Regeneration and assessment of genetic fidelity of the endangered tree *Moringa peregrina* (Forsk.) Fiori using Inter Simple Sequence Repeat (ISSR)

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Abstract *Moringa peregrina* is an endangered species of Moringaceae. *M. peregrina* is a multipurpose tree with a wide variety of potential uses including its medicinal activity. In our study, a rapid and efficient micropropagation protocol for *M. peregrina* has been established. In vitro germinated seedlings were cultured on Murashige and Skoog (MS) medium supplemented with different levels of either 6-benzyladenine (BA) or kinetin (Kin). The maximum shoot proliferation of 6.5 shoots per explant with 100 % shoot proliferation rate was observed on MS medium supplemented with 1.0 mg/l BA. On the other hand, MS medium supplemented with 1 mg/l indole-3-butyric acid (IBA) resulted in the maximum number of roots. Micropropagated plants were successfully acclimatized. Genetic stability of micropropagated plants was assessed using Inter-Simple Sequence Repeat (ISSR). The amplification products were monomorphic in all in vitro grown plants. No polymorphism was detected indicating the genetic integrity of in vitro propagated plants. This micropropagation protocol could be useful for raising genetically uniform plants for plant propagation and commercial cultivation.

Keywords Micropropagation · In vitro · ISSR · Endangered · Genetic stability · *Moringa peregrina*

Introduction

Moringa peregrina (Forsk) Fiori is a green deciduous tree with sweet and oil rich white seeds. It belongs to the

Moringaceae family that has only one genus called *Moringa* with 13 species (Steinitz et al. 2009). The most famous *Moringa* species are *Moringa oleifera* and *Moringa peregrina* (Lalas et al. 2012). *M. peregrina* is distributed in tropical and non-tropical areas. Egypt, Ethiopia to Somalia, Sudan, the Red Sea region, Palestine and Jordan are the main centers of distribution (Lalas et al. 2012). It has been shown that *M. peregrina* has high aridity adaptation with short germination time after irrigation. Traditionally *M. peregrina* has both medicinal and industrial uses. In Greece, Egypt and Romania its seeds' oil is used to make perfume and its wood is used in building because of its resistance to termites. In addition, *Moringa* is used as medicine for abdominal pain, headache, and skin protection as well as a laxative (Steinitz et al. 2009). Recently, it has been shown that *Moringa* seeds contain potential anti-inflammatory and anti-oxidant factors (Koheil et al. 2011).

M. peregrina is becoming an endangered plant species all over the world as a result of unmanaged grazing and slow regeneration rate after browsing (Gomma and Pico 2011; Steinitz et al. 2009). Many recent studies are aiming to find efficient cultivation methods to save *M. peregrina* from extinction (Zaghloul et al. 2010). The loss of plant genetic resources, particularly those of wild species, has led to the development of 'ex situ' conservation methods to maintain genetics diversity. One way to protect rare species is by multiplying plants using tissue culture. In addition to conservation, this method also provides valuable material for amelioration studies. In vitro propagation of plants (micropropagation) is a technique used for plant vegetative multiplication under controlled conditions. This rapid propagation method saves plant biological, physiological and genetic specifications which are usually affected by environmental restrictions especially for endangered plant species (Kant et al. 2010). On the other hand, in vitro propagation provides new ways to improve plant cultivation conditions and plant genetic characteristics. Changing the growing conditions, such as hormone composition and

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concentration, light intensity and temperature, may produce more vigorous plants (Kapai et al. 2010).

To overcome the difficulties involved in *Moringa* species culture and regeneration, many studies were done to find new micropropagation methods of these species. Stephenson and Fahey (2004) used *Moringa oleifera* Lam. immature seeds and liquid medium for in vitro propagation. Although the culturing rate was high, the multiplication rate was only about 4.7 shoot per culture. Islam and his coworkers found that mature *M. oleifera* shoot nodules are a suitable explant source to start with for in vitro propagation on Murashige and Skoog (MS) medium that contains 1.0 mg/l benzylaminopurine and these cultured shoots produced roots at a high rate on MS free medium (Islam et al. 2005). While there are a few reports on in vitro clonal propagation of *Moringa* species, the effect of *Moringa peregrina* micropropagation on the genetic stability of micropropagated plants is yet to be reported. Therefore, the present investigation aimed to develop an efficient and reproducible method for rapid in vitro multiplication, ex vitro establishment and genetic stability analysis of micropropagated *M. peregrina*.

Materials and methods

Plant material and in vitro seed germination

Mature fruits of *M. peregrina* were collected from a wild population near the Dead Sea area/Jordan (31 °74'880"N; 35 °59'378"E) during the summer of 2010 (Fig. 1a, b & c). Fruits were cut open and the seeds (Fig. 1d) were separated from pods and rinsed in running tap water three times then washed in a 2 % (v/v) Tween 20 detergent solution for 8 min. Seeds were surface sterilized with 1 % NaClO solution containing two drops of Tween 20 for 15 min followed by four washings with sterile distilled water under aseptic conditions.

For in vitro seed germination, sterilized seeds were inoculated on half-strength MS medium containing 3 % sucrose and 0.8 % agar. After adjusting the pH to 5.8, 20 ml of medium was dispensed into each 100 ml jar. The culture jars were plugged with nonabsorbent cotton wrapped in cheese-cloth and autoclaved at 1.06 kg cm⁻² and 121 °C for 15 min. The cultures were maintained in the culture room at 16/8 light–dark photoperiod supplied by white fluorescent lights at 25 ± 2 °C until germination. The germination percentage was assessed 4 weeks after inoculation. Forty seeds were used for each treatment and the experiment was repeated three times.

Shoot and root formation

Shoot tips from in vitro germinated seedlings were excised and cultured on MS medium supplemented with different

concentrations of BA or Kin (0.5, 1.0 and 2.0 mg/l). The medium was solidified with 0.8 % agar and supplemented with 3 % sucrose. After 6 weeks of culture, number and height of newly formed shoots and number of leaves were recorded.

Microshoots obtained on MS medium containing the best growth regulator were grown on a growth regulator free MS medium for additional 4 weeks to eliminate growth regulator carry over effect. Subsequently microshoots were incubated on half-strength MS medium supplemented with different concentrations of 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) (0.5, 1.0 and 1.5 mg/l) to induce root formation. In addition the effect of adding activated charcoal (AC) (2 g/l) to culture medium was investigated. After 6 weeks of culture, the percentage of root formation, number and length of roots were assessed.

Acclimatization

Six week old rooted plants were thoroughly washed with sterile water for 10 min to remove the agar, then planted in 15 cm diameter plastic pots filled with autoclaved artificial soil (1 peatmoss: 1 perlite: 1 soil by v/v) and irrigated with sterile distilled water for 2 weeks under the culture room conditions as mentioned above and then moved to the greenhouse. Watering was done when required.

DNA extraction and PCR amplification

Genetic stability of in vitro-propagated plants was tested using ISSR markers (Bhatia et al. 2011). For this purpose, total genomic DNA was extracted from the mother plant and twelve in vitro grown plants chosen randomly. DNA was extracted from young leaf tissue using the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson (1980). DNA concentration was measured using a Genesys 10S spectrophotometer (Thermo Scientific, USA) and DNA quality assessed by gel electrophoresis. Thirty ISSR primers were used for preliminary screening. PCR amplifications were carried out in a total volume of 20 µl containing 1X PCR buffer (75 mM Tris-/HCl, 50 mM KCl, 2.0 mM MgCl₂, and 20 mM (NH₄)₂SO₄); 0.3 mM each of dATP, dTTP, dCTTP, and dGTP; 0.6 mM primer; 1 U *Taq* DNA polymerase (Ampliqon); and 25 ng template DNA and water. Primers showing polymorphic bands were then used for analysing the genetic fidelity of the micropropagated plants. PCR amplification was performed in a DNA thermal cycler (Genepro, Bioer Model TC-E-96 G) with the following cycling profile: an initial denaturation at 92 °C for 5 min, followed by 40 cycles of 1 min at 92 °C, 1 min at 49 °C and 1 min at 72 °C with a final extension for 7 min at 72 °C.

Fig. 1 In vitro propagation of *Moringa peregrina*. **a**: Wild grown *Moringa peregrina* tree near the Dead Sea area, **b**: Flowering tree of *M. peregrina*, **c**: Mature fruit, **d**: Seeds, **e**: germinated seedling after 10 days of inoculation on MS medium, **f**: Shoots after 40 days of inoculation on MS medium supplemented with 1 mg/l BA, **g**: Early stage of rooting, **h**: Later stage of rooting, **i**: Acclimatized plant transplanted into soil in the glasshouse conditions. Scale bar in C= 8 cm, D=1 cm, F=3 cm, I=3 cm

Amplified products were resolved by electrophoresis on 1.5 % agarose gels in 1X TBE buffer at 90 V for 2 h then stained with ethidium bromide and the DNA fragments were visualized under UV light using a gel documentation system. PCR reactions were repeated twice to check the reproducibility of the banding patterns. A 1 kb DNA ladder was used as the molecular standard in order to confirm the appropriate ISSR markers.

Statistical analysis

All experiments were repeated at least twice, for each experiment at least four independent replicates were used. Data were analyzed by analysis of variance (ANOVA) using the statistical program Statistical Package for Social Sciences (SPSS 16.0 for Windows) (SPSS Inc., Chicago, IL, USA) followed by Tukey's t-test for mean comparison.

Results

Seed germination and shoot multiplication

High germination rate (95 %) and low contamination percentage were observed for *Moringa peregrina* culture. Murashige & Skoog (MS) medium (Murashige and Skoog 1962) was used initially to study the effect of medium strength on shoot multiplication. Full strength MS medium proved to be better than half or quarter strength medium since it showed the highest number of shoots and leaves along with the maximum length of shoots (Data not shown). Thus, full strength MS medium was used in the following experiments, to enhance the multiplication rate of *Moringa peregrina* shoot formation.

In the present study, different levels of BA and Kin were used in order to evaluate their effect on shoot formation. Plant response varied as the type and/or level of the

cytokinin changed. While the percentage of explants developing shoots was 100 % in all samples, the control (no plant growth regulators) had the lowest shoot number/explant. The effect of BA on shoot proliferation after 6 weeks of culture was highly significant (ANOVA with $P < 0.05$) (Fig. 1e & f and Fig. 2). Results indicate that using BA for shoot multiplication was better than Kin. Using 1.0 mg/l BA induced shoot formation and resulted in the highest mean number of shoots/explant (6.5) (Fig. 2a). However, there

Fig. 2 Effects of BA and Kin on in vitro shoot proliferation of *M. peregrina* after 6 weeks of growth. (a) Number of shoots, (b) Number of leaves and (c) Shoot height. Data represents mean values $\pm$ standard error of eight replicates and the whole experiment was repeated three times. Means followed by the same letter are not significantly different at the $p \leq 0.05$ according to Tukey's test

was no significant difference between the two BA concentrations on number of shoots (Fig. 2a). On the other hand, using 1 or 2 mg/l Kin resulted in 2.6 and 3.25 mean shoots/explant, respectively. Higher levels of cytokinin (BA or Kin) (2 mg/l) significantly reduced the number of leaves per microshoot (Fig. 2b). However no significant effect was observed between microshoots grown on control medium and microshoots grown on medium supplemented with low levels (1 mg/l) of cytokinins. We found that microshoots grown on MS medium supplemented with BA (either 1 or 2 mg/l) are shorter than the controls. In contrast, microshoots grown on free MS medium and that supplemented with Kin were similar (Fig. 2c).

Rooting and acclimatization

For the rooting experiment, microshoots obtained from the shoot multiplication experiment were inoculated on free MS medium (control) or MS medium supplemented with different levels (0.5, 1.0 or 1.5 mg/l) of NAA or IBA. All of the inoculated microshoots developed roots including those on MS free medium (100 % root induction rate, Data not shown) (Fig. 1g & h). Adding 0.5 or 1.0 mg/l IBA to the culture medium significantly affected number of roots and root length (Fig. 3). In contrast, only the high concentration of NAA (1.5 mg/l) significantly increased number of roots but significantly decreased root length compared to the control (Fig. 3). The maximum number of roots (44) was observed on microshoots inoculated on MS media supplemented with 1.0 mg/l IBA (Fig. 3a). On the other hand, the maximum root length was observed on microshoots inoculated on MS media supplemented with 0.5 mg/l IBA (Fig. 3b). The data shows that using lower levels of auxin (either NAA or IBA) is significantly better than higher levels for root elongation (Fig. 3b). Furthermore, we tested the effect of medium strength (full MS, 1/2 MS and 1/4 MS) on root elongation and number of roots. All the tested media were supplemented with 1.0 mg/l IBA. Data showed that full MS medium is superior to 1/2 or 1/4 MS (Fig. 3c&d). In addition no significant effect of adding charcoal to the rooting medium was observed (Fig. 3c&d).

For plant acclimatization, fifty plants with developed roots (from the rooting experiment) were transferred to the ex vitro conditions after thorough washing with sterile distilled water. A high survival rate was observed (90 %) (Fig. 1i).

Genetic fidelity of micropropagated plants

In order to confirm genetic stability, in vitro micropropagated plants were assessed through ISSR analysis. The amplification profiles were screened for the presence of polymorphisms among the samples (Fig. 4). A total of 12

Fig. 3 Effect of NAA and IBA on in vitro rooting of *M. peregrina* after 6 weeks of growth. (a) Number of roots and (b) Root length. Effect of medium strength and activated charcoal on (c) number of roots and (d) Root length. Data represents mean values $\pm$ standard error of ten replicates and the whole experiment was repeated twice. Means followed by the same letter are not significantly different at the $p \leq 0.05$ according to Tukey's test

microshoots were analyzed from different passages using ten selected ISSR primers which resulted in a total of 756 scorable bands (numbers of plantlets analysed x number of

bands of all ISSR primers) ranging from 150 to 1,600 bp (Table 1). In addition, the mother plant was also analyzed. A variable number of bands was observed for each primer

Fig. 4 DNA fingerprinting patterns generated by ISSR primers of in vitro micropropagated *M. peregrina* plants. Amplification products obtained with primers (a) UBC-828, (b) UBC-810. DNA marker (Lane M), mother plant (MP), randomly selected in vitro grown plants (Lanes 1–8)

ranging from 5 (UBC841 and 860) to 9 (UBC810) bands per primer. The micropropagated plants were found to be phenotypically similar. Furthermore, ISSR analysis using 10 ISSR primers of the micropropagated plants and the mother plant showed an identical banding pattern after more than 8 passages of subculture (Fig. 4). These results indicate that regenerated plants are similar and no genetic variation was detected after in vitro culture. All ISSR profiles were found to be monomorphic among the micropropagated plants and mother plant.

Discussion

Micropropagation of rare species can create a large number of clones from a single explant, thus decreasing the amount of space required for field trials, as well as decreasing the time required, as there is no need to wait the whole life cycle for seed development. Micropropagation of rare species also provides an opportunity to maintain disease-free plants, overcomes seasonal restrictions for seed germination, and enables cold storage of large numbers of viable plants in a

small space. This study investigated the possibility of in vitro propagation of *M. peregrina*, a rare species of tree in Jordan. MS Media was used for shoot multiplication and rooting. This medium has been used widely for in vitro culture of many hard wood species (George and Sherrington 1984). The results of this experiment indicate that there is a high success rate for adopting in vitro propagation methods for conservation and mass propagation of *M. peregrina*.

Different plant growth regulators have been used for plant in vitro propagation. Plant growth regulators have great influence on shoot growth and proliferation. BA and Kin are the main cytokinins used for shoot induction in different plant species (Nas et al. 2010). Data obtained here show that BA was superior to Kin in stimulating shoot proliferation and growth. Moreover, the level of this cytokinin strongly influenced number of axillary shoots developed per explant. Using 1 mg/l BA resulted in the highest number of proliferated shoots/explant, but decreased shoot height compared to the control and no significant effect was observed for number of leaves (Fig. 2). On the other hand, Kin was found to be less effective than BA for shoot multiplication but resulted in taller plants. In *Moringa oleifera* Lam., it has been shown that medium supplemented with BA resulted in the highest number of shoots per explant compared with Kin or TDZ (Marfori 2010). Similarly, Islam et al. (2005) found BA to be the best cytokinin for shoot induction. In contrast, Abdellatif and Khalafalla (2010) found that using a combination of BA and NAA resulted in the best shoot induction rate. These variable responses could be due to different factors including genetic differences, differences in the explant source and the type and/or age of explants used to establish the cultures (Marfori 2010).

It has been shown previously that IBA is more effective than NAA in root induction of a wide variety of species, and it is used in the nursery industry for rooting several plant

Table 1 List of primers, their sequences, number and size of the amplified fragments generated by inter-simple sequence repeat (ISSR) primers

Primer number	Primer name	5'–3' sequence	Annealing temperature (°C)	Number of scorable bands/primer	Total number of bands amplified
1	807	AGAGAGAGAGAGAGAGT	52	6	72
2	810	GAGAGAGAGAGAGAGAT	52	9	108
3	823	TCTCTCTCTCTCTCC	52	6	72
4	826	ACACACACACACACACC	52	7	84
5	828	TGTGTGTGT GTG TGT GA	52	6	72
6	835	AGAGAGAGAGAGAGAGYC	52	7	84
7	841	GAGAGAGAGAGAGAGAYC	52	5	60
8	843	CTCTCTCTCTCTCTRA	52	6	72
9	848	CACACACACACACARG	52	6	72
10	860	TGTGTGTGTGTGTGTGAA	52	5	60
Total				63	756

species worldwide (Epstein and Luduig-Muller 1993). Here, medium supplemented with IBA showed higher rate of root induction and longer roots than control or medium supplemented with different levels of NAA. In agreement, Prathanturug et al. (2012) found that IBA was superior to NAA in terms of root induction percentage and number of roots per shoot with *S. hutanguriana* regenerated shoots after 8 weeks in culture. A similar trend was also observed for other species including *Centella asiatica* (Tiwarei et al. 2000), *Boscia senegalensis* (Khalafalla et al. 2011), *Pappea capensis* (Mngomba et al. 2007) and *Teucrium fruticans* (Frabetti et al. 2009).

In an experiment for in vitro rooting of another species of *Moringa* (*M. oleifera*), when microshoots were inoculated on a medium containing different levels of IAA and IBA, they found that medium supplemented with 0.5 mg/l IAA along with IBA at 1.0 mg/l resulted in the highest number of induced roots (Saini et al. 2012). Stephenson and Fahey (2004) found that 4.7 shoots of *M. oleifera* were produced per explant when inoculated on medium containing 1 mg/l IBA with 1 mg/l GA_3 , in addition, rooting was the best in MS containing 0.5 mg/l NAA. In contrast, Islam et al. (2005) found that hormone-free medium was the best rooting medium.

It has been shown for several species that rooting rate is higher when shoots are rooted on low strength MS medium. Therefore, a preliminary experiment was conducted to test the effect of medium strength on root induction. Results showed a similar rooting frequency between shoots inoculated on full, half or quarter strength MS medium (Data not shown). In addition we found that shoots grown on full MS medium were healthier than the others.

The most important dilemma encountered during plant in vitro propagation is the presence of somaclonal variation among the micropropagated plants. In the present study, ISSR analysis was used to evaluate the genetic stability of *Moringa peregrina* micropropagated shoots after 8 passages of in vitro multiplication. The results obtained suggest that shoot proliferation could be used for clonal propagation with a minimal risk of producing somaclonal variants.

Molecular marker analysis of micropropagated plants has been reported in several plant species (Bhatia et al. 2011; Goto et al. 1998; Salvi et al. 2001; Varshney et al. 2001). Martins et al. (2004) reported the genetic stability of micropropagated almond plants. The genetic stability of in vitro propagated *Picea mariana* was also reported by Isabel et al. (1993) and in vitro-adventitious shoots of *Pinus taeda* (Tang 2001) and chestnut (Carvalho et al. 2004). PCR based molecular markers including ISSR are very simple, fast, reliable, highly discriminative and inexpensive. Due to the absence of variation in the banding profile in ISSR analysis of the all micropropagated plants, we concluded that this protocol could be used efficiently as a means of rapid propagation of this rare species.

The present study provides the first report on in vitro propagation and genetic stability of micropropagated *Moringa peregrina* using ISSR analysis. This study presents a successful and quick protocol for large scale in vitro propagation method of this tree species. Further studies are essential to optimize an in vitro conservation protocol in order to maintain this threatened species.

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