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Anti-ethylenes improve moringa multiplication via the reduction of hyperhydricity and genetic changes resulting from long-term culture

Ahmed M. Hassanein[1], Mohamed M. Azooz[2,3], Amal N. Mohamed[2,3], Naglaa Loutfy[2,3]

[1]Central Lab. Genet. Eng., Botany and Microbiology Dept., Faculty of Science, Sohag University, 82524, Egypt
[2]Molecular Biology and Biotechnology Center, Faculty of Science, South Valley University, 83523 Qena, Egypt
[3]Botany and Microbiology Dept., Faculty of Science, South Valley University, 83523 Qena, Egypt

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CORRESPONDANCE TO:
Naglaa Loutfy
Botany and Microbiology Department, Faculty of Science, South Valley University, Qena, Egypt.
Emails: naglaa.hassan@sci.svu.edu.eg; naglaaloutfy@yahoo.com

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Long-term culture of a fast-growing plant species such as moringa in partially closed vessels led to the appearance of hyperhydricity which retard mass multiplication. Therefore, the overall goal of this study was to know the physiological changes associated with avoiding the phenomenon by antiethylenes ($AgNO_3$, $CoCl_2$, and salicylic acid (SA)) and the effect of these compounds on the genetic changes resulting from long-term culture. Moringa multiplication was successfully obtained when hyperhydricity was successfully avoided using MS medium containing 0.56 mg/l BAP and 1.70mg/L $AgNO_3$ or 6.91mg/L SA and it was associated with a significant increase in photosynthetic pigments, carbohydrates, proteins and Mg^{++} contents. In addition, no significant changes were detected in free amino acids, Na^+, K^+, and Ca^{++} contents with decreasing the activities of CAT, POX, APX and SOD. Therefore, improvement due to antiethylenes application, in particular 1.70mg/L $AgNO_3$, which led to the disappearance of hyperhydricity could be attributed to modulations in some physiological processes and avoidance of genetic changes under the influence of long-term culture (11 subculture), as the genetic changes decreased from 45.2% and 40.3% to 29.6% and 30.8% using RAPD and ISSR, respectively. Application of antiethylenes was recommended for improvement and mass propagation of plants suffering from hyperhydricity under tissue culture conditions.

Keywords: Anti-ethylene, Antioxidants, Micropropagation, Molecular markers, *Moringa oleifera*, Organic and inorganic solute

INTRODUCTION

Partial restriction in gas exchange lowers plant growth rate and development pattern, but inadequate aeration can prevent plant survival (Vartapetian & Jackson, 1997). For instance, waterlogged maize plants experiencing partial oxygen shortage experienced aeration stress and ethylene buildup, which resulted in the formation of aerenchyma (Jackson et al., 1985). Fast-growing plant species, like moringa, are typically cultivated in vitro in sealed vessels under conditions similar to waterlogging, which include high relative humidity (90–100%) and aeration stress (Ziv & Ariel, 1988). In addition to causing changes in plant metabolites (Suresh et al., 2009), plants exposed to decreased gas exchange (O_2, CO_2, and ethylene) between the culture and its immediate surroundings may die from hypoxia (Dat et al., 2004). Even in environments with adequate oxygen, hypoxia can occur when oxygen demand exceeds oxygen supply (Drew et al., 2000). According to Sakihama et al. (2002), low gas exchange always leads to ethylene buildup and oxidative damage through the generation of oxygen superoxide.

Reactive oxygen species (ROS) are significantly

elevated in plants exposed to environmental stressors like hypoxia. This imbalance between ROS generation and antioxidant defense causes oxidative stress. Cellular macromolecules like proteins, lipids, carbohydrates, and DNA are harmed by this imbalance (Mittler et al., 2004; Gill & Tuteja, 2010). Several enzymatic and non-enzymatic antioxidant defense systems need to be strengthened to scavenge ROS. These include SOD, POX, CAT, APX, and other enzymatic antioxidants. Ascorbate, carotenoids, proline, sucrose, and other substances are examples of non-enzymatic antioxidants (Desikan et al., 2005; Foyer & Noctor, 2005; Ahmad et al., 2008; Gill & Tuteja, 2010).

Hypoxia in closed vessels is linked to hyperhydricity (vitrification, glassiness), which makes using in vitro culture methods more difficult by delaying plant cloning and acclimation. Anatomical, morphological, physiological, and molecular abnormalities result from hyperhydricity (Hassanein et al., 2018; Polivanova & Bedarev, 2022). Hypolignification, chlorophyll deficiency, abnormalities in enzyme activity, necrosis of apical buds (van den Dries et al., 2013; Tian et al., 2015; Hassanein et al., 2018), death of the shoot apex, increased shoot and leaf thickness, the development of friable calli on the base of cultured explants (Hassanein et al., 2018), and the buildup of ROS are some signs of hyperhydricity (Polivanova & Bedarev, 2022). Also, they suffer from a decrease in dry weight, lignin, cellulose, calcium, and polar auxin transport but increased water content, peroxidases activity, auxin catabolism, K+, and ethylene accumulation (Gaspari, 1991).

Anti-ethylene compounds like silver nitrate ($AgNO_3$), salicylic acid (SA), and $CoCl_2$ were used in conjunction with low concentrations of cytokinin to eliminate hyperhydricity along with control factors that affect its appearance, such as a lack of oxygen supply (Beyer, 1976; Tamimi, 2015; Hassanein et al., 2018; Drisya Ravi et al., 2019; Salem, 2020). This may be because $AgNO_3$ retards the action of ethylene (Beyer, 1976), $CoCl_2$ inhibits ethylene biosynthesis (Lau & Yang, 1976), inhibits the ethylene forming enzyme (Yang & Hoffman, 1984), or enhances the biosynthesis of polyamines (Freschi, 2013). When a moringa shoot culture was established on MS medium with 2.5µM BA and 2.5µM $AgNO_3$, the regenerated shoots' genetic fidelity was 95% (Drisya Ravi et al., 2019). Application of 10µM $AgNO_3$ was the most effective anti-ethylene substance when compared to the two other anti-ethylenes (200µM $CoCl_2$ or 50µM SA) under long-term cultures of moringa (14 subcultures) on MS medium containing 2.5µM BAP; however, their effects were linked to high DNA polymorphism (Hassanein et al., 2018). In addition to improving stomata function, photosynthetic pigments, adventitious root production, and the survival of micropropagated plantlets, $AgNO_3$ reduced hyperhydricity and enhanced plant regeneration and growth (Tirni et al., 2013; Hassanein et al., 2018; Mani et al., 2024).

Polymorphism data were collected in a previous study by Hassanein et al. (2018) to compare the effects of three different types of anti-ethylene on the genome stability of moringa that was subjected to 14 subcultures. Stated differently, the genetic alteration brought about by prolonged cultivation (as a control) was not considered. Then, there was no response to the following question Was the genome more stable when the hyperhydricity caused by the anti-ethylene application was avoided? The purpose of this effort was to provide an answer to this question. The study also sought to advance our knowledge of the physiological alterations linked to the use of anti-ethylenes, which improved moringa acclimation and in vitro cloning.

MATERIALS AND METHODS

Moringa oleifera seeds were obtained from the Central Administration for Seed Production (8 Gamaa El Kahira St, Agricultural Research Center, Giza, Egypt). For four weeks, seeds were cultivated in clay soil in plastic containers. After being cut, nodal and shoot cuttings were sterilized with 5% (v/v) Clorox for two minutes, 0.2% (w/v) $HgCl_2$ for two minutes, and 70% (v/v) ethyl alcohol for one minute while being constantly shaken. The cuttings were then rinsed three times with sterile distilled water for five, ten, and fifteen minutes. To obtain explants that were between 1.0 and 1.5cm long, the lateral ends of each explant were thrown away (Hassanein et al., 2018). Explants were placed in glass jars with 25ml of MS (Murashige & Skoog, 1962) medium supplemented with 3% (w/v) sucrose, 8g/L agar (Difco Bacto agar), and 0.56mg/L BAP (Benzyl amino purine) at pH 5.8 to generate the initial shoot culture. The vessels were then autoclaved. Vitamin B1-hydrochloride (4), myo-inositol (100), pyridoxal hydrochloride (0.7), nicotinic acid (4), folic acid (0.5), and biotin (0.04) were the vitamin components of this medium, measured in (mg/L). Autoclaving at 121°C for 20min was used to sterilize the media (Hassanein & Mazen, 2001). Moringa shoot

cultures were incubated in tissue culture rooms for 3 weeks at 28 ± 2ºC under a 16-h photoperiod at 100µmol m-2s^{-1}. The number of regenerates, their growth parameters and the appearance of hyperhydricity were investigated according to Hassanein et al. (2018). Application of anti-ethylene compounds to avoid hyperhydricity: Two experiments were conducted to study the effect of three anti-ethylene agents on moringa micropropagation, physiology and genome stability. To carry out the first one, shoot tip and nodal cuttings of disinfected seedlings were multiplied on MS medium containing 0.56mg/L BAP for four subcultures to save enough plant materials. During the fifth culture, explants were cultured in 250ml glass jars containing 25ml of MS medium containing 0.56mg/L BAP in combination with three concentrations of $AgNO_3$ (1.70, 4.25 or 12.74mg/L), salicylic acid (6.91, 13.81 or 27.62mg/L) and $CoCl_2$ (11.90, 23.79 or 47.59mg/L).

Based on the data of the first experiment, 1.70mg/L $AgNO_3$ in combination with 0.56mg/L BAP were selected for the second experiment. Shoot and nodal cuttings of disinfected seedlings were subjected to 11 subcultures (three weeks each) to study its effect as an anti-ethylene during long-term culture of moringa.

The experiment was repeated twice. Thirty explants in six glass jars were used for each treatment. The number of shoots/explant and growth of the obtained shoots (fresh weight/ shoot cluster, fresh weight/ shoot; shoot length and the number of nodes/shoot) were determined. Then, to study the effect of anti-ethylene on in vitro long-term culture of moringa, micro-shoot cuttings were subjected to eleven subcultures on MS medium containing 0.56mg/L BAP in the absence or presence of selected concentration of anti-ethylene (1.70mg/L $AgNO_3$). Experiments were repeated twice.

In addition, in vitro obtained shoots were cultured on MS medium containing 1mg IBA (Indole butyric acid) with or without 1.7mg/L $AgNO_3$ for induction of root formation. Shoots with well-developed root systems were acclimatized in pots containing a mixture of peat and sand (2:1 v/v) for two months. Then, plantlets were transferred to grow in open conditions (Hassanein et al., 2018).

Plant material analysis

Determination of photosynthetic pigments

The spectrophotometric method, as suggested by Lichtenthaler (1987), was used to determine the photosynthetic pigments (chlorophyll a, b, and carotenoids).

Estimation of organic solutes

For the determination of carbohydrate fractions, anthrone sulphoric acid was used (Jermyn, 1975). Water-soluble, insoluble and total proteins were determined according to Lowry et al. (1951). Total free amino acid content was determined by the ninhydrin test, according to the method of Moore & Stein (1948). According to Bates et al. (1973), proline was determined.

Minerals content

Ion concentrations were estimated following the Nitric–perchloric acid digestion recommended by the Association Official Analytical Chemists (AOAC) (1990). The obtained solution was used for (Na^+), (K^+), (Ca^{2+}) and Mg^{2+} determinations (Schwarzenbach & Biedermann, 1948; Munns et al., 2010).

Antioxidant enzymes activity

In 3 milliliters of 50mM phosphate buffer (Na_2PO_4/K_2HPO_4) with 0.1mM Na_2EDTA and 1% (w/v) polyvinylpyrrolidone (PVP) (pH 7.0), 500 milligrams of fresh leaf tissue was ground on ice. For 20min at 4ºC, the homogenate was centrifuged at 13500rpm. The supernatant was collected and stored at -20°C for analysis of peroxidase (POX; EC 1.11.1.7) as described by MacAdam et al. (1992), ascorbate peroxidase (APX; EC 1.11.1.11) according to the technique of Nakano & Asada (1981), catalase (CAT; EC 1.11.1.6) according to the method of Aebi (1984), and superoxide dismutase (SOD; E.C. 1.15.1.1) according to Beauchamp & Fridovich (1971).

Genome analysis

Ten moringa shoots were propagated for eleven subcultures on MS medium supplemented with 0.56mg/L BAP with or without 1.7mg/L $AgNO_3$ and used for DNA extraction as described by Porebski et al. (1997). A total of five 10-mer RAPD primers and ten ISSR primers were used. The genomic DNA of moringa was amplified using Thermal Cycler (Biometra T Personal Combi, Biometra GmbH, and Germany). PCR reaction mixture was prepared in a 25µl volume containing 12.5µl of Go Taq® Green Master Mix (Promega, Madison, USA), 3µl of primer 10 pmol, 6.5µl of free nuclease water and 3µl of 100ng genomic DNA templates. Moringa genome amplification was carried out in a Perkin-Elmer/GeneAmp®

PCR System 9700 (PE Applied Biosystems). PCR device was programmed to carry out 40 cycles after an initial denaturation cycle at 94°C for 5min. Each PCR cycle consisted of denaturation at 94°C for 45sec, annealing at 36°C for 50sec, and elongation at 72°C for 1min. The primer extension was fulfilled for 7 min at 72°C in the final cycle. To separate the amplification products, electrophoresis was carried out using 1.5% or 2% (w/v) agarose gel containing ethidium bromide (0.5µg/ml) in 1X TBE (Tris Borate EDTA) buffer at 70 volts. The PCR products were analyzed by a computer program; Gene Profiler (version 4.03).

Statistical analysis

The experiments were designed as completely randomized with three replications. The experimental data were statistically analyzed by ANOVA. Data were compared using the least significant difference (LSD) test at 5% (*) and 1% (**) levels (Snedecor & Cochran, 1980).

Results

Moringa explants were cultured on MS medium supplemented with 0.56mg/L BAP in closed 250ml glass vessels. In one week, enlargement on the base of the explant was normally detected before the formation of new shoots. Under these conditions, the in vitro obtained shoots on initial explants showed hyperhydricity such as an increase of shoot thickness, formation of callus mass at the base of the explant (Figure 1), and sometimes necrosis of apical buds.

Figure 1. In vitro obtained shoots on nodal explants during the 4th subculture on MS medium supplemented with 0.56mg/L BAP (a), and 0.56mg/L BAP and 1.7mg/L $AgNO_3$ (b) for three weeks

Hyperhydricity symptoms partially or completely disappeared when antiethylene compounds were used. For example, while shoot necrosis of shoot apexes completely disappeared, callus formation at the base of explants was reduced (Figure 1). Data indicated that the lowest concentration of each of $AgNO_3$ (1.70mg/L), SA (6.91mg/L), $CoCl_2$ (11.90mg/L) expressed lowest values of callus formation frequency (26.7%, 30.6% and 33.2, respectively) in comparison to that formed at the base of explant on MS medium supplemented with 0.5mg/L BAP (53.1 %) alone. In addition, the appearance of the large mass of callus was detected when in vitro obtained shoots were cultured on medium supplemented with 1mg/L IBA for induction of root formation (Figure 2), but it disappeared when the combination between 1mg/L IBA and 1.70mg/L $AgNO_3$ was used. The application of 1.70mg/L $AgNO_3$ facilitated the acclimatization processes (Figure 2). On the other side, all plantlets that formed roots on callus at the base of shoot cuttings wilted during the acclimatization period.

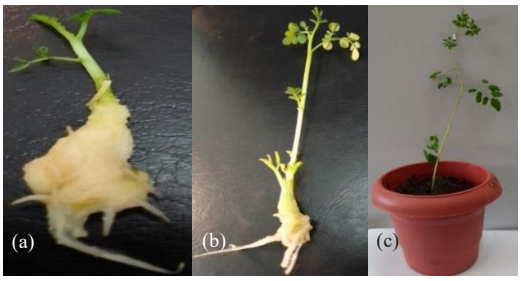

FIgure 2. In vitro root formation of moringa nodal explants on MS medium with 1mg/L IBA (a) or 1mg/L IBA in combination with 1.70mg/L $AgNO_3$ (b) and moringa plantlet acclimatized to ex vitro conditions(c)

Shoot multiplication of the fifth subcultured explants was used to study the effect of anti-ethylene compounds and shoot multiplication of *in vitro* grown moringa plant materials (Tables 1, 2 and 3). Hyperhydricity was reduced when the lowest concentration of each anti-ethylene compound was used; 1.70mg/L $AgNO_3$ was the best where it expressed the highest shoot number and growth parameters (Table 1). In addition, explants resulting from disinfected seedlings were subjected to 11 subcultures; three weeks each. The number of in vitro formed shoots increased with increasing number of subcultures until the eighth subculture, then it began to decrease again (Table 2). Adding the selected concentration of $AgNO_3$ (1.70mg/L) resulted in significant increase in the number of in vitro formed shoots even explants subjected to 11 subculture (Table 3).

When moringa shoots were multiplied on MS medium containing 0.56mg/L BAP, a condition that resulted in the appearance of hyperhydricity, shoots with relatively low chlorophyll contents (0.16mg/g fresh weight) were detected (Table 4). Therefore, the total pigment contents improved

by 131% and 144% due to application of $AgNO_3$ and SA, respectively. The application of $CoCl_2$ decreased chlorophyll and total pigment contents significantly (Table 4, Figure 1). Also, application of $AgNO_3$ and SA improved the carotene contents of moringa shoots cultured in partially-closed glass vessels. Application of antiethylene $CoCl_2$ decreased carotenoid contents. Decreased total chlorophyll and carotene contents led to decrease in total pigment content in plants treated with $CoCl_2$ (Table 4).

Table 1. Moringa in vitro shoot multiplication and growth on MS medium supplemented with 0.56mg/L BAP alone (control) or in combination with different concentration of $AgNO_3$, $CoCl_2$ or SA for three weeks

Treatment (mg/L)	No. of shoots/ explant	F.W/ shoot cluster (g)	Shoot length (cm)	F.W/ one shoot (g)	No. of nodes/ shoot
Control	6.0±1.00	0.70±0.10	1.60±0.20	0.05±0.003	5.0±1.00
1.70 $AgNO_3$	6.3±0.58	0.75±0.08**	2.47±0.15**	0.07±0.001**	4.3±0.58
4.25 $AgNO_3$	3.7±0.58*	0.49±0.03**	1.57±0.12	0.056±0.007	3.0±1.00
12.74 $AgNO_3$	1.7±0.58**	0.28±0.04**	1.00±0.10**	0.031±0.007*	1.7±0.58**
11.90 $CoCl_2$	4.3±0.58	0.43±0.03**	1.60±0.11	0.049±0.008	3.7±0.58
23.79 $CoCl_2$	3.7±0.58*	0.33±0.02**	1.20±0.17*	0.034±0.001*	2.7±0.58*
47.59 $CoCl_2$	3.0±1.00**	0.46±0.04**	1.17±0.15*	0.06±0.0049	4.0±1.00
6.91 SA	5.3±0.58	0.49±0.01**	1.40±0.10	0.033±0.003*	3.7±0.58
13.81 SA	4.3±0.58	0.32±0.03**	0.90±0.10**	0.050±0.001	3.3±0.58
27.62 SA	3.7±0.58*	0.27±0.02**	0.77±0.06**	0.025±0.002**	3.3±0.58

Values are the mean of three replicates ± standard deviation (SD) Statistical significance of differences compared to control (0.56mg/L BAP).

*significant at P<0.05; **significant at P<0.01.

Table 2. Moringa in vitro shoot multiplication and growth on MS medium supplemented with 0.56 mg/l BAP for eleven subcultures

Subculture No.	No. of shoot / explant.	F.W/ shoot cluster (g)	Shoot length (cm)	F.W/one shoot (g)	No. of node/shoot
S1	2.33±.58	0.30±0.01	1.0±0.10	0.10±0.010	2.7±0.58
S2	3.33±0.58	0.34±0.02	0.8±0.10	0.52±0.001	2.0±0.00
S3	5.0±1.00*	0.39±0.03	0.5±0.10*	0.09±0.009	2.3±0.58
S4	7.3±0.58**	1.72±0.13**	1.7±0.58**	0.11±0.006	4.3±0.58
S5	6.0±1.00**	0.73±0.10**	2.1±0.15**	0.13±0.008	4.7±0.58*
S6	5.3±0.58**	0.58±0.04*	2.1±0.20**	0.40±0.500	5.3±0.58**
S7	6.7±0.58**	0.53±0.04	1.6±0.15**	0.11±0.009	5.0±1.00**
S8	6.0±1.00**	0.85±0.10**	2.3±0.15**	0.11±0.134	4.3±0.58
S9	4.0±1.00**	0.43±0.11	1.7±0.15	0.09±0.007	3.3±0.58
S10	4.3±0.58	0.41±0.12	2.3±0.26**	0.09±0.005	3.3±0.58
S11	3.7±0.58	0.53±0.10	1.9±0.15**	0.10±0.010	3.7±0.58

Values are the mean of three replicates ± standard deviation (SD) Statistical significance of differences compared to control (First subculture).

*significant at P<0.05; **significant at P<0.01.

Table 3. Moringa in vitro shoot multiplication and growth on MS medium supplemented with 0.56mg/L BAP and 1.70mg/L $AgNO_3$ for eleven subcultures

Subculture No.	No. of shoot / explant.	F.W/ shoot cluster (g)	Shoot length (cm)	F.W/one shoot (cm)	No. of node/ shoot
S1	2.67±0.58	0.38±0.02	0.90±0.10	0.073±0.058	3.33±0.58
S2	4.33±0.58	0.36±0.02	0.60±0.10	0.037±0.005**	2.66±0.58
S3	5.33±0.58*	0.68±0.04**	1.93±0.15**	0.090±0.003	3.33±0.58
S4	10.0±1.00**	0.72±0.09**	2.17±0.15**	0.040±0.002**	5.00±1.00
S5	8.67±0.58**	1.01±0.09**	2.50±0.1**	0.074±0.010	4.00±1.00
S6	8.00±1.00**	1.19±0.07**	2.67±0.15**	0.10±0.013*	4.00±1.00
S7	7.00±1.00**	1.47±0.10**	2.20±0.20**	0.14±0.014**	4.66±0.58
S8	6.00±1.00**	0.82±0.02	2.40±0.10*	0.065±0.010	3.66±0.58
S9	6.67±0.58**	0.56±0.02	1.57±0.15**	0.058±0.011	3.00±1.00
S10	8.00±1.00**	0.69±0.03**	1.83±0.15**	0.055±0.009	3.33±0.58
S11	6.67±0.58**	0.74±0.13**	1.63±0.10**	0.053±0.008	3.67±0.58

Values are the mean of three replicates ± standard deviation (SD) Statistical significance of differences compared to control (First subculture).
*significant at P<0.05; **significant at P<0.01.

Table 4. Effect of MS medium containing 0.56mg/L BAP and selected concentrations of anti-ethylene compounds on pigment contents of moringa

Treatment (mg/L)	Chl. a (mg/ g^{-1} FW)	Chl. b (mg/ g^{-1} FW)	Chl. a/ Chl. b (mg/ g^{-1} FW)	Carotenoid (mg/ g^{-1} FW)	Total Pigment (mg/ g^{-1} FW)
0.56 BAP	0.07±0.003	0.065±0.003	1.1±0.013	0.020±0.0025	0.16±0.004
1.70 $AgNO_3$	0.12±0.003**	0.070±0.005	1.7±0.098**	0.023±0.0020	0.21±0.006**
6.91 SA	0.13±0.003**	0.076±0.005*	1.7±0.044**	0.030±0.0017**	0.23±0.002**
11.90 $CoCl_2$	0.06±0.003	0.05±0.003**	1.2±0.020	0.013±0.0003**	0.12±0.006**

Values are the mean of three replicates ± standard deviation (SD) Statistical significance of differences compared to control (0.56mg/L BAP).
*significant at P<0.05; **significant at P<0.01.

Application of $AgNO_3$ or SA as an antiethylene increase soluble and insoluble carbohydrate contents leading to significant increase in total carbohydrate contents (Table 5). The total carbohydrates of plants subjected to $CoCl_2$ were relatively the same as plants cultured on MS medium containing only 0.56mg/L BAP. Under the influence of antiethylene, except $CoCl_2$, while soluble proteins increased and insoluble proteins decreased; total proteins showed a significant increase.

Proline contents were not influenced by the presence or absence of $AgNO_3$ as antiethylene compound (Table 6) but it showed a slight increase under the influence of SA or $CoCl_2$. Amino acid contents of moringa shoots slightly increased when they were subjected to $AgNO_3$, SA or $CoCl_2$.

Culture of moringa shoots on MS medium containing 0.56mg/L BAP with or without antiethylenes did not exert any significant change in Na^+, K^+ or Ca^{++} content (Table 7). On the other hand, the application of antiethylenes increased Mg^{++} contents especially when $AgNO_3$ or SA was used.

The application of antiethylene compounds reduced hyperhydricity in moringa and decreased the activity of POX, APX, SOD and CAT (Table 8). The activity of CAT showed significant increase when $CoCl_2$ was used.

When moringa ten shoots of the 11th subculture were grown on MS medium containing 0.56mg/L BAP with or without $AgNO_3$ and subjected to genome amplification using five RAPD primers, the registered polymorphism ranged from 28.6% (OPE-18 primer) to 58.8% (OPG-02 primer), with an average of 45.2% (Table 9 and Figure 3). Polymorphism values decreased when shoots were cultured on MS medium with $AgNO_3$ with an average of 29.6% (Table 10). The registered reduction in polymorphism was 34.5%.

Table 5. The effect of MS medium containing 0.56mg/L BAP and selected concentrations of anti-ethylene compounds on carbohydrates and proteins contents of moringa

Treatment (mg/L)	Carbohydrates			Protein		
	Soluble (mg/g^{-1} DW)	Insoluble. (mg/g^{-1} DW)	Total (mg/g^{-1} DW)	Soluble (mg/g^{-1}DW)	Insoluble (mg/g^{-1} DW)	Total (mg/g^{-1}DW)
0.56 BAP	59.6±2.3	67.1±6.8	127±5.4	81.4±2.1	200.3±2.7	281.7±3.3
1.70$AgNO_3$	83.8±2.3**	111.4±4.3**	195±4.8**	109.9±2.5*	194.2±2.2	304.2±1.7**
6.91 SA	79.2±1.3**	75.6±4.5	155±3.3**	134.2±1.7**	170.9±5.8**	304.9±4.6**
11.90 $CoCl_2$	93.2±3.0**	33.9±7.6**	127±5.7	101.2±2.1	177.1±2.6**	278.3±0.8

Values are the mean of three replicates ± standard deviation (SD) Statistical significance of differences compared to control (0.56mg/L BAP). *significant at P<0.05; **significant at P<0.01.

Table 6. Effect of MS medium containing 0.56mg/L BAP and selected concentrations of anti-ethylene compounds on proline and amino acid contents of moringa

Treatment (mg/L)	Proline (mg/g^{-1} DW)	Amino acids (mg/g^{-1} DW)
0.5 BAP	5.9±0.18	84.7±5.1
1.70 $AgNO_3$	5.9±0.03	93.6±2.8
6.91 SA	6.1±.0.17	93.6±2.8
11.90 $CoCl_2$	6.3±0.50	91.8±5.1

Values are the mean of three replicates ± standard deviation (SD)Statistical significance of differences compared to control (0.56mg/L BAP). *significant at P<0.05; **significant at P<0.01.

Table 7. Effect of MS medium containing 0.56mg/L BAP and selected concentrations of anti-ethylene compounds on mineral contents of moringa

Treatment (mg/L)	Na^+ (mg/g^{-1} DW)	K^+ (mg/g^{-1} DW)	Ca^{++} (mg/g^{-1} DW)	Mg^{++} (mg/g^{-1} DW)
0.56 BAP	1.95±0.16	71.5±2.2	1.4±0.04	0.42±0.02
1.70$AgNO_3$	2.03±0.05	69.5±1.8	1.1±0.05	0.94±0.05**
6.91 SA	1.8±0.024	67.6±3.1	1.1±0.05	0.81±0.02**
11.90 $CoCl_2$	2.05±0.05	67.3±1.5	1.3±0.07	0.43±0.04

Values are the mean of three replicates ± standard deviation (SD). Statistical significance of differences compared to control (0.56mg/L BAP). *significant at P<0.05; **significant at P<0.01.

Table 8. Effect of MS medium containing 0.56mg/L BAP and selected concentrations of anti-ethylene compounds on CAT (mM H_2O_2/g^{-1} FW), POX (mM guaicol /g^{-1} FW), APX (mM ascorbate /g^{-1} FW) and SOD (mM NBT/g^{-1} FW) contents in moringa

Treatment (mg/L)	CAT	POX	APX	SOD
0.56 BAP	12.3±1	58.1±2.2	22.5±2.2	6.0±0.7
1.70$AgNO_3$	11.2±0.3	39.4±0.8**	17.6±2.3*	4.3±0.4*
6.91 SA	11.8±.5.0	50.1±3.0*	19.1±1.3	4.9±0.15
11.90 $CoCl_2$	15.4±0.8**	52.1±2.7	18.0±1.9	5.0±0.8

Values are the mean of three replicates ± standard deviation (SD). Statistical significance of differences compared to control (0.56mg/L BAP). *significant at P<0.05; **significant at P<0.01.

Table 9. RAPD and ISSR - PCR analysis of genome of moringa plants grown for long term culture on MS medium containing 0.56mg/L BAP [Mb= monomorphic band, Pb= polymorphic bands, Ub= unique bands, Tb= total bands, bp= base pair, and poly= polymorphism]

Primer type	Primer code	Sequence (5'→3')	Mb	Pb	Ub	Tb	Size range (bp)	Poly (%)
RAPD	OPA-02	5'-TGCCGAGCTG-3'	8	5	0	13	157-1392	38.5
	OPA-16	5'-AGCCAGCGAA-3'	8	9	1	18	160-2025	55.6
	OPE-18	5'-GGACTGCAGA-3'	10	4	0	14	133-873	28.6
	OPG-02	5'-GGCACTGAGG-3'	7	10	0	17	107-2095	58.8
	OPH-16	5'-CAAGGTGGGT-3'	7	4	0	11	205-1049	36.4
Total			**40**	**32**	**1**	**73**	**107-2095**	**45.2**
ISSR	834	5'-(AG)7ACYT-3'	8	10	0	18	201-1469	55.6
	835	5'-(AG)8YC-3'	13	2	0	15	153-1696	13.3
	900	5'- ACTTCCC(CA)2GG TTAA(CA)2-3'	6	12	0	18	217-1601	66.7
	844 B	5'-(CT)8GC-3'	12	2	0	14	180-1803	14.3
	17898 A	5'-(CA)6AC-3'	9	3	0	12	232-984	25
	17898 B	5'-(CA)6GT -3'	5	9	1	15	130-1595	66.7
	ISSR-3	5'- GTGGTGGTGGC-3	6	5	0	11	159-2010	45.5
	ISSR-5	5'- CTCCTCCTGGC-3'	4	3	1	8	290-1702	50
	ISSR-9	5'- (GA)6CC-3'	10	5	2	17	174-1622	41
	ISSR-13	5'- GAGGAGGAGGC-3'	9	0	1	10	126-1626	10
Total			**83**	**51**	**5**	**139**	**126-1804**	**40.3**

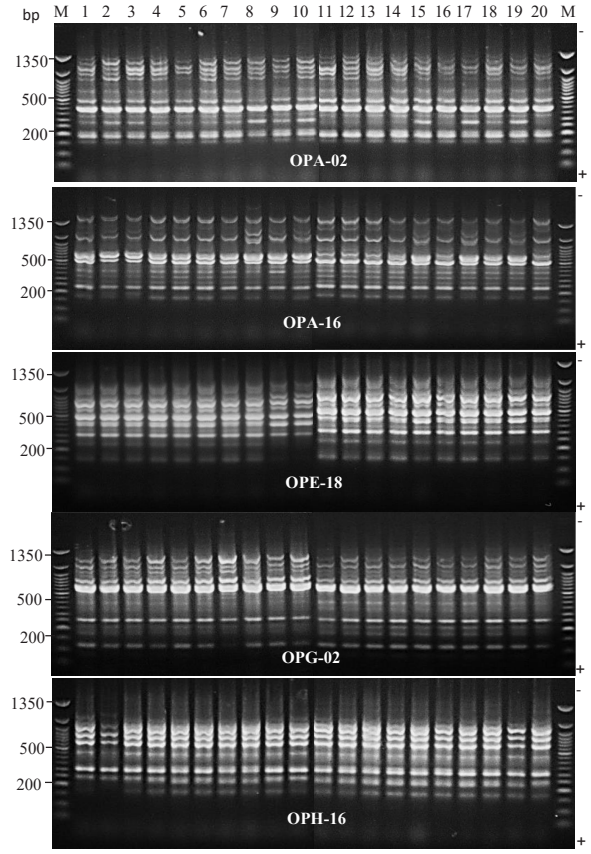

Figure 3. RAPD-PCR profiles generated by genomic DNA of 20 different microshoots of *Moringa oleifera* from the 11th subculture on MS medium + 0.56mg/L BAP without (lanes 1-10) or with (lanes 11-20) $AgNO_3$

Table 10. RAPD and ISSR - PCR analysis of genome of moringa plants grown for long term culture on MS medium containing 0.56mg/L BAP and 1.7mg/L $AgNO_3$ [Mb= monomorphic band, Pb= polymorphic bands, Ub= unique bands, Tb= total bands, bp= base pair, and poly= polymorphism]

Primer type	Primer code	Sequence (5'→3')	Mb	Pb	Ub	Tb	Size range (bp)	Poly (%)
RAPD	OPA-02	5'-TGCCGAGCTG-3'	10	3	0	13	151-1301	23
	OPA-16	5'-AGCCAGCGAA-3'	9	6	1	16	178-2299	43.8
	OPE-18	5'-GGACTGCAGA-3'	12	3	0	15	144-896	20
	OPG-02	5'-GGCACTGAGG-3'	10	6	0	16	112-2000	38.5
	OPH-16	5'-CAAGGTGGGT-3'	9	2	0	11	291-912	18.2
Total			**50**	**20**	**1**	**71**	**112-2299**	**29.6**
ISSR	834	5'-(AG)7ACYT-3'	8	7	1	16	207-1538	50
	835	5'-(AG)8YC-3'	13	2	0	15	158-1686	13.3
	900	5'- ACTTCCC(CA)2GG TTAA(CA)2-3'	6	7	0	13	229-1524	53.8
	844 B	5'-(CT)8GC-3'	12	2	0	14	182-1744	14.3
	17898 A	5'-(CA)6AC-3'	11	1	0	12	189-1017	8.3
	17898 B	5'-(CA)6GT -3'	6	7	1	14	140-1587	57.1
	ISSR-3	5'- GTGGTGGTGGC-3	9	2	1	12	159-2025	23.1
	ISSR-5	5'- CTCCTCCTGGC-3'	4	4	0	8	286-1734	50
	ISSR-9	5'- (GA)6CC-3'	12	4	0	16	186-1649	25
	ISSR-13	5'- GAGGAGGAGGC-3'	9	1	0	10	127-1612	10
Total			**90**	**37**	**3**	**130**	**127-2025**	**30.8**

Using ISSR primers, amplification of the genome of moringa shoots cultured on MS medium supplemented with 0.56mg/L BAP (Table 9). The size of DNA fragments ranged from 107 to 2299 bp using RAPD primers but it ranged from 126 to 2025 bp using ISSR primers. The polymorphism ranged from 10% (ISSR-13 primer) to 66.7% (900 primer and 17898 B), with an average of 40.3%. When AgNO3 was used, polymorphism ranged from 10% (ISSR-13 primer) to 53.8% (900 primer), with an average of 30.8% (Figure 4). The registered reduction in polymorphism was 23.6%. Under long-term culture, the reduction in polymorphism ranged between 34.5% and 23.6% when RAPD and ISSR were used.

DISCUSSION

For mass moringa propagation, nodal or shoot cuttings were multiplied on MS medium supplemented with low concentration of BAP (0.56mg/L). Moringa is a fast-growing plant species even under tissue culture conditions (Hassanein et al., 2018; Olaborode et al., 2022). Therefore, moringa cloning in partially closed vessels suffered from restricted gas exchanges which led to hypoxia (Dat et al., 2004) and ethylene accumulation (Adkins et al., 1993; Tamimi, 2015). Under these conditions, divided plant cells lack sufficient lignin and cellulose (Kevers & Gaspar, 1986), and result in the formation of highly vacuolated cells (Safrazbekyan et al., 1990) which leadis to the of appearance of hyperhydricity. The symptoms of this phenomenon were the enlargement of plant leaves and shoots, necrotic of shoot tips, and formation of callus at the base of explant which retard organogenesis and acclimatization (Hassanein et al., 2018; Drisya Ravi et al., 2019).

Enlargement of the base of the explant normally proceeds the initiation of shoot formation in moringa and other plant species (Hassanein & Soltan, 2000; Avila-Treviño et al., 2017; El-Nagish et al., 2019; Salem, 2020). On anti-ethylene free medium, visible enlargement at the base of the moringa explant was formed and continuously increased to form callus mass. This phenomenon and other hyperhydricity symptoms were disappeared or reduced when antiethylene compounds were used at low concentrations. The appearance of callus mass on shoot cuttings was detected on MS medium containing 1mg/L IBA for root formation. Under these conditions, plantlets formed roots on callus surfaces but they wilted during the acclimatization period. The combination of 1mg/L IBA and 1.70mg/L $AgNO_3$ reduced callus formation at the base of shoot cuttings and established fast contact between the formed adventitious roots and micro-shoot tissues which facilitated the acclimatization process.

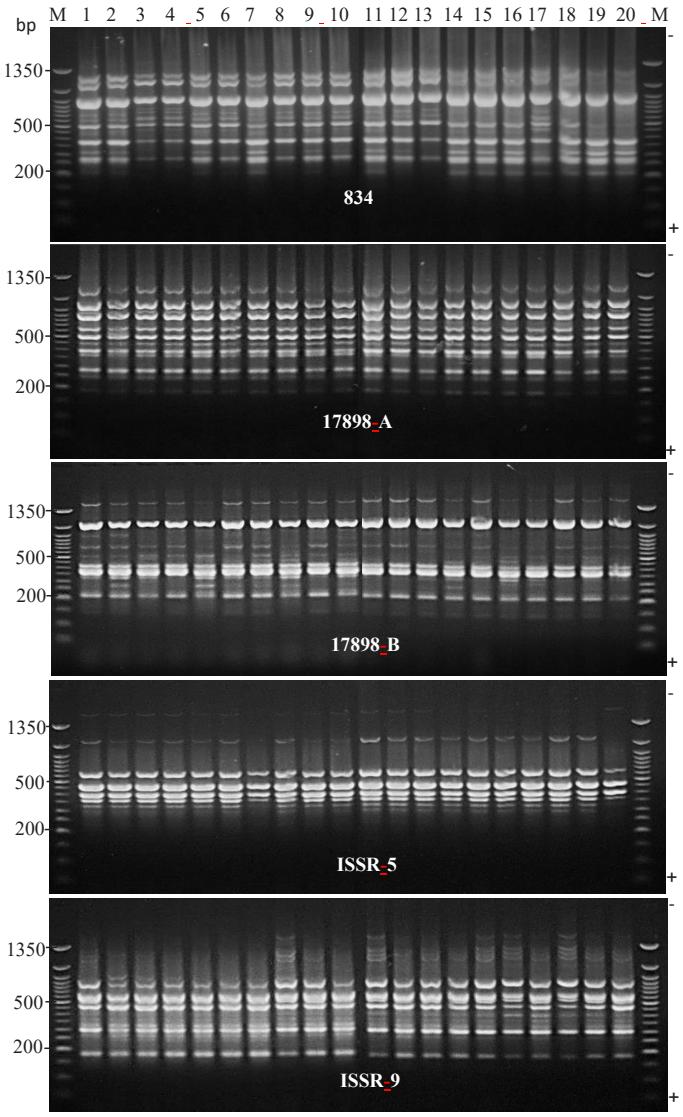

Figure 4. ISSR-PCR profiles generated by genomic DNA of 20 different microshoots of *Moringa oleifera* from the 11th subculture on MS medium + 0.56mg/L BAP without (lanes 1-10) or with (lanes 11-20) $AgNO_3$

Data from this work and others (Sarropoulou et al., 2016) indicated that the effect of antiethylene compounds depends on the antiethylene type and concentration (Salem, 2020). Since mass multiplication is needed for commercial application, it requires repeat subculture. It was effectively accomplished when antiethylene compounds were applied; 1.7mg/L $AgNO_3$ was the best where it reduced hyperhydricity stress, and increased shoot number/explant and growth/shoot even under long term culture (11 subcultures).

In vitro culture of moringa in closed glass jars decreased total chlorophyll contents in moringa and other plant species (Tirni et al., 2013; Mani et al., 2024). The application of antiethylene compounds such as $AgNO_3$ and SA improved plant cloning via enhancement of the biosynthesis of photosynthetic pigments (Beyer, 1976; Tamimi, 2015; Mani et al., 2024). The concentration of chlorophyll and carotenes was influenced by the type and concentration of antiethylene (Sarropoulou et al., 2016). In moringa, total pigment contents improved by 131% and 144% due to application of $AgNO_3$ and SA, respectively. The application of $CoCl_2$ decreased chlorophylls, carotenoids and total pigment contents significantly and exerted the lowest effect on avoidance of callus formation on the base of explant, one of the severe hyperhydricity symptoms in moringa.

Carotenoids increase under different oxidative stresses to scavenge ROS and they are the major

antioxidants for membrane protection against lipid peroxidation (Helaly & El-Hosieny, 2011). The application of $AgNO_3$ and SA as antiethylenes increased carotenoid contents in moringa to scavenge ROS obtained from hypoxia due to the existence of moringa shoots in closed glass jars. In addition, carotenoids protect the photosynthetic apparatus by quenching harmful ROS radicals, establishing PSI and stabising of thylakoid membrane (Gill & Tuteja, 2010). Therefore, the increase in both chlorophyll and carotene contents shared in biological events that are needed for avoidance of hyperhydricity and hypoxia stress leads to improved plant multiplication and growth.

Carbohydrate biosynthesis reduces in vitro cultivated plant materials due to inactive photosynthetic apparatus and low photosynthetic enzyme activity (Grout & Donkin, 1987). (Capellades et al., 1990). In moringa, the application of antiethylene compounds, especially SA or $AgNO_3$, improved shoot contents of carbohydrates and proteins, due to increase in the soluble fraction of both compounds. Normal leaves had a higher total protein content than vitrified leaves. (Ziv, 1991). Lack of nitrogen and carbohydrates led to decrease in the cell wall pressure and an increase water absorption (Kevers et al., 2004), which explains the low effect of $CoCl_2$ on avoidance of hyperhydricity. Osmoprotection refers to accumulation of osmotic adjustment components such as free amino acids and proline under stress conditions (Patade et al., 2012) and hyperhydric shoots (Franck et al., 2004). The presence of antiethylenes in the growth medium improved the growth and multiplication of shoot and it was related to nonsignificant increase of these compounds, which avoided progressive increase in water absorption and the appearance of hyperhydricity (Kevers et al., 2004).

MS medium was used to multiply moringa shoots, where NH_4^+ and Ca^{++} are the main components. These components are the major agents involved in hyperhydricity in many plant species (Ziv et al., 1987; Kevers et al., 2004). Therefore, using media with low mineral content, or only half strength MS medium resulted in improvement of carnation and cucumber plant development (Ziv et al., 1987). In this work, the application of antiethylenes showed nonsignificant changes of Na^+, K^+ or Ca^{++}. Low potassium content in the culture medium caused hyperhydricity (Singha et al., 1990). On the opposite side, antiethylenes increased Mg^{++} ᶜontent which necessitated chlorophyl biosynthesis.

Oxygen availability to the fast-growing tissues, such as moringa, was reduced in partially closed culture vessels, leading to hypoxia stress (Asada, 1992; Gasdaska & Baker, 1997). These conditions led to the appearance of hyperhydricity in moringa (Hassanein et al., 2018) and other plant species (Salem, 2020; Polivanova & Bedarev, 2022). The rate of ethylene release and ROS concentrations were shown to be closely correlated. (Ke & Sun, 2004). Other authors reported that while ROS affected metabolism of ethylene (Li et al., 2015), ethylene enhanced the accumulation of ROS (Chakrabarty et al., 2006; Wi et al., 2010). Then, accumulation of ROS under the influence of antiethylene compounds could be deduced which led to reduction in the activity of SOD, POX, CAT and APX as well as hyperhydricity. CAT activity was significantly increased under the influence of $CoCl_2$ where hyperhydricity was partially reduced.

Generally, When shoots were started from plant cuttings with preexisting meristems, such as nodal segments or meristematic domes, the amount of genetic variation found was low (Joshi & Dhawan, 2007) as was used in this work. In moringa, the detected high genetic variation was attributed to the long-term culture (Hassanein et al., 2018); where the genome was investigated using plant materials after 11 subcultures, three weeks each. In moringa and other plant species, these conditions may trigger DNA lesions that activate transposons, chromosome breakage and rearrangement, polyploidy and aneuploidy, epigenetic variations and point mutations (Kaeppler et al., 2000; Isah, 2015; Hassanein, 2018; Salem, 2020). Application of RAPD or ISSR techniques to detect the effect of antiethylene on genome stability of moringa under long term culture indicated that genetic polymorphism of shoots cultured on MS medium containing 0.56mg/L BAP and 1.7mg/L $AgNO_3$ was lower than that of others growing on MS medium containing 0.56mg/L BAP only. The decrease in polymorphism values ranged between 23.6% and 34.5% using RAPD and ISSR, respectively. This and other previously reported effects of antiethylenes on in vitro cultured moringa tissues expressed a positive role in avoidance of hyperhydricity and improvement of plant multiplication under long term culture.

CONCLUSION

In moringa, the application of antiethylene compounds especially $AgNO_3$ (1.70mg/L) or salicylic acid (6.91mg/L) retard hyperhydricity and improved in vitro moringa replication

through significant increase in photosynthetic pigments, carbohydrates, proteins and Mg^{++} contents. In general, application of antiethylene was associated with nonsignificant changes in proline, free amino acids, Na^{+}, K^{+} and Ca^{++} but the enzymatic antioxidants (CAT, POX, APX and SOD) decreased significantly, according to our findings. Also, the application of RAPD or ISSR techniques to investigate the effect of antiethylene on genome stability of *in vitro* grown moringa indicated that the application of $AgNO_3$ reduced genetic polymorphism resulted under the effect of long-term culture.

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