Journal of Agricultural Science; Vol. 11, No. 1; 2019
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Pomegranate Micropropagation, Callogenesis and Genetic Integrity Assessment Using Simple Sequence Repeat Markers

Tebogo Stimela[1], Remmy W. Kasili[2] & Edward G. Mamati[3]

[1] Pan African University Institute for Basic Sciences Technology and Innovation, Nairobi, Kenya

[2] Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, USA

[3] Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya

Correspondence: Remmy W. Kasili, Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, 70803, USA. Tel: 1-225-247-9900. E-mail: rkasili@gmail.com

Received: September 15, 2018 Accepted: October 19, 2018 Online Published: December 15, 2018

doi:10.5539/jas.v11n1p237 URL: https://doi.org/10.5539/jas.v11n1p237

Abstract

In recent years, the awareness of pomegranate health benefits has grown exponentially; nonetheless the existing propagation methods remain a challenge to supply adequate suitable planting materials needed for commercial production. Micropropagation can lead to mass production of plantlets and callus-mediated *in vitro* regeneration can open avenues for the use of genetic engineering to improve this crop. The aim of this study was to evaluate appropriate conditions for pomegranate micropropagation, callogenesis and use Simple Sequence Repeat markers to screen for somaclonal variation. Cytokinins (Benzylaminopurine, Kinetin and Thiadiazol-5ylurea) were tested for shoot induction from nodal explants while auxins (1-Naphthaleneacetic acid, Indole-3-butyric acid and Indole-3-acetic acid) were tested for root induction of *in vitro* regenerated shoots. 1-Naphthaleneacetic acid combined with Benzylaminopurine was assessed for their ability to induce callus from cotyledon and leaf explants. Genetic integrity between mother plant, callus and *in vitro* regenerated shoots were assessed using eight Simple Sequence Repeat markers. Maximum number of shoots and leaves were obtained on full strength Murashige and Skoog media with 6.9 µM kinetin. The highest number of roots was achieved on half strength Murashige and Skoog media with 4.9 µM Indole-3-butyric acid and the longest root was got on half strength Murashige and Skoog media with 5.3 µM Indole-3-acetic acid. Leaves and cotyledons demonstrated to be potential explants for callus formation at all hormonal combination levels tested. Eight out of 13 amplified alleles were polymorphic. A wider genetic variation was found with similarity coefficient range of 0.46-0.92. More somaclonal variation was in regenerated shoots compared to callus.

Keywords: callogenesis, micropropagation, pomegranate, simple sequence repeats, somaclonal variation

1. Introduction

Pomegranate (*P. granatum* L.) is a native plant in Iran and the Himalayas of northern India. It has been cultivated and naturalized over the Mediterranean and the Caucasus region of Asia since ancient times. The plant adapts well to different kinds of soil, climate and is tolerant to drought. Pomegranate fruits are high in fiber and are a good source of vitamin A and C (Glozer & Ferguson, 2011). The fruit, flowers, bark and leaves contain bioactive phytochemicals which are antimicrobial, reduce blood pressure and act against diseases such as diabetes and cancer (Kahramanoglu & Usanmaz, 2016). There is an increasing worldwide demand for pomegranate fruit owing to its superior pharmacological and therapeutic properties. However there are challenges in pomegranate propagation which include poor seed germination due to dormancy. Pomegranate seed coat is hard and makes water penetration and gaseous exchange difficult resulting in low germination percentage (Materechera & Seeiso, 2013). Cuttings have poor root development and this propagation method is season dependent, labour intensive, time consuming and results in slow establishment (Kaji, Ershadi, & Tohidfar, 2013a; Kaji, Ershadi, & Tohidfar, 2013b; Singh, Patel, & Kadam, 2013). This is a setback for commercial pomegranate production. Therefore an *in vitro* regeneration protocol can be used to produce a suitable pomegranate planting material en mass which will make commercialization achievable.

Several studies have been carried out and protocols developed for *in vitro* regeneration of pomegranate over the past years (Patil, Dhande, Thigale, & Rajput, 2011) but appropriate regeneration conditions for pomegranate

growing in Kenya have not been established. Increased awareness of pomegranate health benefits in Kenya has created an increased demand against very poor supply leading to a net importation of this fruit and therefore making it fetch a high market price.

The objective of this study was to develop an appropriate direct *in vitro* regeneration protocol of pomegranate which can be used for mass production of planting material to meet the increased demand by Kenyan farmers. Appropriate conditions for callus induction were also investigated as callus-mediated regeneration will open avenues for improvement of the fruit tree using modern biotechnology approaches such as genetic engineering. The study was designed to determine optimum plant growth regulator (PGRs) concentrations for plant regeneration, callus induction and determine the genetic integrity of callus and *in vitro* regenerated plantlets using SSR markers.

2. Materials and Methods

2.1 Explant Source and Surface Sterilization

Ripe pomegranate fruits were obtained from a farm in Eldoret-Kenya and washed with running tap water for five minutes followed by surface sterilization with 70% ethanol for one minute. They were then rinsed three times with sterilized distilled water. The fruit was cut open using sterilized scalpels and forceps to remove the seeds. Seeds were pressed between filter papers on the bench to remove the juicy pulp and washed in running tap water for ten minutes. Subsequently they were treated with 100 mg/L ridomil solution for 45 minutes and rinsed with distilled water three times. Seeds were further treated in 100 mg/L streptomycin solution for 20 minutes and rinsed three times with sterilized distilled water. After streptomycin treatment, they were immersed in 70% ethanol for ten seconds followed by rinsing thrice with sterilized distilled water. They were then soaked for ten minutes in 20% NaOCI and rinsed again thrice with sterilized distilled water under the lamina flow cabinet. In each treatment and rinsing, seeds (explants) were agitated to ensure complete exposure of explants to disinfectants and removal of any traces of disinfectant from explants.

2.2 Basal Media Preparation

Murashige and Skoog (MS) media was prepared by weighing 4 g (for full strength) or 2 g (for half strength) of MS powder (Duchefa Biochemie) and mixing with 30 g sucrose, then dissolving the mixture in 200 ml distilled water. The solution was then poured in 1 L volumetric flask and topped up to 1 L with distilled water. The media pH was adjusted to a range of 5.70-5.80 using 0.1 M NaOH and HCI followed by addition of 2.8 g gelrite with constant stirring to ensure uniform distribution of gelrite. Using a measuring cylinder, 50 ml media were dispensed in 350 ml culture vessels. The vessels were then sealed and the media sterilized by autoclaving at 121 °C, 120 kPa for 20 minutes.

2.3 Culturing

All the sterilization of explants, culturing and subculturing was done under aseptic conditions in the laminar flow cabinet. The laminar flow cabinet bench was wiped with 70% ethanol and allowed to run for 15 minutes before using it. The forceps and scalpels were washed first, autoclaved and put in a glass bead sterilizer inside the laminar flow cabinet and sterilized at 250 °C. After use, they were wiped, returned to a sterilizer, cooled and used again.

2.4 Growth/Culture Room Conditions

Cultures were incubated in a culture room maintained at 25±2 °C temperatures with a photoperiod cycles of 16 hours light and 8 hours dark. Light was emitted by fluorescent Philips tubes of 36 W.

2.5 Seed Germination and Explant source

Surface sterilized seeds were cut open to remove embryos which were then germinated on hormone free full strength MS media. Two weeks old axenic seedlings were used as a source of leaf explants for callus induction whilst four weeks old seedlings served as a source of nodal explants.

2.6 Direct Shoot Induction

The experiment was conducted in a completely randomized design (CRD) with three replications. Full strength MS shoot induction media was supplemented with three cytokinins; Benzylaminopurine (BAP), Kinetin (KN) and Thiadiazol-5ylurea (TDZ). The hormones were evaluated to determine the best cytokinin type and concentration for direct pomegranate shoot induction. These hormones were each evaluated at five different levels; BAP at 2.2, 4.4, 6.6, 8.8, 11.0 µM, KN at 2.3, 4.6, 6.9, 9.2, 11.5 µM and TDZ at 1.0, 2.0, 3.0, 4.0, 5.0 µM. Each treatment (hormonal level) had three culture vessels containing 50 ml shoot induction media and one single nodal explant. Culturing of explants involved removal of leaves from seedling stems and cutting them into single

nodal explants of 2.0-2.5 cm length with two axillary buds. The explants were then inoculated on shoot induction media in an upright orientation and cultures incubated under light conditions. The number of shoots per explant and the number leaves developed on shoots per explant were recorded after a period of three weeks. The regenerated shoots were then transferred on root induction media.

2.7 Root Induction

Root induction experiment was carried out in a CRD with three replications using half strength MS media. The media was supplemented with three auxins; 1-Naphthaleneacetic acid (NAA), Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA). These auxins were assessed to determine the best hormone and concentration for root induction and growth. Each hormone was tested at five different levels; NAA at 0.6, 3.0, 5.4, 7.8, 10.2 µM, IBA at 2.9, 3.9, 4.9, 5.9, 6.9 µM and IAA at 0.5, 1.7, 2.9, 4.1, 5.3 µM. Each treatment had three culture vessels containing 50 ml half strength MS rooting media and one shoot. Cultures were incubated under light conditions for a period of six weeks. Number of roots that developed and length of the longest root in (mm) were recorded.

2.8 Callus Induction

The experiment was performed in a CRD with three replications using full strength MS media supplemented with combination of two PGR; NAA and BAP. We tested the potential of PGR combination to induce callus from cotyledon and leaf explants. One set had five levels of NAA while BAP was kept constant; 4.4 µM BAP combined with NAA at 2.8, 5.4, 8.0, 10.6, 13.2 µM. In the other set; BAP had five levels while NAA was kept constant; 5.4 µM NAA combined with BAP at 2.2, 4.4, 6.6, 8.8, 11.0 µM. Each hormone combination had three culture vessels containing 50 ml callus induction media and one explant.

Zygotic embryos with cotyledons were removed from sterilized seeds and cut into half then inoculated on callus induction media. The first pair of true leaves from germinated seedlings was excised; leaf edges cut off and the explant placed flat with the adaxial surface in contact with the media. Cultures were incubated for four weeks under light conditions. Data taken after four weeks included; percentage explants forming callus, percentage callus forming roots, percentage callus forming shoot and roots as well as the colour of formed callus.

2.9 DNA Extraction

Total genomic DNA was extracted from young leaves of the mother plant, cotyledon callus, leaf callus and leaves from regenerated shoots on nodal explants. Four of each (callus and plantlets) were randomly selected. DNA was extracted using Cetyl trimethylammonium bromide (CTAB) method by (J. J. Doyle & J. L. Doyle, 1987). The DNA quality was evaluated on ethidium bromide (EtBr) stained 1.8% (w/v) agarose gel run for 1 hour at 100V. DNA concentration was determined using a Nano drop spectrophotometer (Bibby Scientific Ltd, UK).

2.10 Evaluation of Somaclonal Variation

Assessment of genetic integrity was accomplished using eight SSR markers shown in Table 1. Markers were selected based on the previous *P. granatum* SSR analysis studies by (Hasnaoui et al., 2010; Sinjare, 2015). PCR amplification was carried out in a final reactions volume of 10 µl comprised of; 2 µl of 5× My Taq reaction buffer containing 5 mM dNTPs and 15 mM $MgCI_2$, 0.5 µl of 10 µM forward primer, 0.5 µl of 10 µM reverse primer, 0.2 µl of 1 U My Taq DNA polymerase (Bioline), 0.5 µl of DNA template equivalent to 30 ng and 6.3 µl of PCR water. Amplification was performed using the following temperature profile; 5 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 45 °C and 45 s at 72 °C ending with a final extension of 5 min at 72 °C. PCR products (for allele size identification) were separated using 2% (w/v) agarose gels run for one hour 30 minutes and visualized under UV light in a gel documentation system. The photographs of resolved DNA bands and 1kb ladder were captured for analysis.

Table 1. SSR markers for DNA amplification

Locus name (marker)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
POM 10	CCTCATTGCTGATGAATCTT	ACTCGAGAAGCTCTGTGAAG
POM 13	CACACCCTTCATCAAAAGAT	GGACTAACAACCAGCCATAG
POM 46	CTTCCTCCTACCGAACTATG	CCCACTTTGACACTTCTACC
POM 55	GAGACAATTGGGATCAGAAA	AGTCGACGAACTGTGAAATC
PGCT033b/ FN677553	TAATAAGCTGCCCCGAAGTC	CGGTGATGTCCCTATTGGAG
PGCT038b/ FN677558	CGTGCCAAATGGGTAAATAA	AGAACTCCACGACCCATAAA
PGCT091b/ FN677611	ATCAGAATTGGAATCGGAAC	ACCGAGGTCATCGAACTAAA
PGCT101b/ FN677621	GAACGCCAAATTCAAGAACC	GACGATTCTTTCCTGCCTTG

2.11 Data Analysis

Comparison of the effect of three cytokinins and their levels on shoot induction was done using analysis of variance (ANOVA) from Statistical Package for Social Sciences (SPSS) software version 19. The means ± S.E. significance between treatments was determined using the multiple comparison test performed by least significant difference (LSD) at $P \leq 0.05$. Data on the effect of different auxins and their levels on root induction were subjected to ANOVA using SPSS version 19. LSD multiple comparison test at $P \leq 0.05$ was also computed to determine significance between treatments.

Based on gel electrophoresis results, number of alleles per marker was determined by counting maximum number of bands in the PCR product of each sample. PCR amplification profiles of samples were then compared with that of the mother plant for polymorphism. Amplified DNA fragments were scored as 1 denoting allele presence or 0 denoting allele absence. Using this binary data, Jaccard's similarity coefficient was computed by subjecting the data to SPSS software version 19.

2.12 Determining the Best Hormone and Hormonal Level Using Grey Relational Analysis (GRA)

Grey relational analysis method (Ertugrul, T. Oztas, Ozcil, & G. Z. Oztas, 2016) was used to make a decision on which cytokinin and cytokinin level is suitable for shoot induction. Regardless of the difference between treatments in number of shoots and number of leaves, grey relational analysis computation aggregates the two variable parameters (number of shoots and number of leaves) into a single value of each treatment. Using this method, the experimental data was initially normalized to a scale varying between 0 and 1.0 followed by grey relational coefficients and grades computation. The grades were then ranked with the highest grade being the best and given the lowest rank number. The same method was used to determine the best auxin and auxin level on root induction and growth.

3. Results

3.1 Direct Shoot Induction

Shoots were induced on nodal explants cultured on MS media supplemented with cytokinins after three weeks of culture. No shoot induction was observed on hormone free MS (control) cultures. The maximum mean number of shoots formed per explant was two, achieved on MS media supplemented with KN in all treatments (Figure 1). The least mean number of shoots formed per explant was observed on media with 3.0 µM TDZ. Total number of leaves formed on shoots per explant increased with increasing concentrations for both BAP and KN. Further increase in KN concentration above 6.9 µM and BAP concentration above 8.8 µM led to a decrease in number of leaves (Figure 1).There was no significant difference in number of leaves per explant among TDZ treatments (Figure 1) at $P \leq 0.05$. The highest mean number of leaves on shoots per explant was found on media supplemented with 6.9 µM KN. The lowest mean number of leaves on shoots per explant was on media with 2.0 and 3.0 µM TDZ. TDZ was inferior in terms of number of shoots and leaves formed compared to BAP and KN since it had the highest rank number (Table 2). Shoot induction appeared to be favoured on MS media supplemented with 6.9 µM KN followed by 9.2 µM KN as the treatments showed the highest number of shoots and leaves. This was depicted in Figure 1 and Table 3(by the lowest ranks).

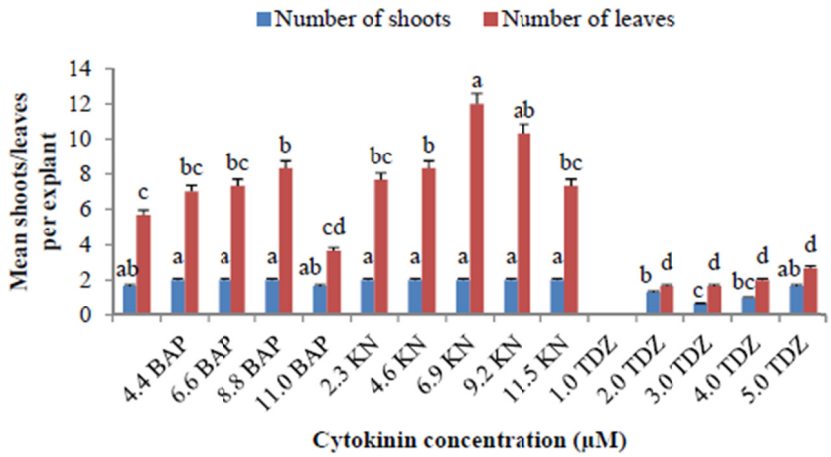

Figure 1. The effect of BAP, KN and TDZ on number of induced shoots and number of leaves on shoots per explant

Note. Columns with different letters are significantly different at $P \leq 0.05$ (LSD multiple comparisons test).

Table 2. Correlation between cytokinin, number of shoots and number of leaves

Cytokinin	Normalized scores		GRA coefficient		R_i	Rank
	Shoots	Leaves	Shoots	Leaves		
BAP	0.88	0.64	0.80	0.58	0.69	2
KN	1.00	1.00	1.00	1.00	1.00	1
TDZ	0.00	0.00	0.33	0.33	0.33	3

Note. R_i (Grey relational analysis grade).

Table 3. Correlation between KN level, number of shoots and number of leaves

KN concentration (µM)	Normalized scores	GRA coefficient	R_i	Rank
	Leaves	Leaves		
2.3	0.07	0.35	0.35	4
4.6	0.21	0.39	0.39	3
6.9	1.00	1.00	1.00	1
9.2	0.64	0.58	0.58	2
11.5	0.00	0.33	0.33	5

Note. R_i (Grey relational analysis grade).

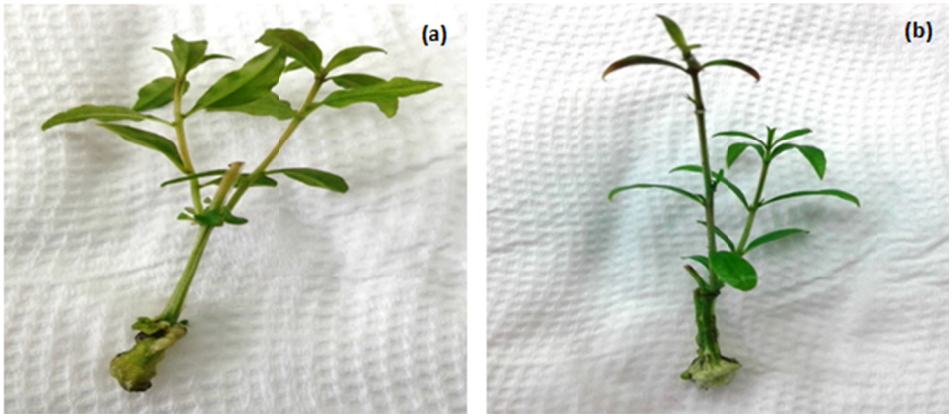

Figure 2. Shoots developed from nodal explants cultured on MS supplemented with 8.8 µM BAP (a); 9.2 µM KN (b)

3.2 Rooting of Shoots

Cultures on half strength MS medium devoid of hormones and all those supplemented with NAA failed to induce roots after six weeks of culture. Supplementation of half strength MS media with IBA and IAA appeared vital for both root induction and root growth. The highest number of roots was on media supplemented with 4.9 µM IBA and the lowest on media supplemented with 1.7 µM IAA (Figure 3). ANOVA showed no significant difference in number of roots on media with 5.3 µM IAA and 4.9 µM IBA at $P \leq 0.05$. Significant difference in number of roots was observed with increasing IBA level up to 4.9 µM, concentrations above this resulted in a significant decrease in number of roots between treatments (Figure 3). There was no significant difference in mean number of roots between IAA treatments except at 5.3 µM IAA where the highest number of roots was recorded (Figure 3). The longest root was observed on media supplemented with 5.3 µM IAA. Both IBA and IAA treatments showed no significant difference in mean length of the longest root with exception at 5.3 µM IAA and 6.9µM IBA (Figure 3). Using grey relational analysis method, both auxins appeared to have the same influence on root induction and root growth from shoots (Table 4). The correlation between auxin type, root induction and root growth was the same for both auxins. Despite this, the difference was found at hormonal levels of each auxin (Table 5). On rankings (Table 5), the optimum IAA and IBA concentration for rooting of shoots was 5.3 µM and 4.9 µM respectively. Shoots which were cultured on MS media supplemented with 4.1 and 5.3 µM IAA, formed callus at the base where the explant was in contact with the media. Multiple shoots were formed from the callus (Figure 5(b)).

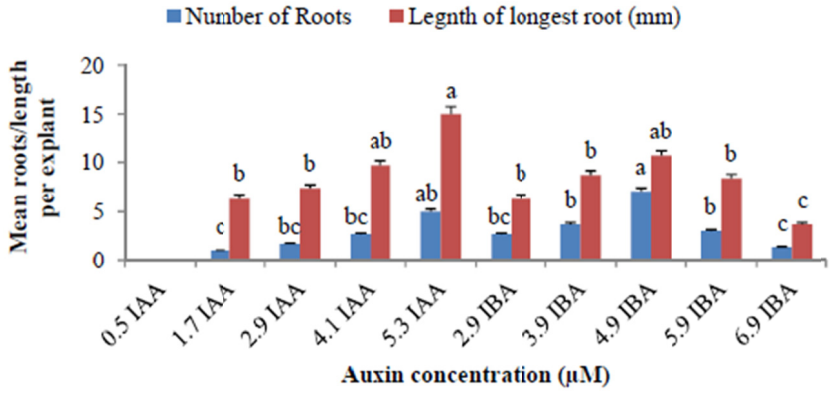

Figure 3. The effect of IAA and IBA on number of induced roots and the length of the longest root developed per shoot

Note. Columns with different letters are significantly different at $P \leq 0.05$ (LSD multiple comparisons test).

Table 4. Correlation between auxin, number of roots and length of the longest root

Auxin	Normalized scores		GRA coefficient		R_i	Rank
	RN	RL	RN	RL		
IAA	0	1	0.33	1.00	0.67	1
IBA	1	0	1.00	0.33	0.67	1

Note. RN (roots number); RL (length of the longest root); R_i (Grey relational analysis grade).

Table 5. Correlation between auxin level, number of roots and length of the longest root

Auxin	Concentration (μM)	Normalized scores		GRA coefficient		R_i	Rank
		RN	RL	RN	RL		
IAA	0.5	0.00	0.00	0.33	0.33	0.33	5
	1.7	0.20	0.42	0.38	0.46	0.42	4
	2.9	0.33	0.49	0.43	0.49	0.46	3
	4.1	0.53	0.64	0.52	0.58	0.55	2
	5.3	1.00	1.00	1.00	1.00	1.00	1
IBA	2.9	0.24	0.38	0.40	0.45	0.43	4
	3.9	0.41	0.71	0.46	0.64	0.55	2
	4.9	1.00	1.00	1.00	1.00	1.00	1
	5.9	0.29	0.67	0.41	0.60	0.51	3
	6.9	0.00	0.00	0.33	0.33	0.33	5

Note. RN (roots number); RL (length of the longest root); R_i (Grey relational analysis grade).

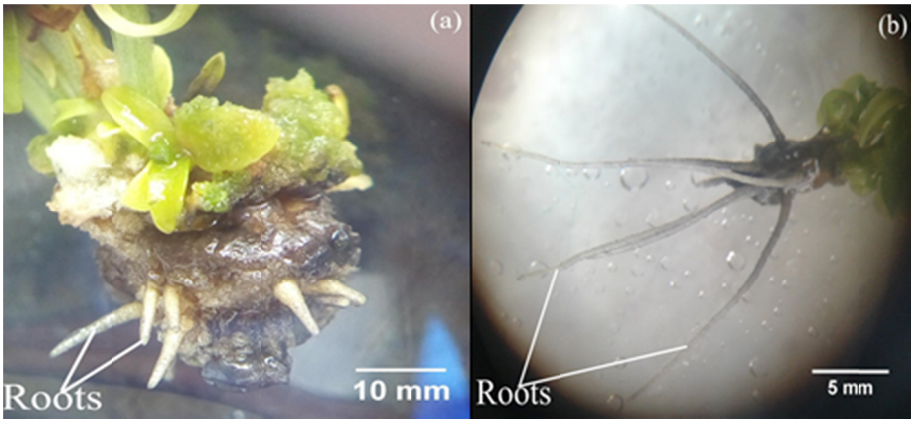

Figure 4. Roots developed from shoots cultured on half strength MS media supplemented with 4.9 μM IBA (a); 5.3 μM IAA (b)

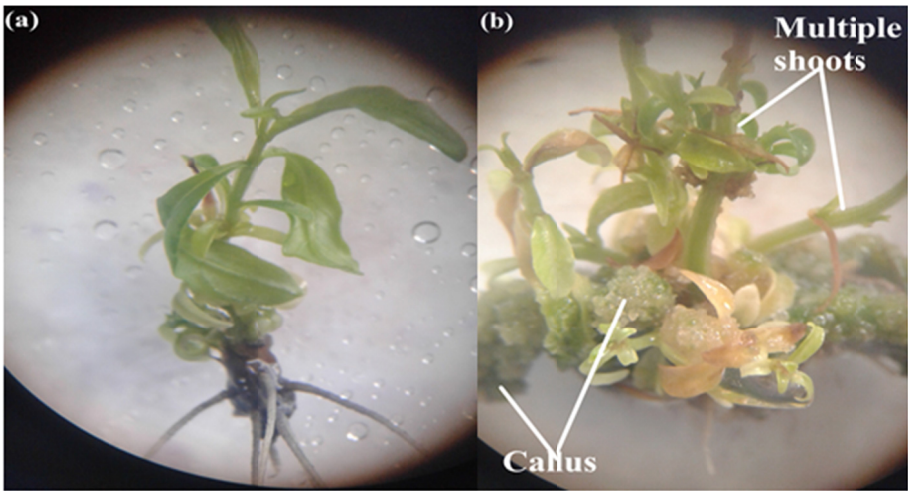

Figure 5. Plantlet on half strength MS media supplemented with 5.3 μM IAA (a); callus development at the base of a shoot with multiple shoots (b)

3.3 Callus Induction

None of the explants cultured on MS media devoid of hormones (control) formed callus. All the explants which were cultured on MS media supplemented with PGR combinations formed callus. Both explants formed callus and a few callus from cotyledon explants formed roots and shoots (Table 6 and Figure 6(b)).

Table 6. The effect of BAP-NAA combination on callus induction from leaf and cotyledon explants of *P. granatum* after four weeks of culture

Treatment/Explant		Cotyledons				Leaves			
BAP (µM)	NAA (µM)	% EFC	% CFR	% CFSR	CC	% EFC	% CFR	% CFSR	CC
0	0	0	0	0	GC	0	0	0	G
2.2	5.4	100	66	0	GC	100	0	0	G
4.4	5.4	100	0	33	GC	100	0	0	G
6.6	5.4	100	0	0	GC	100	0	0	G
8.8	5.4	100	0	0	GC	100	0	0	G
11.0	5.4	100	0	0	GC	100	0	0	G
4.4	2.8	100	0	0	GC	100	0	0	G
4.4	5.4	100	66	0	GC	100	0	0	G
4.4	8.0	100	33	0	GC	100	0	0	G
4.4	10.6	100	0	0	GC	100	0	0	G
4.4	13.2	100	0	0	GC	66	0	0	G

Note. EFC = explant forming callus; CFR = callus forming roots; CFSR = callus forming shoot and roots; CC = Callus colour.

Callus colour: GC (Green and Cream), G (Green).

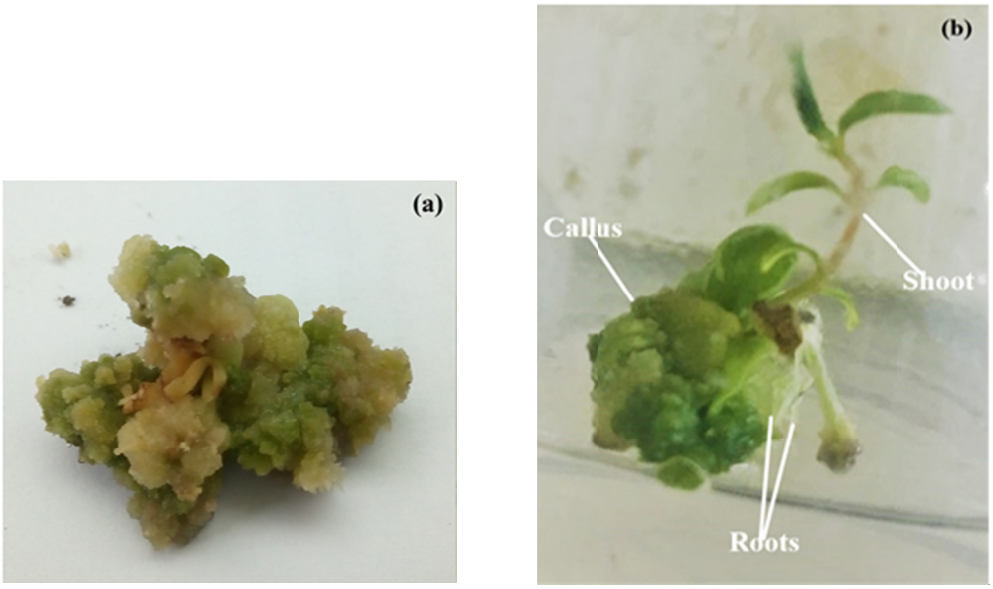

Figure 6. Callus generated from cotyledon explant (a); shoot and roots formed from callus (b)

3.4 Genetic Integrity of Derived Callus and in Vitro Regenerated Shoots

Eight SSR markers generated a total of 13 alleles with an average of 1.75 alleles per marker (Table 7). The allele sizes ranged between 200 and 2000bp. Marker POM 10, POM13, POM 46, PGCT033b/FN677553 and GCT038b/FN677558 gave one allele whilst marker PGCT101b/FN677621 gave two alleles. POM 55 and PGCT091b/FN677611 gave 3 alleles each (Figure 6 and Table 7). Out of 13 alleles, 8 were polymorphic and 5 were monomorphic (Table 7).

Jaccard's similarity coefficient was computed using SPSS version 19 to evaluate genetic similarity between the mother plant, callus and *in vitro* regenerated shoots. The similarity coefficients ranged from 0.46 to 1.00 (Table

8). The lowest genetic similarity 0.46 (46%) was found when P4SL was compared with the MP. The highest genetic similarity of 1.00 (100%) was observed when CC1, CC3, CC4, LC2, LC3 and P1SL were compared to the MP (Table 8). Less genetic similarity was obtained after comparison between MP and (P2SL, P3SL, P4SL). P2SL, P3SL, P4SL had genetic similarity coefficients 0.77, 0.69 and 0.46 to the MP (Table 8).

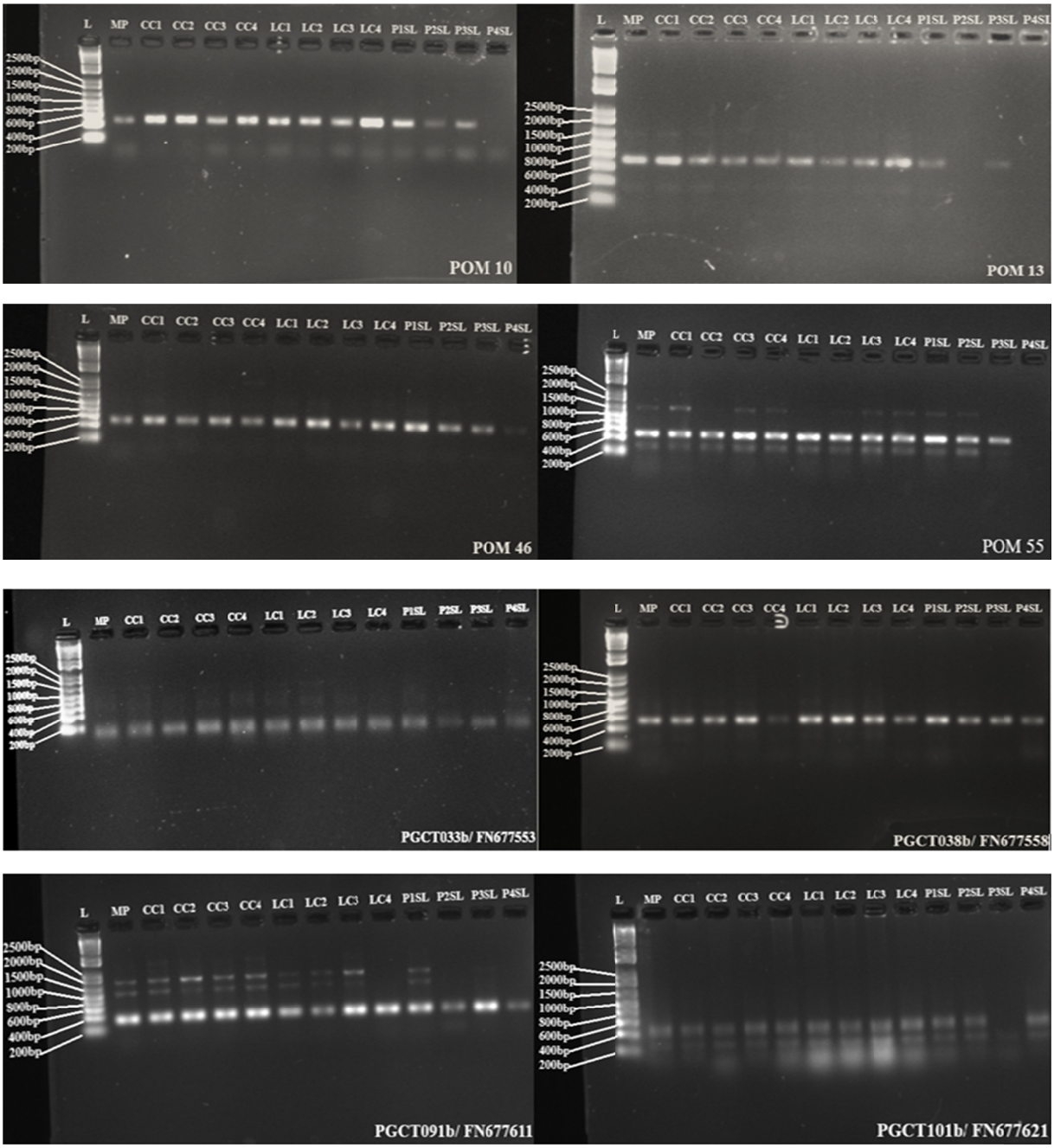

Figure 6. PCR products from mother plant, callus (cotyledons and leaves) and regenerated shoots using SSR markers

Note. MP-mother plant, CC1-cotyledonary callus 1; CC2-cotyledonary callus 2, CC3-cotyledonary callus 3; CC4-cotyledonary callus 4; LC1-leaf callus 1; LC2-leaf callus 2; LC3-leaf callus 3; LC4-leaf callus 4; P1SL-Plantlet 1 shoots leaves; P2SL-Plantlet 2 shoots leaves; P3SL-Plantlet 3 shoots leaves; P4SL-Plantlet 4 shoots leaves.

Table 7. Polymorphism between the mother plant and somaclones as revealed by SSR markers

Marker	A. alleles	M. alleles	P. alleles	Allele size (bp)
POM 10	1	0	1	600
POM 13	1	0	1	800
POM 46	1	1	0	600
POM 55	3	0	3	400-1500
PGCT033b/FN677553	1	1	0	200
PGCT038b/FN677558	1	1	0	600
PGCT091b/FN677611	3	1	2	400-2000
PGCT101b/FN677621	2	1	1	200-400
Total	13	5	8	

Note. A. alleles (Amplified alleles); M. alleles (Monomorphic alleles); P. alleles (Polymorphic alleles).

Table 8. Genetic similarity between *P. granatum* mother plant, derived callus (cotyledons and leaves) and regenerated shoots based on SSR markers

	MP	**CC1**	**CC2**	**CC3**	**CC4**	**LC1**	**LC2**	**LC3**	**LC4**	**P1SL**	**P2SL**	**P3SL**	**P4SL**
MP	1.00												
CC1	1.00	1.00											
CC2	0.92	0.92	1.00										
CC3	1.00	1.00	0.92	1.00									
CC4	1.00	1.00	0.92	1.00	1.00								
LC1	0.92	0.92	1.00	0.92	0.92	1.00							
LC2	1.00	1.00	0.92	1.00	1.00	0.92	1.00						
LC3	1.00	1.00	0.92	1.00	1.00	0.92	1.00	1.00					
LC4	0.85	0.85	0.77	0.85	0.85	0.77	0.85	0.85	1.00				
P1SL	1.00	1.00	0.92	1.00	1.00	0.92	1.00	1.00	0.85	1.00			
P2SL	0.77	0.77	0.69	0.77	0.77	0.69	0.77	0.77	0.91	0.77	1.00		
P3SL	0.69	0.69	0.75	0.69	0.69	0.75	0.69	0.69	0.82	0.69	0.73	1.00	
P4SL	0.46	0.46	0.50	0.46	0.46	0.50	0.46	0.46	0.55	0.46	0.60	0.50	1.00

Note. MP-mother plant, CC1-cotyledonary callus 1; CC2-cotyledonary callus 2, CC3-cotyledonary callus 3; CC4-cotyledonary callus 4; LC1-leaf callus 1; LC2-leaf callus 2; LC3-leaf callus 3; LC4-leaf callus 4; P1SL-Plantlet 1 shoots leaves; P2SL-Plantlet 2 shoots leaves; P3SL-Plantlet 3 shoots leaves; P4SL-Plantlet 4 shoots leaves.

4. Discussion

4.1 Direct Shoot Induction

PGRs are not only important in tissue culture of pomegranate but also in other plants as well. Studies have been done to identify the best hormone and hormonal levels for shoot and root induction as well as for callus formation in pomegranate and other plant species (Patil el al., 2011; Bonyanpour & Khosh-Khui, 2013; Kaji et al., 2013a, 2013b; Parmar, Kanwar, & Thakur, 2013; Geetha, Harathi, & Naidu, 2016; Guranna, Hosamani, Sathyanarayana, Hegde, & Hipparagi, 2017). In this study, it was found that the presence of cytokinins (BAP and KN) in the culture media significantly induced shoots on nodal explants. The response varied between treatments of each cytokinin with KN giving an elite hormonal level for optimum shoot induction and number of leaves on shoots. The difference in response between cytokinins and cytokinin levels may be due to the difference in physiological activity of each hormone and the degree at which they are metabolized and translocated through the explant. This could also be the reason Naik, Pattnaik, and Chand (2000); found contrary research results where KN was less effective compared to BAP in shoot development from pomegranate cotyledon nodal explants. Contrasting results from other authors such as Golozan and Shekafandeh (2010); Kaji et al. (2013b); Thombare, Tiwari, Sapre, and Dattgonde (2017) could have been due to the difference in cultivar, explant age, explant type and type of culture media. Kaji et al. (2013b) observed variation in shoot induction response

between two pomegranate cultivars and attributed the cause to the difference in levels of internal PGRs in each cultivar. This finding was also corroborated by Bonyanpour and Khosh-Khui (2013) who reported that contrary results obtained in shoot induction response was caused by differences in cultivar and explant type. Different results were also obtained from other plant species as well (Khateeb, Bahar, Lahham, Schroeder, & Hussein, 2012; Baskaran, Moyo, & Van Staden, 2013). Idowu, Ibitoye and Ademoyegun (2009) reported that in general explants obtained from different organs vary in their regeneration capabilities and juvenile tissues have higher micropropagation potential than mature tissues.

4.2 Rooting of Shoots

Though there was no response in rooting of shoots on half strength MS media supplemented with NAA and hormone free media, other researchers have successfully used NAA in half strength media for root induction in pomegranate (Naik et al., 2000; Patil el al., 2011; Kaji et al., 2013a; Kaji et al., 2013b; Guranna et al., 2017). Patil el al. (2011); Thombare et al. (2017); Desai et al. (2018) obtained roots from pomegranate nodal explants cultured on media devoid of hormones which is contrary to our results. The reason could be due to explant age, culture media type and cultivar. Also it might be because of the fact that explants of different ages have different levels of endogenous hormones, making the explant age a critical factor in successful rooting. It was proven in this study that IBA and IAA are essential for rooting pomegranate shoots. Roots were obtained in media supplemented with either IBA or IAA. There was a significant difference between auxin levels in terms of number of roots and length of the longest root. The highest root number was obtained on media supplemented with IBA and the longest root was from media supplemented with IAA. The significant difference between treatments could be due to the correlation between auxin level and nodal segment position on the shoot where it came from. Hence causing root induction from shoots cultured on media with low hormonal level. Nor Aini, Guanih and Ismail, (2010) observed that the bottom position node (from the seedling base) of *Gonystylus bancanus* produced many short and thick roots whilst the top position node (towards seedling tip) yielded single thin and long roots.

4.3 Callus Induction

No callus was formed on explants cultured on MS medium devoid hormones. Both leaf and cotyledon explants formed callus when cultured on callus induction media supplemented with a combination of NAA and BAP. This implies that both hormones are essential for callus formation by both explants. Deepika and Kanwar (2010); Kanwar, Joseph, and Deepika (2010); Parmar, Kanwar, and Thakur (2012); Guranna et al. (2017); observed similar results; they induced callus from different pomegranate explants cultured on media supplemented with BAP and NAA combination. Guranna et al. (2017) reported poor quality callus when pomegranate nodal, shoot tip and leaf explants were cultured on MS media supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) alone. Generally, most studies suggest that a combination of auxins and cytokinins are necessary for callus induction in pomegranates.

4.4 Genetic Integrity of Derived Callus and Plantlets

Polymorphism was detected by five out of eight SSR markers. Analysis of results revealed somaclonal variation between the mother plant, callus and regenerated shoots from nodal cuttings. The genetic similarity range obtained signified a wider genetic variation among the tested samples and mother plant. It was shown that it is possible for the variation to occur in callus after four subcultures (subculturing after three weeks in culture) and also after long culture periods of *in vitro* regenerated shoots. However the chances of getting this genetic variation in callus after four subcultures are minimal and cannot be of concern as the genetic similarity was still high at 77% and above. More genetic variation was revealed between the mother plant and *in vitro* regenerated shoots since minimum genetic similarity obtained was 46%.

It was reported that culture initiation and subsequent subculture can induce mutation by exposing explants to oxidative stress with the stress level being dependent on the technique of tissue culture being used. Protoplast culture and callus formation impose more stress than other techniques (Krishna et al., 2016). Callus formation is likely to induce more genetic variation than regeneration from nodal explants. However our results contradict this suggestion because more genetic variation came from leaves of *in vitro* regenerated shoots compared to callus. This may be due to the fact that DNA was extracted from callus and callus is mere proliferation of cells that does not involve differentiation to form organs. Unlike callus, organ formation involves a lot of metabolic and mechanistic rearrangements coupled with complex cell cycle activities to eventually result into organs. The fact that we extracted DNA from leaves of *in vitro* regenerated shoots which were formed by first germinating seeds *in vitro,* getting nodal explants and inducing them to shoot, followed by rooting of shoots implies that the plants were kept *in vitro* for close to four months. Depending on culture conditions, this could have resulted in

somaclonal variation. Using explants from *in vitro* raised seedlings of *Moringa peregrina* (Forsk.) Fiori, Khateeb et al. (2012) obtained no genetic variation among regenerated plants. Similar results were also reported by Mallaya and Ravishankar (2012), where there was no genetic variation found between micropropagated plants of eggplant (*Solanum melongena* L.) cv. Arka Shirish using hypocotyl explants from *in vitro* germinated seedlings. This was contrary to our findings hence suggesting that the variation was due to tissue culture. Kanwar, Thakur, Verma and Sharma (2010) observed genetic variation among *in vitro* raised pomegranate plants using RAPDs and attributed the variation to somaclonal variation. Collin and Edwards (1998) observed that the proportion of abnormal cells can be reduced by minimum period of cultures since tissue culture initiation seems to cause disruption in the cell cycle. Tissue culture involves cells being reprogrammed to acquire an embryonic state sometimes occurring very fast and as a result cause unwanted genetic changes. They also reported that the rate of cell division in explant can be decreased by reducing temperature or PGRs supply hence slowing down processes by tissue culture because fast process can cause genetic changes.

Exposure to hormones for long periods could have also contributed to somaclonal variation in regenerated shoots. Matsuda et al. (2014) reported an increase in genetic variation following addition of plant growth regulators to the culture medium on African violet. They further reported that 2,4-D and BAP are associated with induction of somaclonal variation and that the presence of cytokinins in the media disrupts the cell cycle.

5. Conclusion

Despite more somaclonal variation being found in regenerated shoots, the results indicate that pomegranate growing in Kenya can be regenerated *in vitro*. The established conditions can be used to produce planting materials en mass from nodal explants and induce callus from both leaf and cotyledon explants which can be used in genetic engineering. However further studies are necessary using larger sample size to assess the genetic integrity of plantlets.

Acknowledgements

Authors would like acknowledge Pan African University Institute for Basic Sciences Technology and Innovation (PAUSTI) for providing financial support to undertake the study as well as Institute of Biotechnology Research (IBR), Jomo Kenyatta University of Agriculture and Technology for providing their facilities and technical staff for making our work possible.

References

Baskaran, P., Moyo, M., & Van Staden, J. (2013). *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. *South African Journal of Botany, 90*, 74-79. https://doi.org/10.1016/j.sajb.2013.10.005

Bonyanpour, A., & Khosh-Khui, M. (2013). Callus Induction and Plant Regeneration in *Punica granatum* L. *Nana* from Leaf Explants. *Journal of Central European Agriculture, 14*(3), 928-936. https://doi.org/10.5513/JCEA01/14.3.1285

Collin, H. A., & Edwards, S. (1998). *Introduction to Biotechniques: Plant cell culture*. UK, Bios Scientific Publishers.

Deepika, R., & Kanwar, K. (2010). *In vitro* regeneration of *Punica granatum* L. plants from different juvenile explants. *Journal of Fruit and Ornamental Plant Research, 18*(1), 5-22.

Desai, P., Patil, G., Dholiya, B., Desai, S., Patel, F., & Narayanan, S. (2018). Development of an efficient micropropagation protocol through axillary shoot proliferation for pomegranate variety 'Bhagwa'. *Annals of Agrarian Science*. https://doi.org/10.1016/j.aasci.2018.06.002

Doyle, J. J., & Doyle, J. L. (1987). A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochemical Bulletin, 19*, 11-15.

Ertugrul, I., Oztas, T., Ozcil, A., & Oztas, G. Z. (2016). Grey Relational Analysis Approach in Academic Performance Comparison of University: A Case Study of Turkish Universities. *European Scientific Journal*, 128-139.

Geetha, G., Harathi, K., & Naidu, C. V. (2016). Influence of silver nitrate on *in vitro* callus induction and indirect shoot organogenesis of *Solanum nigrum* (L.)—An important antiulcer medicinal plant. *International Journal of Pharmacy and Biological Sciences, 6*(2), 89-99.

Glozer, K., & Ferguson, L. (2011). *Pomegranate production in Afghanistan*. UCDavis.

Golozan, A. B., & Shekafandeh, A. (2010). Effects of Plant Growth Regulators on Pomegranate (*Punica granatum* L. cv. Rabbab) Shoot Proliferation and Rooting. *Advances in Horticultural Science, 24*(3), 207-211.

Guranna, P. Hosamani, I., Sathyanarayana, R., Hegde, R., & Hipparagi, K. (2017). Micropropagation in Pomegranate (*Punica granatum* L.) cv. 'Bhagwa' through Indirect Organogenesis and Assessment of Genetic Fidelity by RAPD Marker. *Biotechnology Journal International, 20*(3), 1-8. https://doi.org/10.9734/BJI/2017/38806

Hasnaoui, N., Buonamici, A., Sebastiani, F., Mars, M., Trifi, M., & Vendramin, G. G. (2010). Development and characterization of SSR markers for pomegranate (*Punica granatum* L.) using an enriched library. *Conservation Genetics Resources, 2*, 283-285. https://doi. 10.1007/s12686-010-9191-8

Idowu, P. E., Ibitoye, D. O., & Ademoyegun, O. T. (2009). Tissue culture as a plant production technique for horticultural crops. *African Journal of Biotechnology, 8*(16), 3782-3788.

Kahramanoglu, I., & Usanmaz, S. (2016). *Pomegranate production and marketing* (1st ed.). New York, London CRC Press. https://doi.org/10.1201/b20151

Kaji , B. V., Ershadi, A., & Tohidfar, M. (2013b). *In vitro* propagation of two Iranian commercial pomegranates (*Punica granatum* L.) cvs. 'Malas Saveh' and 'Yusef Khani'. *Physiol Mol Biol Plants, 19*(4), 597-603. https://doi. 10.1007/s12298-013-0193-3

Kaji, B. V., Ershadi, A., & Tohidfar, M. (2013a). *In vitro* propagation of pomegranate (*Punica granatum* L. Cv. 'Males Yazdi'. *Albanian J. Agric. Sci, 12*(1), 1-5.

Kanwar, K., Joseph, J., & Deepika, R. (2010). Comparison of *in vitro* regeneration pathways in *Punica granatum* L. *Plant Cell Tiss Organ Cult, 100*, 199-207. https://doi.10.1007/s11240-009-9637-4

Kanwar, K., Thakur, K., Verma, V., & Sharma, R. K. (2010). Genetic Variability of *in vitro* Raised Plants of *Punica granatum* L. by RAPDs. *Fruit, Vegetable and Cereal Science and Biotechnology, 4*(2), 144-147.

Khateeb, W. A., Bahar, E., Lahham, J., Schroeder, D., & Hussein, H. (2012). Regeneration and assessment of genetic fidelity of the endangered tree *Moringa peregrina* (Forsk.) Fiori using Inter Simple Sequence Repeat (ISSR). *Physiol Mol Biol Plants, 19*(1), 157-164. https:// doi.10.1007/s12298-012-0149-z

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M., & Sadh, R. K. (2016). Somaclonal variations and their applications in horticultural crops improvement. *Biotech, 6*, 54. https://doi.10.1007/s13205-016-0389-7

Mallaya, N. P., & Ravishanka, G. A. (2012). *In vitro* propagation and genetic fidelity study of plant regenerated from inverted hypocotyl explants of eggplant (*Solanum melongena* L.) cv. Arka Shirish. *3 Biotech, 3*, 45-52. https://doi.10.1007/s13205-012-0068-2

Materechera, S. A., & Seeiso, T. M. (2013). Seed Treatment to Improve Water Imbibition and Germination of Pomegranate (*Punica granatum*). *Acta Hort., 979*, 713-722. https://doi.org/10.17660/ActaHortic.2013.979.80

Matsuda, S., Sato, M., Ohno, S., Yang, S., Doi, M., & Hosokawa, M. (2014). Cutting Leaves and Plant Growth Regulator Application Enhance Somaclonal Variation Induced by Transposition of *VGs1* of *Saintpaulia*. *J. Japan. Soc. Hort. Sci, 83*(4), 308-316. https://doi.10.2503/jjshs1.MI-009

Naik, S. K. Pattnaik, S., & Chand, P. K. (2000). High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (*Punica granatum* L.). *Scientia Horticulturae, 85*, 261-270. https://doi.org/10.1016/S0304-4238(99)00149-1

Nor Aini, A. S., Guanih, V. S., & Ismail, P. (2010). Effect of cutting positions and growth regulators on rooting ability of *Gonystylus bancanus*. *African Journal of Plant Science, 4*(8), 290-295.

Parmar, N., Kanwar, K., & Thakur, A. K. (2012). *In Vitro* Organogenesis from Cotyledon Derived Callus Cultures of *Punica granatum* L. cv. Kandhari Kabuli. *Natl. Acad. Sci. Lett., 35*(3), 215-220. https://doi.10.1007/s40009-012-0041-y

Parmar, N., Kanwar, K., & Thakur, A. K. (2013). Direct Organogenesis in *Punica granatum* L. cv. Kandhari Kabuli from Hypocotyl Explants. *Proc. Natl. Acad. Sci. India, Sect. B Biol. Sci*. https://doi.10.1007/s40011-013-0178-6

Patil, V. M., Dhande, G. A., Thigale, D. M., & Rajput, J. C. (2011). Micropropagation of pomegranate (*Punica granatum* L.) 'Bhagava' cultivar from nodal explant. *African Journal of Biotechnology, 10*(79), 18130-18136. https:// doi.10.5897/AJB11.1437

Singh, P., Patel, R. M., & Kadam, S. (2013). *In vitro* mass multiplication of pomegranate from cotyledonary nodal explants cv. *Ganesh*. *African Journal of Biotechnology, 12*(20), 2863-2868. https://doi.org/10.5897/AJB2013.11962

Sinjare, D. Y. (2015). Application of Microsatellite SSR Markers in a Number of Pomegranate (*Punica granatum* L.) Cultivars in Kurdistan Region/Duhok Province. *International Journal of Chemical and Biomolecular Science, 1*(3), 117-122.

Thombare, D., Tiwari, S., Sapre, S., & Dattgonde, N. (2017). An efficient regeneration protocol for pomegranate (*Punica granatum*) cv. bhagwa from nodal and meristem explants. *The Bioscan, 11*(0), 1-4.