treatments effects were tested with a two-way analysis of variance (ANOVA). The Duncan's multiple range test was used to test mean separation between treatments.

RESULTS AND DISCUSSION

Nucellus tissues from ovular halves of immature mango cv Baramasi fruits (size 3.5 to 5.00 cm length) responded differently in different media compositions for induction of somatic embryogenesis.

Callus Induction and PEC initiation stage

The data in Table 4 shows the effect of media formulation on callus induction of Baramasi cultivar via somatic embryogenesis (Figure 1d). The results revealed that media formulations varied significantly in inducing Baramasi nucellar tissues for callus induction. Average number of callused explants ranged between 0.67 to

12.6, where IND1 media having 1 mg/l 2,4-D, 0.25 mg/l BAP, 400 mg/l L-Glutamine and 500 mg/l Malt extract gave the highest number (12.6 explants), followed by IND4 (6.3 explants) and IND3 (5 explants), while, IND2 was the lowest (0.67 explants). Similarly, Ara et al. (2000) found that 2, 4-D 1 mg/l stimulated the callus initiation and induction of pro-embryonic calli (PEC) in cultured nucellar tissues of monoembryonic cultivars Amrapali and Chausa while Malabadi et al. (2011) used 4.52 μ M 2, 4-D and 2.27 μ M thidiazuron (TDZ) with full strength MS as the basal medium and were able to induce somatic embryo formation in Ratnagiri cv after 4 to 9 weeks of culture. All of these studies indicate that auxin 2, 4-D is the foremost requirement during induction stage of somatic embryogenesis in monoembryonic mango cultivars though prolonged presence of 2, 4-D in induction media inhibits the growth of somatic embryos beyond globular stage (Hare Krishna and Singh, 2007).

In the present study, callusing percentage ranged from 7.9 to 37.2, whereas maximum (32.1%) callusing (Table 4) was observed in media IND1 (Table 1) after 120 days of culture. Media IND3 with 2 mg/l 2, 4-D and 0.5 mg/l BAP induced more callusing (37.2%) than media IND1 but after that, none or very rare PEC and SE formation observed where mostly non-embryogenic callus that was formed. Media IND4 containing activated charcoal (500



Figure 1. Nucellar embryogenesis in Baramasi (a) Baramasi tree with fruits, (b) Immature fruit, (c) Ovular half, explant for somatic embryogenesis, (d) Callus formation, (e) Somatic embryos, (f) Cotyledonary SEs for germination, (g) Regenerated plantlet, (h) Plantlets transferred in pots with sand and soil, (i) Acclimatization of plantlets.

mg/l) with 1 mg/l 2,4-D and 0.25 mg/l BAP induced callusing only 14.5% of explants but browning problem was controlled at some extent and, also took longer time (about 150 days) for callusing (data not shown). Explants cultured in media IND1 produced embryogenic callus mostly and initial globular shaped somatic embryos can be observed as early as 100 days of culture period. These initial globular somatic embryos will be transferred in conversion media for further proliferation and maturation.

Conversion and formation of somatic embryo

Data in Table 5 presents number of PEC, number of primary somatic embryos formed and average of somatic embryos per PEC of Baramasi variety as results of media formulations effect. The results showed that response of transferred PEC to produce somatic embryos (SEs) was significantly affected by conversion media viz; CM1 and CM2 (Table 2). After 4 weeks of culture almost none or very less number of somatic embryos (SEs) of different stages have been observed in media CM1, while media containing 0.5 mg/l BAP (CM2) was filled with numerous SEs of all the stages that is, globular, heart shape, torpedo and cotyledonary stage within a month of transfer

into CM2 media. In CM2, 49.6% of PEC was formed from induced callus and significantly higher about 396.4 somatic embryos, all the stages have been formed (Table 5) after 150 days from first culture. Also, in the same media, 69.7 somatic embryos were developed from each embryogenic callus, while only 11.1 somatic embryos of all the stages were developed from each embryogenic callus in CM1 media. It is evident from observed data, that low concentration of BAP (0.5 mg/l) and withdrawal of 2,4-D from conversion media was necessary for conversion of PEC and globular embryos into heart shaped embryos and early cotyledonary stage somatic embryos. Similar to our results, Ara et al. (2000) also reported that the presence of 2, 4-D in the medium inhibited the progression of development of SEs to next stages.

Somatic embryo maturation and germination

In this study, somatic embryos of different developmental stages were transferred to maturation media (M1) for maturation and further germination of matured cotyledonary embryos (Figure 1e). This step is necessary because development of bipolarity in globular somatic embryos and differentiation of cotyledons are initiated

Table 5. Effect of media formulation on number of PEC, Primary somatic embryos and average of somatic embryos per PEC of Baramasi variety.

Media	Number of callus cultured	Number of pro-embryonic calli formed (PEC)	% of PEC formation (SE induction)	Number of primary somatic embryos	Average of somatic embryos / embryogenic callus
CM 1	7.25	1.8 ^a	26.0 ^a	20.3 ^a	11.1 ^a
CM 2	11.2	5.4 ^b	49.6 ^b	396.4 ^b	69.7 ^b

Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range.

Table 6. Effect of media formulation on % of somatic embryos germination of Baramasi variety via somatic embryogenesis.

Media	Number of matured embryos cultured	Number of germinated somatic embryos	% germination
GM 1	26.2	5.2 ^b	20.9 ^c
GM 2	37.25	4.0 ^b	10.6 ^e
GM 3	35.00	9.6 ^b	27.5 ^{ab}
GM 4	37.4	7.7 ^b	21.8 ^d
GM 5	52.6	17.8 ^a	35.9 ^a

Different letters within a column indicate significant differences at P = 0.05 by Duncan's multiple range.

only when somatic embryos were transferred into maturation media (Hare Krishna and Singh, 2007). Similarly, Singh et al. (2001) reported that enhanced embryogenesis and normal bicotyledonary somatic embryos were produced by using 100 µM ABA in hormone free regeneration media, while Thomas, (1999) accounted that maturation of heart stage and early cotyledonary stage somatic embryos required 1.0 mg/l ABA with reduced concentration of sugar.

One month old matured cotyledonary stage somatic embryos having shoot and root primordia were transferred into germination media and were needed 4 weeks to develop into plantlets with variable response to germination media as shown in in Table 6. In this regard, the results revealed that the maximum percentage of germination (35.9%) was observed in germination media GM5 containing 0.1 mg/l IAA, 1 mg/l Kinetin and 0.5 mg/l GA3 (Table 6). About 27.5% germination was observed in GM3 media containing 0.1 mg/l IAA and 0.5 mg/l GA3 with all the other components of basal media. There was no significant change in germination percentage by using activated charcoal; however, sucrose concentration has been reduced to 20 g/l in all five germination media as recommended by Litz, (2003) and Laxmi et al., (1999) who found that reduced sugar concentration was important for germination and plantlet formation. Significantly (P ≤ 0.05) higher about 20.9% germination was recorded in GM1 media devoid of any plant growth regulators indicates that germination and plantlet formation can be achieved without any phytohormone, only in presence of basic nutrient composition with L-

glutamine and malt extract. In similar experiment, Patena and Barba, (2011) regenerated mango plantlets of Carabao in mango medium for plantlet regeneration (MMPR) without adding any phytohormone. However, when we added 0.5 mg/l BAP in GM3 media (now GM4 media), germination percentage has been decreased and 1 mg/l GA3 alone in GM2 media didn't induce germination and plantlet formation significantly. The mature somatic embryos were germinated into well-developed seedlings and subsequently transferred to soil.

In the present study, first true plant (containing well developed shoot, leaves and roots) was formed after 9 months of culture (Figure 1 h and i). The *in vitro* raised plantlets of Baramasi have been transplanted successfully in plastic pots containing sand and soil (3:1) mixture. About 65% of transplanted plants are still surviving in green house conditions even after 4 months of transfer.

Conclusion

Micropropagation protocol for mango cv. Baramasi was successfully developed through nucellar embryogenesis. Gamborg's B5 major salts, MS minor salts, iron source and organics were used as basal media with varying hormone concentrations at each stage. Basal media containing 1 mg/l 2, 4-D, 0.25 mg/l BAP, 400 mg/l L-glutamine and 500 mg/l malt extract is best for callus induction while addition of 0.5 mg/l BAP only in basal media is sufficient for somatic embryo formation and proliferation from embryogenic callus. Germination of

matured cotyledonary somatic embryos can be achieved in germination media having 0.1 mg/l IAA, 1 mg/l Kinetin and 0.5 mg/l GA3 along with basal media. High frequency somatic embryogenesis and plantlet regeneration was achieved by using modified basal media and different concentration of plant growth regulators at each stage in Mango cv Baramasi. Plant growth regulator 2, 4-D is essential in inducing callus and PEC formation in nucellar embryogenesis in Baramasi. Also, 2, 4-D concentration more than 1 mg/l was detrimental in nucellar embryogenesis and led to the formation of non-embryogenic callus mostly. Malt extract played a significant role for getting PEC in short duration (less number of days) and large number of somatic embryos which can be converted into complete plantlets successfully. Use of malt extract and optimum concentration of 2, 4-D and BAP were beneficial in improving the embryogenic response and enhancing regeneration rate from nucellar explants. An effective acclimatization strategy and further refinement at each stage is required to increase the survival rate of regenerated plantlets and apply for mass propagation of this cultivar. Further refinement and standardization at each stage is required to scale-up at mass production level.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

2,4-D, 2,4-Dichlorophenoxyacetic acid; **ABA**, Abscisic acid; **BAP**, 6-Benzylaminopurine; **GA3**, Gibberellic acid; **HgCl₂**, mercuric chloride; **IAA**, Indole-3-acetic acid; **NAA**, 1-Naphthaleneacetic acid; **PEG**, Polyethylene glycol; **PVP**, Polyvinylpyrrolidone; cv, cultivar; hrs, hours; **MS**, Murashige and Skoog; **IND**, Induction media; **CM**, Conversion media; **M**, Maturation media; **GM**, Germination Media.

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