Plant Cell Tiss Organ Cult (2018) 132:201–212
DOI 10.1007/s11240-017-1326-0

ORIGINAL ARTICLE

Effect of anti-ethylene compounds on isoenzyme patterns and genome stability during long term culture of *Moringa oleifera*

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Received: 21 March 2017 / Accepted: 19 October 2017 / Published online: 30 October 2017

Abstract Multiplication of *Moringa oleifera* shoots on MS medium supplemented with 2.5 µM BAP for 3 weeks resulted in shoot vitrification which led to chlorosis, retardation of shoot formation, reduction in shoot length, necrosis of shoot tips and formation of friable calli on the base of cultured explants. Vitrification symptoms decreased when MS medium containing 2.5 µM BAP in combination with 10 µM $AgNO_3$, 50 µM salicylic acid (SA) or 200 µM $CoCl_2$ was used. Studying isoenzyme patterns of SOD, POX, CAT, GOT and EST indicated that moringa shoots multiplied without obvious variation in isoenzyme patterns up to 7 subcultures. Moringa shoots subjected to 14 subcultures and anti-ethylene compounds showed variation in isoenzyme patterns and were associated with the disappearance of vitrification which facilitated root formation and acclimatization. Under long term cultures, RAPD, ISSR and SSR indicated that $AgNO_3$ was the optimal anti-ethylene substance for avoidance of vitrification in moringa but it resulted in high somaclonal variation. Application of SA decreased vitrification as well as somaclonal variation compared to $CoCl_2$ under long term culture. Consequently, SA was recommended for moringa clonal multiplication.

Keywords Anti-ethylene · Gene expression · Isoenzymes · Molecular markers · Moringa micropropagation

Communicated by Sergio J. Ochatt.

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Abbreviations

BAP	Benzyl amino purine
SA	Salicylic acid
EST	Esterases
GOT	Glutamate oxaloactate transaminases
SOD	Superoxide dismutases
CAT	Catalases
POX	Peroxidases
ISSR	Simple Sequence Repeat
RAPD	Random Amplified Polymorphic DNA
SSR	Simple Sequence Repeat technique

Introduction

Plant tissues were cultured under controlled conditions for clonal multiplication, induction of genetic variation or study of plant metabolism and differentiation (Hassanein 2004; Hassanein et al. 2005, 2008). During in vitro culture, dehydration of the cultured plant tissue is avoided, sterile conditions are conserved, and physical and chemical conditions are well determined, conditions that cannot be exactly achieved in the greenhouse or field. On the other side, under in vitro conditions, cultured plant tissue faces an artificial environment leading to growth aberrations (vitrification) and genetic instability (Larkin and Scowcroft 1981; Kaeppler et al. 2000). While vitrification should be avoided during clonal multiplication, somaclonal variation should be induced for plant improvement (Jainet al. 1998).

Vitrification or hyperhydricity of cultured tissues occurs due to stress factors imposed by in vitro culture conditions. These factors include high relative humidity, accumulation of ethylene (Chen and Ziv 2001) and limitation of oxygen leading to hypoxia (Rojas-Martínez et al. 2010). Vitrification results in slowing down of the cell

cycle and an increasing of water content of the cultured tissue. Also, vitrification expresses abnormal lignification, disorganized cell wall, fragile leaves, loss of apical dominance, reduction of shoot multiplication, necrosis of shoot tips, poor acclimatization, and impaired stomatal function. In addition, vitrification causes reduction of some metabolic components such as chlorophyll, protein and phenolics, alteration of ion composition, and inhibition of H_2O_2 detoxification enzymes but it induces the expression of lipid peroxidation enzymes (Gaspar 1991; Franck et al. 1998; Kei-ichiro et al. 1998; Cassells and Curry 2001; Machado et al. 2014; Frank et al. 2004; Isah 2015). Vitrification can be avoided by reducing the relative humidity, improving the aeration within culture vessels (Saez et al. 2012; Salem 2016), increasing the concentration of agar (Debergh et al. 1981) and application of anti-ethylene compounds such as $CoCl_2$, $AgNO_3$ or salicylic acid (Wang et al. 2002; Mirza et al. 2015; Isah 2015).

Isoenzyme patterns were used to test the role of anti-ethylene compounds to control vitrification and determine the number of subcultures that should be applied to avoid somaclonal variation during in vitro mass propagation (Hassanein 2004; Balen et al. 2004; Hassanein et al. 2008; Salem 2016).

Abnormalities on in vitro grown plants increased with increasing of culture age or long term culture and lead to genetic instability (Kaeppler et al. 2000). Genetic instability resulted from chromosomal changes in number or structure, transposable elements, or possibly pre-existing genetic changes in the donor plant (Isah 2015). To detect somaclonal variation, molecular techniques such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) were recommended (Isah 2015; Butiuc-Keul et al. 2016; Saha et al. 2016).

Vitrification of the in vitro cultured moringa due to ventilation deficiency and accumulation of ethylene in cultured jars was reported by Salem (2016). Mild ventilation and anti-ethylene compounds were used to avoid this phenomenon (Isah 2015; Mirza et al. 2015; Salem 2016). Vitrification and the associated somaclonal variation are two aspects retarding the application of in vitro techniques as effective vegetative micropropagation tools in moringa (Hassanein et al. 2008; Mirza et al. 2015; Salem 2016). There are no studies to evaluate which anti-ethylene compounds can be used to avoid severe vitrification and somaclonal variations in moringa. Consequently, the objective of the current study was to evaluate the effect of anti-ethylene compounds on vitrification, isoenzymes and somaclonal variation during long term culture of moringa plants.

Materials and methods

Plant source

Moringa oleifera seeds were deprived of their coats and sterilized in 70% ethyl alcohol for 3 min, and 0.1% mercuric chloride for 5 min. After three successive rinses in sterile deionized water for 5 min each, seeds were germinated on cotton dampened by tap water in 250 ml glass jars for 6 days. Shoot segments were cut and transferred for in vitro culture on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 8 g l^{-1} agar and 2.5 µM BAP. Cultures were incubated at 28 ± 1 °C under 16 h of daily light at 100 µmol m^{-2} s^{-1} (initial culture). Shoots obtained from initial cultures were transferred to a new medium (first subculture) for further experiments.

Shoot multiplication under the influence of different concentrations of BAP and anti-ethylene compounds

Nodal segments of the initial culture were cut and subcultured on MS medium containing two concentrations of BAP (2.5 or 10 µM) in combination with three concentrations of each anti-ethylene compound: $AgNO_3$ (10, 25 and 75 µM), Salicylic acid (50, 100 and 200 µM) or $CoCl_2$ (50, 100 and 200 µM). Thirty explants for each treatment were cultured in three glass jars. Cultures were incubated for three weeks at 28 ± 1 °C under 16 h of daily light at 100 µmol m^{-2} s^{-1}. Number of shoots/explant, shoot length, number of leaves/shoot, number of nodes/shoot and fresh weight/shoot were estimated.

Influence of BAP concentrations, anti-ethylene compounds and multiple subcultures on shoot multiplication, isoenzyme patterns and genome stability

Nodal or shoot cuttings obtained from shoots of the initial culture were subcultured 14 times on MS medium supplemented with 2.5 or 10 µM of BAP in combination with 10 µM $AgNO_3$, 200 µM $CoCl_2$ or 50 µM SA. For shoot multiplication, ten explants were cultured in 250 ml glass jars containing 30 ml MS medium solidified with 8 gm l^{-1} agar. Plant cuttings were subcultured at 3 weeks interval for 3 months, followed by once a month for 10 months. Generally, shoots of the first subculture on MS media supplemented with 2.5 µM BAP were used as the control. Shoots subjected to 7 and 14 subcultures were used for protein extraction and isoenzymes analysis. Shoot cultures subjected to 14 subcultures were used for genomic DNA extraction and PCR analysis using different molecular

markers (RAPD, ISSR and SSR) to detect somaclonal variation under the influence of the applied conditions.

Protein extraction and isoenzyme analysis

For isoenzyme analysis, one gram of in vitro grown shoots was ground in 1 ml of extraction buffer (0.1 µM Tris-HCl, pH 7.0 and 0.002 M cysteine) at 4 °C. The homogenate was centrifuged for 15 min at 13,500 rpm and 4 °C. Supernatants were transferred for immediate electrophoresis using 7.5% polyacrylamide slab gels in running buffer (0.025 M Tris + 0.129 M glycine at pH 8.9) at 24 mA for 6 h at 10 °C. Superoxide dismutase (SOD) was stained according to Beauchamp and Fridovich (1971), peroxidase (POX) according to the method of Siegel and Galston (1967), catalase (CAT) according to Woodbury et al. (1971), and glutamate oxaloacetate transaminase (GOT) and esterase (EST) according to the method of Brewer (1970).

Genomic DNA extraction

Total genomic DNAs were extracted from ten shoot cultures subjected to 14 subcultures on MS medium supplemented with 2.5 µM BAP and 10 µM AgNO3, 200 µM CoCl2 or 50 µM SA as described by Porebski et al. (1997). Genomic DNA extracted from shoots of the first culture on MS medium supplemented with 2.5 µM BAP was used as a control.

RAPD analysis

Twelve RAPD primers (OPA-02, OPA-08, OPad-6, OPaf-20, OPC-02, OPC-05, OPD-01, OPE-02, OPG-09, OPK-02, OPK-03 and OPP-13) were tested to amplify the template DNA extracted from shoots grown on MS medium containing 2.5 µM BAP and 10 µM $AgNO_3$ for 14 subcultures. PCR reaction mixtures (6.5 µl deionized H_2O, 12.5 µl master mix, 3 µl primer and 3 µl template DNA) were used for DNA amplification. Amplification was carried out in a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle at 94 °C for 5 min. Each cycle consisted of a denaturation at 94 °C for 45 s, annealing at 36 °C for 50 s, and elongation at 72 °C for 1 min. The primer extension segment was extended to 7 min at 72 °C in the final cycle.

Five of twelve RAPD primers were selected for DNA amplification of plant materials cultured on 2.5 µM BAP and 200 µM $CoCl_2$ or 50 µM SA.

ISSR analysis

A total of ten ISSR primers (ISSR1, ISSR2, ISSR3, ISSR4, ISSR5, ISSR6, ISSR7, ISSR8, ISSR9 and ISSR10) were used to amplify the template DNA extracted from shoots grown on MS medium containing 2.5 µM BAP and 10 µM $AgNO_3$ for 14 subcultures. The ISSR-PCR method was carried out according to Nagaoka and Ogihara (1997). Amplification reactions were carried out in 25 µl volumes containing the same components of RAPD reactions except that primers were exchanged by ISSR primers. The amplification conditions were the same as RAPD conditions except that the annealing temperature varied among primers.

Out of the ten ISSR primers, five were selected based on their amplification products to apply for plant materials cultured on 2.5 µM BAP and 200 µM $CoCl_2$ or 50 µM SA.

SSR analysis

A total of five SSR primers (SSR1, SSR2, SSR3, SSR4 and SSR5) were used to amplify the template DNA extracted from shoots grown on MS medium containing 2.5 µM BAP and 10 µM $AgNO_3$, 200 µM $CoCl_2$ or 50 µM SA for 14 subcultures. Amplification mixtures were prepared in 25 µl volumes containing the same components of RAPD reactions except that primers were exchanged by SSR primers (1.5 µl of forward and 1.5 µl reverse primer). The amplification conditions were the same as mentioned above on RAPD amplification conditions except that the annealing temperature varied among primers. The primer extension was extended to 10 min at 72 °C in the final cycle (Senior et al. 1998).

Visualization of DNA fragments

Amplification products were resolved by horizontal gel electrophoresis using 1.5% agarose gel containing ethidium bromide (0.5 µg ml^{-1}) in 1× TAE buffer in the case of RAPD products and 2% agarose in the case of ISSR and SSR. Electrophoresis was carried out under constant voltage of 70 V for 1 h. Data matrices of RAPD, ISSR and SSR banding patterns were scored for clear and distinct amplification products as present (1) or absent (0). Cluster analysis was performed using Un-weighted Pair Group Method with Arithmetic Averages (UPGMA; Sneath and Sokal 1973).

Statistical analysis

Experiments were designed in a completely randomized design and data were statistically analyzed using standard deviation (SD) according to the method described by Snedecor and Cochran (1980).

Results

Moringa shoots were initiated from shoot cuttings of aseptic grown seedlings on MS medium supplemented with 2.5 µM BAP (initial subculture). Enlargement on the base of shoot cuttings was observed at day 7 from which several shoots were initiated. Nodal segments were cut and subcultured (first subculture) on the same medium. Moringa shoots vitrification such as chlorosis, necrosis of some shoot tips and formation of friable calli at the base of explants was observed within 3 weeks (Fig. 1). Increasing the concentration of BAP to 10 µM increased vitrification which resulted in decreasing the number of formed shoots and their length and increasing the callus size at the base of the cultured plant segments (Fig. 1).

Fig. 1 Photograph shows in vitro shoot multiplication and growth on MS medium supplemented with 2.5 µM BAP for 3 weeks, arrow refers to vitrified shoot

For mass multiplication of moringa, shoots obtained from the first subculture were subjected to 13 subcultures on MS medium containing two concentrations of BAP (2.5 and 10 µM). The number of obtained shoots on MS medium with 2.5 µM BAP increased during the 2nd, 3rd and 4th subcultures but decreased when the number of subcultures was more than five (Table 1). This reduction in shoot number was associated with reduction of all the estimated parameters such as the number of nodes/shoot. The number of shoots/explant on MS medium with 10 µM BAP had the same trend but lower than those on MS with 2.5 µM BAP.

To study the effect of several anti-ethylene compounds on in vitro multiplication of moringa, plant cuttings were subcultured on MS medium supplemented with 2.5 µM BAP and different concentrations of SA, $AgNO_3$ and $CoCl_2$ for 3 weeks (Table 2). Anti-ethylene compounds, especially 10 µM $AgNO_3$, 50 µM SA or 200 µM $CoCl_2$ decreased the appearance of verification aspects on cultured moringa shoots. These concentrations of anti-ethylene compounds expressed the highest number of formed shoots/explant and nodes/shoot, $AgNO_3$ was the best one (Table 2 and Fig. 2). Friable calli at the base of the cultured explants were reduced under the influence of $AgNO_3$ or SA, and associated

Table 1 Moringa in vitro shoot multiplication and growth on MS medium supplemented with 2.5 µM BAP and subjected for several subcultures

Subculture	No. of shoots/explant	Shoot fresh weight/cluster (gm)	Shoot length (cm)	Shoot fresh weight/shoot (gm)	No. of nodes/shoot
S1	4.7 ± 0.6	0.49 ± 0.04	3.83 ± 0.29	0.36 ± 0.47	3.0 ± 0.00
S2	6.3 ± 0.5**	0.61 ± 0.02*	2.80 ± 0.36**	0.07 ± 0.02**	1.9 ± 0.50**
S3	7.3 ± 0.5**	0.89 ± 0.08**	2.58 ± 0.15**	0.05 ± 0.01**	1.9 ± 0.50**
S4	5.8 ± 1.2**	0.77 ± 0.10**	2.24 ± 0.34**	0.05 ± 0.01**	1.7 ± 0.52**
S5	3.8 ± 0.5**	0.51 ± 0.14	1.68 ± 0.30**	0.02 ± 0.01**	1.0 ± 0.00**
S6	1.7 ± 0.4**	0.33 ± 0.06**	1.45 ± 0.26**	0.03 ± 0.03**	1.5 ± 0.58**
S7	1.4 ± 0.4**	0.28 ± 0.08**	1.42 ± 0.51**	0.04 ± 0.02**	1.5 ± 0.55**
S8	1.5 ± 0.5**	0.26 ± 0.06**	1.00 ± 0.25**	0.02 ± 0.00**	1.3 ± 0.55**
S9	1.4 ± 0.5**	0.13 ± 0.05**	1.63 ± 0.71**	0.03 ± 0.02**	1.2 ± 0.46**
S10	1.3 ± 0.5**	0.20 ± 0.06**	1.52 ± 0.50**	0.04 ± 0.03**	1.1 ± 0.38**
S11	2.3 ± 0.5**	0.18 ± 0.03**	1.32 ± 0.36**	0.06 ± 0.03**	1.5 ± 0.55**
S12	2.4 ± 0.5**	0.19 ± 0.03**	1.32 ± 0.43**	0.05 ± 0.04**	1.6 ± 0.55**
S13	2.2 ± 0.4**	0.16 ± 0.02**	1.06 ± 0.32**	0.04 ± 0.01**	2.0 ± 0.00**
S14	2.2 ± 0.4**	0.18 ± 0.01**	1.10 ± 0.27**	0.04 ± 0.01**	2.0 ± 0.00**

Values are means of three replicates ± standard deviation (SD)

Statistical significance of differences compared to control (S1)

*Significant at $P < 0.05$; **significant at $P < 0.01$

Table 2 Moringa in vitro shoot multiplication and growth on MS medium supplemented with 2.5 µM BAP and different concentrations of SA, $AgNO_3$ and $CoCl_2$ for 3 weeks

Treatments (µM)	No. of shoots/explant	Shoot fresh weight/ cluster (gm)	Shoot length (cm)	Shoot fresh weight/ shoot (gm)	No. of nodes/shoot
2.5 BAP	5.3 ± 0.6	0.80 ± 0.12	2.77 ± 0.25	0.08 ± 0.00	3.3 ± 0.6
10 $AgNO_3$	8.3 ± 0.6**	0.89 ± 0.33	2.67 ± 0.29	0.05 ± 0.01	4.7 ± 0.6
25 Ag NO_3	6.0 ± 1.0	0.48 ± 0.08	2.37 ± 0.32	0.04 ± 0.00	4.7 ± 0.6
75 Ag NO_3	2.0 ± 1.0**	0.20 ± 0.03**	1.90 ± 0.46	0.19 ± 0.03	2.0 ± 1.0
50 $CoCl_2$	5.0 ± 1.7	0.44 ± 0.09*	1.87 ± 0.55	0.03 ± 0.02	2.3 ± 0.6
100 $CoCl_2$	4.7 ± 1.2	0.48 ± 0.08	2.73 ± 0.25	0.27 ± 0.19*	3.7 ± 0.6
200 $CoCl_2$	6.3 ± 0.6	0.72 ± 0.44*	3.37 ± 0.51	0.05 ± 0.01	3.0 ± 1.0
50 SA	5.3 ± 0.6	0.41 ± 0.18	2.20 ± 0.52	0.07 ± 0.03	4.0 ± 2.0
100 SA	4.3 ± 0.6	0.34 ± 0.16*	2.40 ± 0.53	0.04 ± 0.02	4.0 ± 0.0
200 SA	4.7 ± 0.6	0.39 ± 0.09*	2.67 ± 0.57	0.05 ± 0.01	3.7 ± 0.6

Values are means of three replicates ± standard deviation (SD)

Statistical significance of differences compared to control (56 BAP)

*Significant at $P < 0.05$; **significant at $P < 0.01$

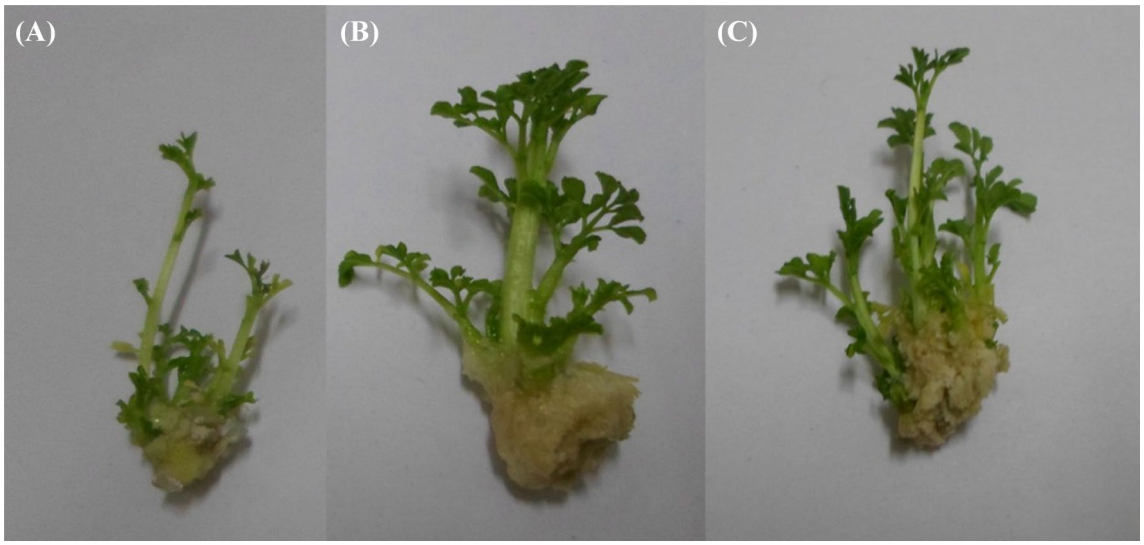

Fig. 2 Photographs show in vitro shoot multiplication and growth on MS medium supplemented with 2.5 µM BAP and 50 µM SA (**a**), 200 µM CoCl2 (**b**) and 10 µM AgNO3 (**c**) for 3 weeks

with normal phenotypes of the formed shoots which facilitated root formation on shoot cuttings and their transfer to open conditions (Fig. 3). On the other hand, some aspects of verification such as thick shoots did not disappear when 200 µM $CoCl_2$ was used (Fig. 2).

The number of shoots/explant, shoot length and the number of nodes/shoot improved when the selected concentration of anti-ethylene compounds was used (Table 3). This improvement depended on the number of subcultures and type of anti-ethylene compound. Salicylic acid during the 12th and 13th subcultures expressed the highest number of shoots/explant and shoot length compared to $AgNO_3$ and $CoCl_2$. The improvement of anti-ethylene compounds on shoot multiplication decreased if the number of subcultures was increased more than 11 (Table 3).

Shoots of the first and seventh subcultures expressed the same number of SOD bands (Fig. 4a, lanes 1 and 2). Generally, the number and intensity of SOD bands increased with the increasing number of subcultures on MS medium containing high or low BAP concentrations in combination with anti-ethylene compounds. Two SOD bands (SOD-6 and SOD-7) were detected when shoots were cultured for 7 or 14 subcultures on MS medium supplemented with 2.5 or 10 µM BAP in combination with 200 µM $CoCl_2$ or 50 µM

Fig. 3 Acclimatized moringa shoot grown in soil under lab conditions for 8 weeks

SA. These two bands were not detected in shoots of the first (control) or 7th subculture without anti-ethylene compounds or with 10 µM $AgNO_3$.

Peroxidase patterns were influenced by the concentration of BAP in MS medium, number of subcultures and anti-ethylene type (Fig. 4b). Shoots of the first subculture (lane 1) and shoots of 7th or 14th subculture with 10 µM $AgNO_3$ (lanes 6 and 7) expressed the same pattern. Under the influence of other treatments (200 µM $CoCl_2$ or 50 µM SA), the number of peroxidase bands decreased especially when SA was used.

The catalase banding patterns of moringa shoots (Fig. 4c) of the first subculture (lane 1) and 7th subculture (lane 2) included three bands (CAT-5, CAT-6 and CAT-7). Application of 10 µM BAP or anti-ethylenes increased CAT expression under the influence of long term culture. It was higher in shoots of 14 subcultures than that of 7 subcultures.

GOT banding patterns of moringa shoots subcultured for the first time on MS medium with 2.5 µM BAP and the shoots of 7th subculture expressed the same pattern (Fig. 4d). Two bands (GOT-1 and GOT-2) disappeared under the influence of all the applied anti-ethylene compounds in MS medium. In addition, GOT-5 disappeared under the influence of long term culture irrespective the type of anti-ethylene compounds. Generally, long term culture resulted in decreasing the intensity of the detected bands.

On MS medium with 2.5 µM BAP, moringa shoots of the first and 7th subcultures expressed the same banding pattern of esterase isoenzyme forms (Fig. 4e). Isoenzyme forms ES-1, EST-2 and ES-3 disappeared but EST-8 appeared under the influence of high or low concentrations of BAP in combination with any type of anti-ethylene compounds. The number of esterase isoenzyme forms and the intensity of some bands of shoots subcultured for 14 subcultures were higher than shoots subcultured seven times.

The dendrogram (Fig. 5) obtained from the banding pattern of the five investigated isoenzymes was studied. The first clade indicated that shoots subcultured on MS medium with 2.5 µM BAP for the first and 7th subculture expressed

Table 3 Percentage of increase in number of formed shoots/explant, length of shoot and number of nodes/shoot of explants subjected for 14 subcultures on MS medium with 2.5 µM BAP + anti-ethylene compounds (10 µM $AgNO_3$, 200 µM $CoCl_2$ or 50 µM SA) in comparison to those grown on MS medium supplemented with 2.5 µM BAP only (Table 1)

Sub. no.	$AgNO_3$			SA			$CoCl_2$		
	Increase in shoot no. (%)	Increase in shoot length (%)	Increase in node no. (%)	Increase in shoot no. (%)	Increase in shoot length (%)	Increase in node no. (%)	Increase in shoot no. (%)	Increase in shoot length (%)	Increase in node no. (%)
S1	214	105	211	143	71	67	136	84	122
S2	174	157	248	214	120	267	116	103	171
S3	164	117	267	158	139	248	95	106	190
S4	168	111	180	114	119	200	74	89	180
S5	200	93	267	121	146	233	115	72	233
S6	367	120	200	131	152	133	283	135	200
S7	233	95	156	350	213	133	200	157	200
S8	217	348	225	350	125	125	200	188	200
S9	280	169	160	217	282	213	200	135	160
S10	325	139	175	260	202	204	225	129	175
S11	214	224	133	350	141	133	129	158	133
S12	100	126	125	157	212	146	100	105	104
S13	100	133	100	129	154	100	100	145	83
S14	117	209	100	100	185	100	100	134	100

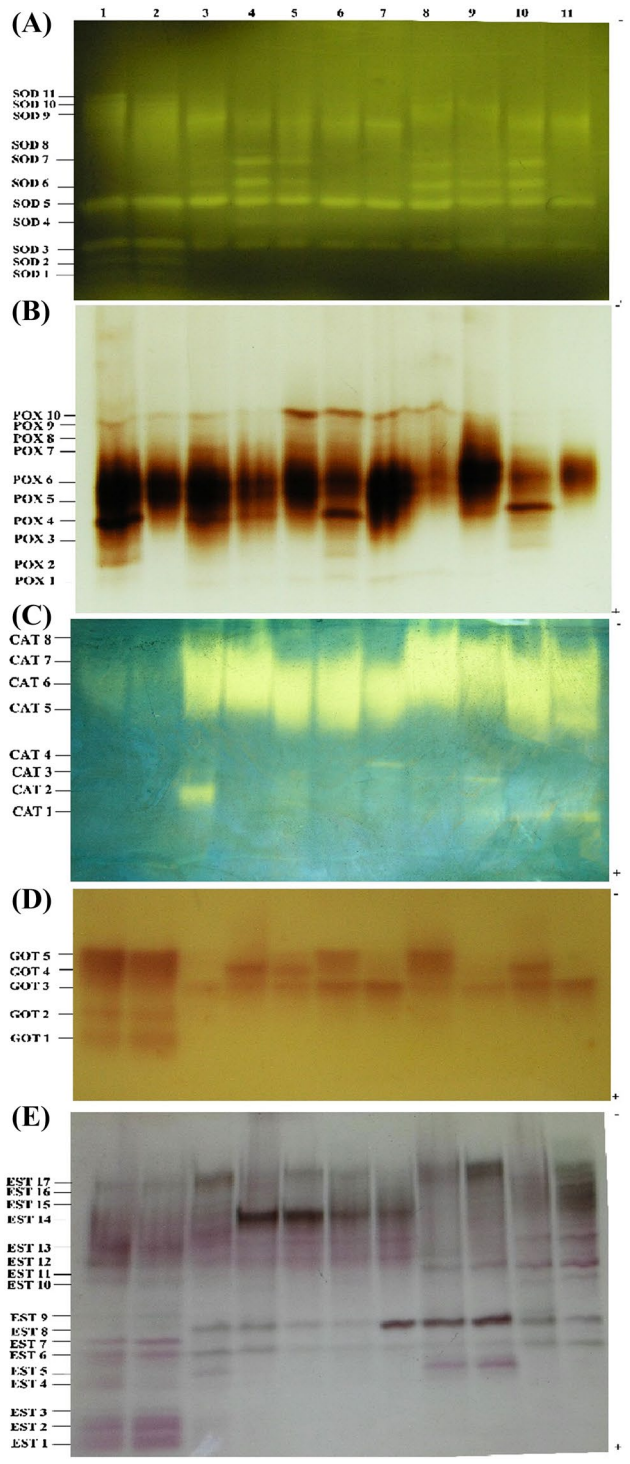

Fig. 4 Native PAGEs of SOD (**a**), POX (**b**), CAT (**c**), GOT (**d**) and Esterase (**e**) show the effect of different concentrations of anti-ethylene compounds on moringa shoots subcultured on MS medium containing 2.5 µM BAP for the 1st (lane 1), 7th (lane 2) and 14th subcultures (lane 3), on MS containing 10 µM BAP for the 7th (lane 4) and 14th subcultures (lane 5), on MS containing 2.5 µM BAP + 10 µM AgNO3 for the 7th and (lane 6) and 14th subcultures (lane 7), on MS containing 2.5 µM BAP + 200 µM $CoCl_2$ for the 7th (lane 8) and 14th subcultures (lane 9), or on MS containing on 2.5 µM BAP + 50 µM SA for the 7th (lane 10) and 14th subcultures (lane 11)

the same banding patterns. The highest variation in protein patterns was detected when shoots were subjected for long term culture (lane 3) or with high concentrations of BAP (lanes 4 and 5). Salicylic acid (lanes 10 and 11) was better than $AgNO_3$, (lanes 6 and 7) or $CoCl_2$ (lanes 8 and 9).

Ten RAPD primers were used to amplify the genomic DNA of ten moringa shoots subjected to 14 subcultures. In addition, shoots of the first subculture were used as a control. The shoots were subcultured on MS medium containing 2.5 µM BAP and different anti-ethylene compounds. In case of 10 µM $AgNO_3$, forty out of 110 polymorphic fragments (36.36%) were obtained using 10 primers. No amplicons were obtained using OPC-05 and OPK-02 primers. Polymorphism ranged from 33.33% (OPG-08) to 64.3% (OPP-13) with an average of 48.82%. The highest polymorphic bands were obtained when OPP-13 was used (Fig. 6a). Five primers were selected to amplify genomic DNA of shoot cultures subcultured on MS with 2.5 µM BAP in combination with 200 µM $CoCl_2$ or 50 µM SA. Amplification of DNA extracted from in vitro grown shoots for 14 subcultures on MS medium supplemented with $CoCl_2$ shows DNA polymorphism ranging from 42.86% using OPA-08 to 64.71% using OPP-13, with an average of 53.97%. The RAPD primer OPP-13 gave the highest number of polymorphic bands (Fig. 6b). In the case of SA, five primers gave DNA polymorphism ranging from 40% (OPA-02) to 70% (OPA-08), with an average of 58.85% (Fig. 6c).

Shoot lines grown for 14 subcultures on MS medium containing 2.5 µM BAP and 10 µM $AgNO_3$ were analyzed using ISSR primers. DNA polymorphism of the studied moringa lines ranged from 28.6% (ISSR5) to 71.5% (ISSR6) with an average of 50.0% (Fig. 7). Five primers were selected to study DNA polymorphism of shoots subjected to $CoCl_2$ or SA for 14 subcultures. Under the influence of $CoCl_2$, polymorphism among shoot cultures ranged from 30.0% (ISSR3) to 53.85% (ISSR9), with an average of 41.41%. Polymorphism among moringa shoots due to SA ranged from 22.22% (ISSR10) to 62.50% (ISSR6) with an average of 38.99%.

Five SSR primers were used to detect polymorphism between ten shoots under the influence of anti-ethylene compounds for 14 subcultures. On $AgNO_3$ containing medium, the polymorphism between cultures varied between 0% (SSR1, SSR3, SSR4, and SSR5) and 50% (SSR2) with an average of 25.0% (Fig. 8). Under the influence of $CoCl_2$, DNA polymorphism was 0% with SSR1 or SSR5, or 50% with SSR2 or SSR4 with an average of 26.66%. DNA polymorphism within shoot lines obtained on MS with SA was 0% when SSR1, SSR3, SSR4, SSR5 were used but it was 66.67% using SSR2 with an average of 13.33%.

Cluster trees based upon UPGMA analysis of RAPD, ISSR and SSR markers for moringa shoots subjected to 14 subcultures under the influence of SA (Fig. 9) as well as $CoCl_2$ and $AgNO_3$ (data not shown) indicated that all lines

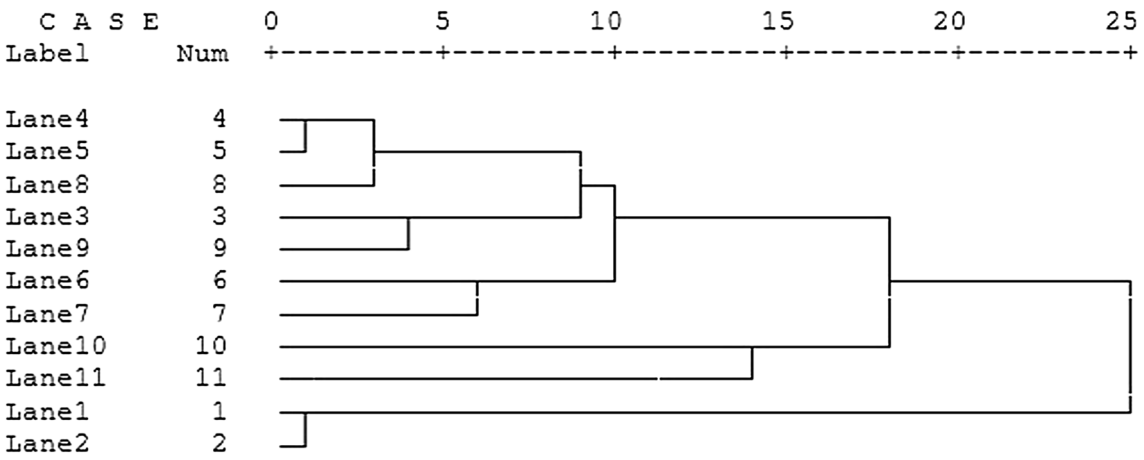

Fig. 5 Dendrogram obtained from total bands of native PAGEs of SOD, POX, CAT, GOT and EST isoenzymes

were discriminated from shoots of the first subculture; the lowest variation was detected when SA was used as an anti-ethylene agent.

Discussion

Moringa shoots were initiated and multiplied on MS medium supplemented with 2.5 µM BAP. Conservation of an aseptic environment in glass jars resulted in high humidity, depletion of oxygen and accumulation of ethylene (Isah 2015). These conditions caused high water content and water logging of the intercellular spaces of the cultured tissues, which led to chlorophyll deficiency and necrosis of shoot tips as well as the formation of friable calli at the base of cultured shoot segments in moringa and other plant species (Gaspar 1991; Gaspar et al. 1995; Frank et al. 2004; Rojas-Martínez et al. 2010). Fast shoot subcultures or improvement the aeration within the cultured jars was used to overcome vitrification (Saez et al. 2012; Salem 2016).

Shoot vitrification of shoots cultured on MS medium with 10 µM BAP was higher than others on MS medium with 2.5 µM BAP. Vitrification resulted in decreasing the number and length of shoots but increased the callus size at the base of the cultured plant segments. The detected negative correlation between plant shoot lengths and vitrification confirmed the results previously obtained by Kharrazi et al. (2011), it may be due to ethylene accumulation in culture vessels. Ethylene accumulation in culture vessels depends on age, type and physiological state of cultured tissue as well as nature and concentration of the added growth regulators (Isah 2015).

In moringa, to improve shoot multiplication and reduce vitrification, different concentrations of anti-ethylene compounds (SA, $AgNO_3$ and $CoCl_2$) in combination with the low concentration of BAP (2.5 µM) were investigated. Symptoms of vitrification such as chlorophyll degradation, retardation of shoot formation, death of shoot tips and formation of a large masses of friable calli at the base of cultured plant segments decreased when 10 µM $AgNO_3$ or 50 µM SA were used. When moringa shoots were cultured on MS medium containing 2.5 µM BAP and 200 µM $CoCl_2$, the base of explants showed a large mass of calli but in compact form. Silver nitrate and $CoCl_2$ in combination with the low concentration of cytokinin were previously used to avoid ethylene accumulation and vitrification symptoms (Beyer 1976; Pimenta et al. 2013; Mirza et al. 2015; Tamimi 2015). In the current work, reduction of the vitrification symptoms in moringa was associated with increased the number of formed shoots/explant and shoot growth even under long term culture. Inhibition of ethylene action by $AgNO_3$ (Beyer 1976) or ethylene biosynthesis by $CoCl_2$ (Lau and Yang 1976) explain reduction of vitrification due to incorporation of these compounds during moringa in vitro culture. Cobalt chloride increased shoot regeneration and decreased ethylene accumulation in sunflower (Chraibi et al. 1992). Application of $AgNO_3$ improved shoot multiplication in moringa and other plant species such as banana (Tamimi 2015), cherry (Sarroppulou et al. 2016) and sunflower (Mirza et al. 2015). In moringa, SA was the best applied anti-ethylene compound where it resulted in healthy shoots formation which facilitated root formation and plantlets acclimatization.

In comparison to SOD expression of shoots cultured on MS medium with 2.5 µM BAP, shoots cultured on 10 µM BAP or anti-ethylene compounds showed an increase in SOD expression, where the number of bands and staining intensity of some bands increased. SOD patterns of shoots cultured on MS medium without anti-ethylene compounds were similar to that of shoots cultured on $AgNO_3$ containing medium where two bands (SOD-6 and SOD-7) were not detected. Hypoxia stress due to conservation of cultured plant materials under aseptic conditions in glass jars

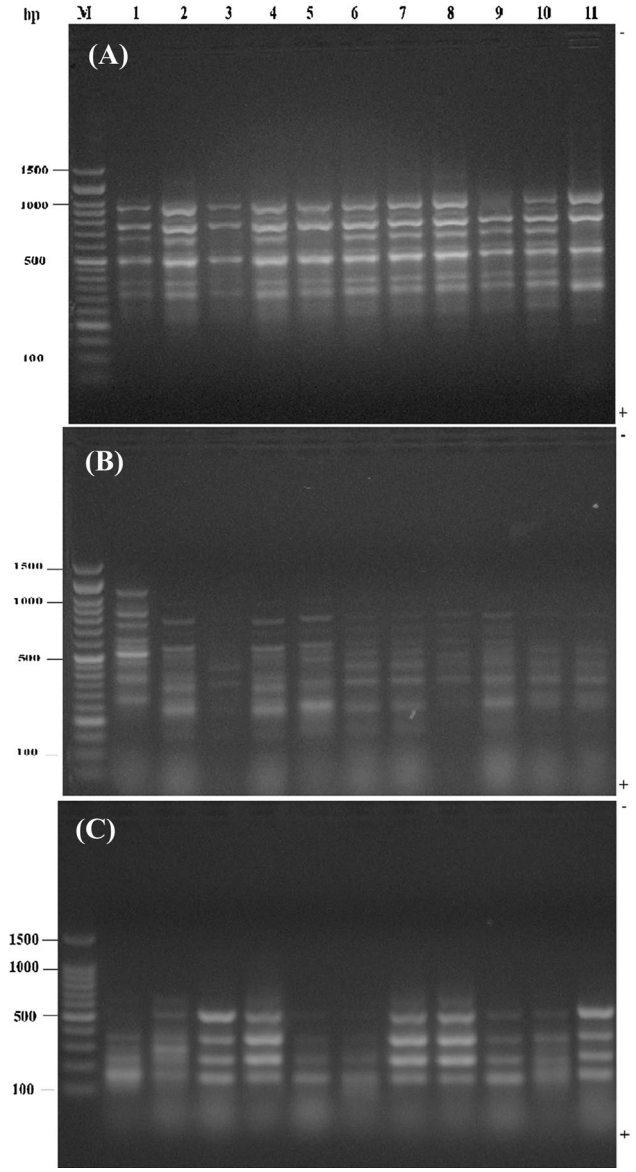

Fig. 6 RAPD-PCR profiles of genomic DNA of moringa shoots generated by primers OPP-13 on MS containing 10 µM AgNO3 (**a**); OPP-13 on MS containing 200 µM $CoCl_2$ (**b**); OPA-08 on MS containing 50 µM SA (**c**), obtained for 1st subculture "as control (lane 1) and long term micropropagated shoots; 14th subculture" (lanes 2–11), M: DNA ladder

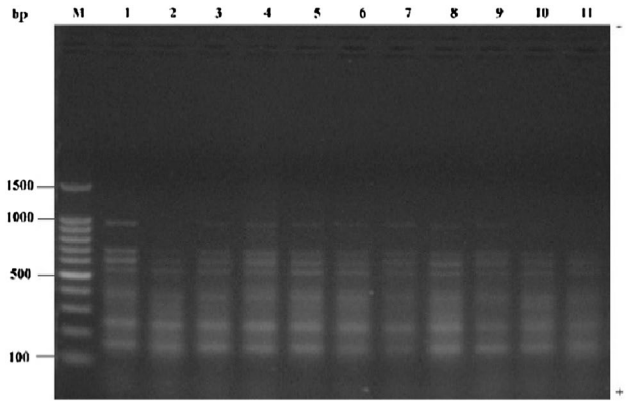

Fig. 7 ISSR-PCR profiles of genomic DNA of moringa shoots generated by primers ISSR-09 on 200 µM $CoCl_2$ (*b*) obtained for control "1st subculture" (lane 1) and long term micropropagated shoot cultures "14th ". (lanes 2–11), M: DNA ladder

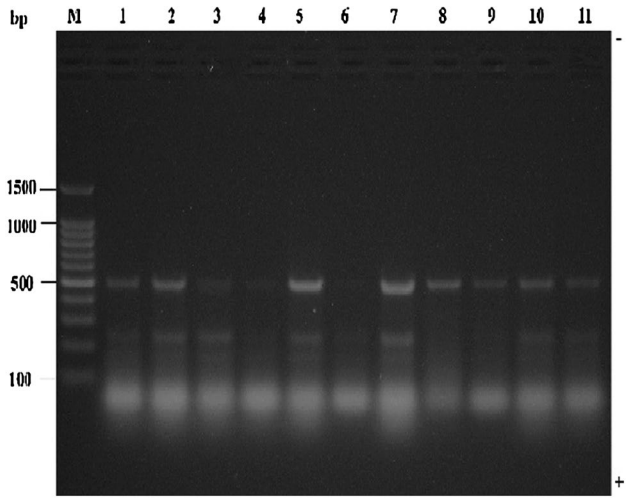

Fig. 8 SSR-PCR profiles of genomic DNA of moringa shoots generated by primers SSR-04 on 200 µM $CoCl_2$ (**c**), obtained for control "1st subculture" (lane 1) and long term micropropagated shoot cultures "14th " (lanes 2–11), M: DNA ladder

required SOD for H_2O_2 detoxification (Rojas-Martínez et al. 2010). While the number of SOD bands under the influence of $AgNO_3$ was lower than those of $CoCl_2$ or SA, $AgNO_3$ was the best anti-ethylenes compound for moringa shoot multiplication and growth. $AgNO_3$ was reported to overcome exerted stress on explants following their excision and prevent ethylene accumulation (Sarroppulou et al. 2016).

Under the influence of low BAP concentration, peroxidase and catalase patterns of the first subculture were similar to that of the 7th subculture. After 14 subcultures, 10 µM BAP in combination with 200 µM $CoCl_2$ or 50 µM SA decreased the number of peroxidase bands. Both SOD and CAT followed the same trend where they increased under long term culture. Peroxidase band pattern of the first subculture were similar to others shoots grown on MS containing 10 µM $AgNO_3$. In comparison to shoots subjected to long term culture with or without anti-ethylene compounds, shoots subjected to 10 µM $AgNO_3$ expressed the lowest staining intensity and number of catalase isoenzyme forms. The influence of AgNO3 on SOD, CAT and POX overcame the appearance of hyperhydricity malformations leading to normal shoot formation (Scandalios 1993; Gaspar et al. 1995; Mittler 2002; Pimenta et al. 2013). POX controlled ethylene and IAA accumulation

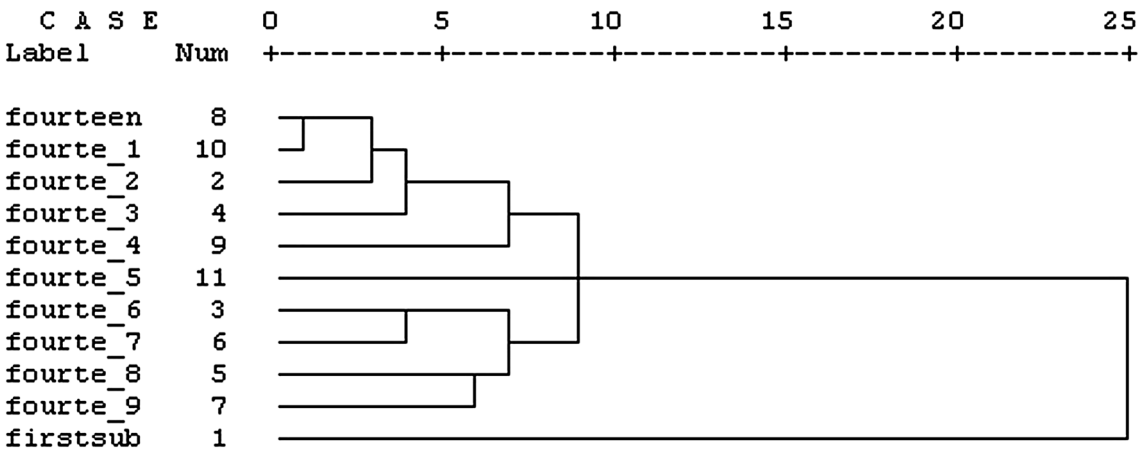

Fig. 9 UPGMA based cluster tree of 1st subculture on MS medium with 2.5 µM BAP (number 1) and different lines (number 2–11) of 14th subculture on MS medium with 2.5 µM BAP and 50 µM SA with five primers of each RAPD, ISSR or SSR

which regulated tissue development (Gaspar et al. 1985; Hassanein et al. 1999; Wang et al. 2002).

There is an enzymatic system, such as GOT, which is responsible for fast conversion of NH_4^+ to organic forms to sustain plant survival, growth and development (Zhang et al. 2006). The expressions of these enzymes are differentially influenced by duration and intensity of the applied stress condition (Ramanjulu et al. 1994; Wang et al. 2007). Increasing the concentration of BAP or the number of subcultures with or without anti-ethylene compounds decreased GOT expression especially in shoots subjected to 14 subcultures. GOT expression patterns indicated that production of organic nitrogen compounds from NH_4^+ of the medium may be negatively influenced by long term culture leading to reduction of shoot multiplication in moringa.

Under the influence of anti-ethylene compounds, the number of esterase isoenzyme forms and/or staining intensities of some bands of shoots subjected to 14 subcultures were higher than others subjected to 7 subcultures. Esterases play major roles in developmental processes in several plant species (Hassanein et al. 1999; Balen et al. 2004). An essential part of their role is linked to cell wall metabolism and metabolism of many pollutants (Cummins et al. 2001; Tamás et al. 2005). Consequently, esterases may play a role in the detected improvement of shoot multiplication due to application of anti-ethylene compounds upon long term culture.

The dendrogram based on the variation of the obtained isoenzymes indicated that moringa shoots were safely multiplied up to 7 subcultures where isoenzyme expression patterns of shoots cultured for 7 subcultures was similar to those of the first subculture. Under long term culture, incorporation of salicylic acid in culture media was recommended to avoid vitrification and variation in isoenzyme expressions.

To confirm which anti-ethylene compounds may be used to conserve the genetic fidelity of the multiplied shoots, fingerprinting profiles of the obtained regenerants after 14 subcultures in comparison to those of the first subculture were assessed using RAPD, SSR and ISSR. Those assays were previously used (Zietkiewicz et al. 1994; Ganesana et al. 2014). Under long term culture, somaclonal variation was registered in moringa and other plant species, and they expressed variation in morphological and biochemical traits (Hassanein et al. 2008; Kumar and Prabha 2016; Tripathy et al. 2016). In sorghum, somaclonal variation could be avoided if the number of subcultures was less than seven (Hassanein et al. 2008). In the current work, the expected somaclonal variation was not high, where the used protocol depended on nodal cuttings. Generally, low genetic variation was detected when shoots were initiated from or around axillary buds and meristematic dome (Devarumath et al. 2002; Joshi and Dhawan 2007). In our study, the number of multiplied fragments using SSR primers was low as reported by Ganesana et al. (2014), but in contrast to our data, they found that SSR as a co-dominant marker and locus specific was better than RAPD and ISSR to detect genetic diversity in moringa. Application of anti-ethylene compounds, especially silver nitrate, improved plant multiplication and reduced vitrification. The question was: which anti-ethylene expressed safe multiplication under long term culture? The answer was deduced from dendrograms depending on all the applied molecular markers. SA was the best where it resulted in improvement of shoot multiplication, decrease verification and expressed the lowest somaclonal variation.

Conclusion

In moringa, for in vitro multiplication without severe verification or retardation of root formation and successful acclimatization, SA as anti-ethylene compound was recommended. Also, under the influence of long term culture, ISSR was recommended to test genome stability of moringa.

Acknowledgements The authors would like to thank Prof. Moneim Shamloul of Fraunhofer USA Center for Molecular Biotechnology, Newark, DE, USA for English language revision.

Authors' contributions AMH proposed the idea and the experimental design, revised the manuscript and acted as a corresponding author; JMS participated in the experimental design, interpretation and acquisition the data; FAF participated in the experimental design and drafted the manuscript; AE performed the measurements, drafted the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors whose names are listed in this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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