

# Vegetative micro-cloning to sustain biodiversity of threatened *Moringa* species

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**Abstract** Efficient vegetative cloning *in vitro* requires definition of plant growth regulator regimes for each genotype, and therefore formulation of a uniform culture protocol for a genetically heterogeneous wild or uncultivated plant population is often impossible. The likelihood of cloning a wide array of plant genotypes by avoiding the use of plant growth regulator(s) was explored with *Moringa oleifera* Lamk., *Moringa stenopetala* (Baker f.) Cufod, and *Moringa peregrina* Forssk. ex Fiori tree seedlings. Propagation was achieved by multiple shoot regeneration from the cotyledonary node of decapitated seedlings, followed by axillary shoot growth from single node shoot segments and rooting of excised shoots. All steps were accomplished on basal Murashige and Skoog medium without plant growth regulator supplements. The results revealed competence for generation of multiple shoots from cotyledonary node tissue, stimulated by repeated shoot harvest, in seedlings of all three tree species. Tens of plants per seedling were regenerated in about 4 mo from culture initiation. In a given species clone size was seedling-dependent, which presumably stems from genotypic variability among seedlings in regeneration ability *in vitro*. By this means the laborious search for a plant growth regulator regime suitable for organogenesis induction and adapted per genotype became redundant, and biodiversity of the seed germplasm could be

maintained. The approach ideally suits establishment of clones of wild plants of endangered species, like those of the Moringaceae, species with high ability for producing supplementary shoots, and without the need to add plant growth regulators, including the rooting stage.

**Keywords** Biodiversity · Cloning · Conservation · Moringaceae · Endangered plant

## Introduction

The Moringaceae comprises 13 tropical and sub-tropical trees species. Our study involved three species: (a) *Moringa oleifera* Lamk., which grows in many tropical and subtropical moist through dry areas of the world (Morton 1991; Duke 2001). *M. oleifera* is used for food (Bennett et al. 2003; Gidamis et al. 2003), animal feed (Sánchez et al. 2006), as a natural coagulant for treatment of turbid water (Suarez et al. 2003), and as a source of phytomedicine compounds (Anwar et al. 2006). (b) *Moringa stenopetala* (Baker f.) Cufod, a tree endemic in south Ethiopia and north Kenya, largely uninvestigated, and carries comparable beneficial traits to those found in *M. oleifera* (Bennett et al. 2003; L alas et al. 2003). (c) *Moringa peregrina* Forssk. ex Fiori, occurs from the Dead Sea to southern Arabia and northern Somalia (Olson 2002). There is no documentation of the use of this species.

*M. oleifera* is the sole species from the Moringaceae for which cultivation practices are being developed (Veeraragava Thatham 1998; Sánchez et al. 2006). In absence of cultivation practices of other species, and growing demands by local populations, wild-harvest and over-browsing is decimating natural tree resources. Genetic diversity of *Moringa arborea*, *Moringa borziana*, *Moringa longituba*,

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*Moringa rivae*, *Moringa ruspoliana*, and *Moringa stenopetala* is endangered (Odee et al. 2001; Shibru 2003; Stephenson and Fahey 2004). *M. peregrina* is a rare species with a low rate of regeneration following herbivorous animal browsing (Hakham and Ritte 1993), *M. arborea* is listed in the 2006 IUCN Red List of Threatened Species (World Conservation Monitoring Centre 1998), and *Moringa hildebrandtii* is extinct in the wild (Olson and Razafimandimbi-son 2000; Hammer and Khoshbakht 2005).

Plant tissue culture technologies support endeavors to alleviate extinction threats (Fay 1994; Sarasan et al. 2006), yet *Moringa* spp. have received little attention. Multiplication of *M. oleifera* from immature embryo-, seedling-, and mature tree-derived explants was reported (Mohan et al. 1995; Islam et al. 2005), but Stephenson and Fahey (2004) encountered difficulties to repeat such an accomplishment. Achievement of plant regeneration and multiplication *in vitro* of any other *Moringa* species have not been reported.

The inception of an *in vitro* micro-cloning approach for endangered plants may differ from a micropropagation approach developed for cultivated crop plants in several respects: (a) the intent is initiation and establishment of small vegetative clones that will serve subsequent vegetative and reproductive propagation. The aim is not mass micropropagation on the scales employed in horticulture or forestry; (b) the method should maintain biodiversity found in a plant or seed population; (c) due to paucity of specimens, inconsistent year-to-year seed production or seed availability, inconsistent seed germination, or due to the dynamics of current events requiring swift plant rescue, the amount of plant material available for experimenting and formulating proper culture conditions could be limited.

Numerous studies demonstrate that very often plant growth regulator (PGR) regimes in culture media must be defined for each genotype to succeed in plant regeneration and micropropagation, which means that valuable plants may be sacrificed for this purpose. Moreover, due to genotype-specific PGR requirements, formulation of a uniform culture protocol valid for a genetically heterogeneous wild or non-cultivated plant population is often impossible. The aim of the present study was to examine the possibility to initiate micro-cloning *in vitro* of seedlings in a way that will maintain biodiversity of the plant population and that will avoid the need to search for PGR regimes mediating plant regeneration.

## Materials and Methods

**Plant material.** Seeds were collected in the wild or in gardens, from trees that have not been subjected to any selection or breeding. *M. oleifera* seeds were harvested in gardens located in the Mediterranean area of Israel (Rishon Lezion,

E31.963661071 N34.8032788652; Ta'oz E31.801758723 N34.9750517248). *M. peregrina* seeds were from Ein Gedi nature reserve, near the Dead Sea (E31.451016122 N35.3820021444), and from Kibbutz Samar in the desert Arava rift valley (E29.833716579 N35.0210710114), Israel. *M. stenopetala* seeds were obtained from ECHO Seed Bank, North Fort Myers, FL, and were the progeny of seeds collected in Ethiopia.

**Culture conditions for shoot regeneration and plant establishment.** Seeds were surface sterilized twice, before and after peeling off the shell. Sterilization was for 20 min with a 1% sodium hypochlorite solution containing a drop of Tween-20, followed by rinsing several times with sterile distilled water. Seed germination was in the dark for 2–3 d at  $25\pm 1^\circ\text{C}$ , in Petri plates (90×20 mm), on filter paper wetted with sterile distilled water. Germinating seeds and seedlings were planted in GA7 Magenta boxes, one seedling per box, on 30-ml medium, and covered with a Magenta lid with a 1.5 cm diameter filter-vent. Growth medium for seedlings, shoot regeneration (Figs. 1 and 2) and for shoot multiplication (Fig. 3) consisted of MS (Murashige and Skoog 1962) basal salt medium (Duchefa, Haarlem, The Netherlands, catalog no. M 0221) fortified with 1.0 mM  $\text{MgCl}_2$ , 1.5 mM  $\text{CaCl}_2$ , Gamborg B5 vitamins (Gamborg et al. 1968) (Sigma Co., St. Louis, MO, catalog no. G1019), 90 mM sucrose, and 3 g/L Phytigel (Sigma Co., St. Louis, MO, catalog no. P8169), at pH 5.8. The pH of the medium was adjusted after addition of Phytigel, before autoclaving at 120 kPa for 20 min at  $121^\circ\text{C}$ . Filter-sterilized vitamins were added to the cooling autoclaved medium. Cultures were exposed in all stages to  $10\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  fluorescent cool white light (Osram 36W10), 16 h photoperiod, at  $25\pm 1^\circ\text{C}$ . Contaminated seedling cultures were discarded.

The main shoot and shoots regenerated thereafter from decapitated seedlings were excised at the border between shoot base and cotyledonary node, and planted onto growth medium. Single-node segments prepared from elongated shoots were subcultured to obtain one new elongating axillary shoot per segment. Recurrent multiplication by virtue of single-node segments was at 2–3 wk intervals, for a total of 7 wk (Fig. 3). By the end of the multiplication stage, all shoots of a given clone were transferred to a rooting medium with MS salts reduced to half strength, B5 vitamins, 45 mM sucrose, 3 g/L Phytigel, at pH 5.8. Rooting percentage was scored 1 mo after planting shoots on rooting medium. Rooted plantlets (Fig. 4a) were first transplanted to Jiffy-7 Peat Pellets® (Fig. 4b), later potted into a peat-vermiculite (1:1, v/v) soil mixture (Fig. 4c), and were acclimatized to *ex vitro* conditions in a growth room at  $25\pm 2^\circ\text{C}$ , 16 h photoperiod,  $30\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$  fluorescent white light and relative humidity of 70–80% (Fig. 4b-d). Following



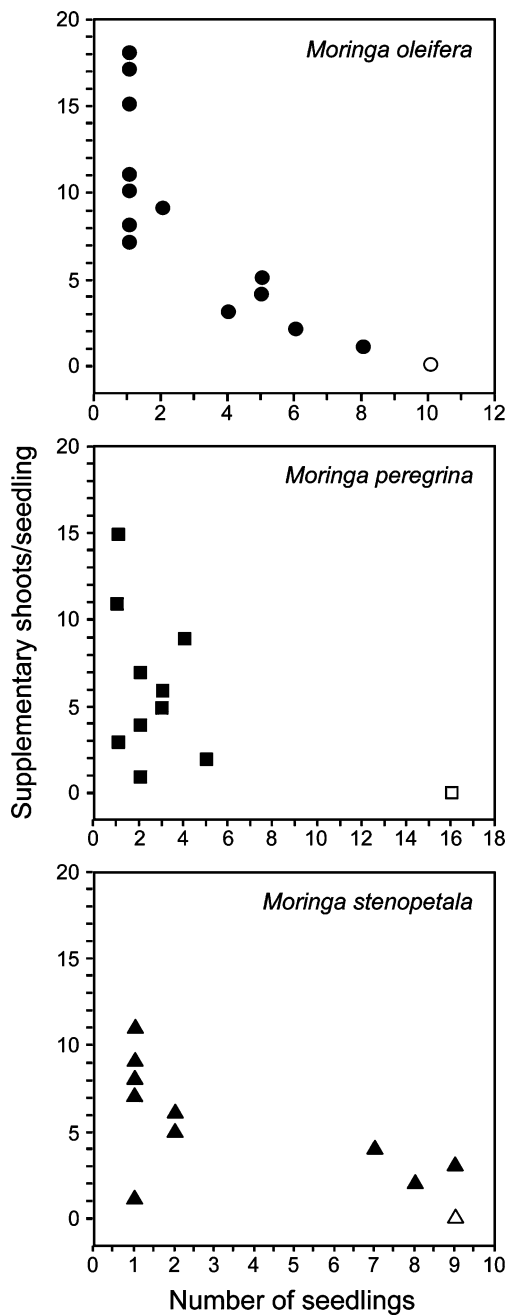
**Figure 1.** Types of shoots formed in *Moringa* spp. seedling cultures. *Moringa stenopetala* (a, b, c), *M. peregrina* (d, e, f). Main shoot (a, d), axillary shoots growing from cotyledons axils (b, e), and multiple

supplementary shoots developing on the cotyledonary node (c, f). Bars indicate 2 cm.

acclimatization, plants were transferred and grown in a greenhouse, shaded and cooled in the summer, or heated in the winter. Typical temperatures were  $28 \pm 3^\circ\text{C}$  and maximum photosynthetic photon flux was  $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Plants were irrigated periodically with tap water.

**Experimental design.** *Moringa* species were examined separately. Thirty seeds were sown from a species three times (three repeats) on different dates. Germination

percentage was calculated from repeats in a species. For the assessment of biodiversity in a plant (seed) population in terms of cloning ability, the identity of every seedling and its derived clone were traced throughout the examination. Some initiated clones could not be subcultured and multiplied throughout the experiment because of tissue-borne bacterial contamination of the medium or premature senescence of cultures. Figures 2 and 3 are only from



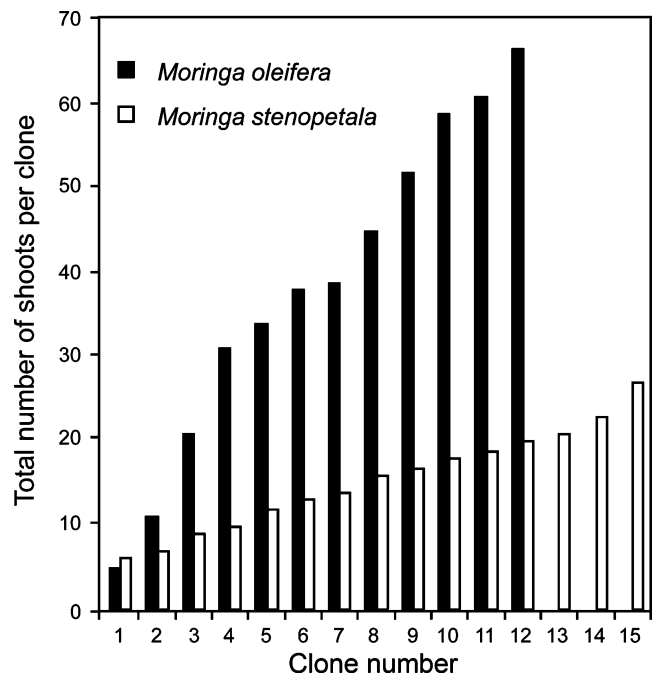
**Figure 2.** Regeneration of supplementary shoots in decapitated *Moringa oleifera*, *M. peregrina* or *M. stenopetala* seedlings. Open symbols (circle, square, triangle) represent seedlings that produced no supplementary shoot. Closed symbols (circle, square, triangle) represent seedlings that produced the indicated total number of supplementary shoots per seedling. Supplementary shoots were collected by repeated harvests during 7 wk from removal of the axillary shoot that grew out from cotyledons' axil.

cultures that showed no visible symptoms of senescence, therefore the number of clones from which data were recorded was from a fraction of the germinated seeds. In all repeats together, a total of 47, 42, and 40 clones were generated in *M. oleifera*, *M. stenopetala* and *M. peregrina*,

respectively. Data from all repeats per species were assembled for presentation in Figs. 2 and 3. In Fig. 2, an indicated value (point) of “supplementary shoots/seedling” in a species represents all seedlings, from all repeats together, that produced the indicated number of shoots; this value is identical for each of the individual clones as well as for the mean of these clones and for the mean of repeats. In *M. oleifera*, *M. stenopetala*, and *M. peregrina*, 4, 5, and 1 clones, respectively, were selected per repeat for multiplication by single-node segments (Fig. 3). A column in Fig. 3 represents a clone derived from a seedling. Forty *M. oleifera* or *M. stenopetala* shoots, and 15 *Moringa peregrina* shoots were rooted per repeat. Rooting percentage was calculated from all clones of a species in a repeat, and the mean of repeats $\pm$ SE in a species is specified.

## Results

Seed germination percentage (mean $\pm$ SE) was 77 $\pm$ 15, 72 $\pm$ 8, and 69 $\pm$ 12 for *M. oleifera*, *M. stenopetala*, and *M. peregrina*, respectively. Two problems were observed in seedling cultures of all species: (a) seed-borne bacterial contamination of the medium of 100% of cultures. No seed surface sterilization measure eliminated the difficulty. (b) Precocious organ senescence that eventually led to culture



**Figure 3.** Proliferation of axillary shoots by single-node culture in *Moringa oleifera* and *M. stenopetala*. The main, cotyledonary axillary and supplementary shoots were used for propagation. Data were recorded following 7 wk of propagation. Results were ordered in an ascending number of shoots per clone.

**Figure 4.** Rooting and transplanting to soil of *Moringa* spp. culture-derived plants. (a) Abnormal *in vitro* adventitious roots (arrow) (*M. stenopetala*). (b) Established plants in Jiffy-7 Peat Pellets® (*M. peregrina*). (c) Established plants in pots (*M. stenopetala*). (d) Normal adventitious roots (arrow) regenerated in soil substituting *in vitro* roots, one mo after transplantation to soil (*M. stenopetala*). Bars indicate 2 cm.



decay. This phenomenon was partially reduced by replacing standard lids of Magenta box GA7 with vented lids.

**Shoot formation by seedlings.** Seedlings were decapitated 2 or 3 wk after sowing in *M. oleifera* and *M. peregrina* or *M. stenopetala*, respectively, after the main shoot reached a length of at least 2 cm. An axillary shoot emerged from each cotyledon axil within 7 or 10 d after decapitation of the main shoot in *M. oleifera* and *M. peregrina* or *M. stenopetala*, respectively (Fig. 1a,b,d,e). Following removal of both axillary shoots, most seedlings generated one or more shoots from the cotyledonary node (Fig. 1c,f). Regeneration of additional shoots took about 10–14 d in *M. oleifera* and *M. peregrina* or 10–20 d in *M. stenopetala*. Excision of additional shoots sometimes stimulated appearance of another wave of shoots, but shoot production gradually declined with repeated harvests until eventually it ceased 6–8 wk from first decapitation.

Figure 2 summarizes the accumulative number of supplementary shoots harvested per seedling (excluding main shoot and two axillary shoots from cotyledons axils) during 7 wk following removal of the pair of cotyledon axillary shoots. We found a similar general response pattern in all species: no supplementary shoot regeneration (Fig. 2) was observed in a substantial fraction of seedlings (23, 38 or 19% in *M. oleifera*, *M. peregrina*, or *M. stenopetala*, respectively); one to six supplementary shoots appeared in a second large fraction of seedlings (55, 52, or 72% in *M. oleifera*, *M.*

*peregrina*, or *M. stenopetala*, respectively); and a minority of seedlings produced more than eight supplementary shoots.

**Multiplication from node segments and plant establishment.** The accumulated number of axillary shoots per clone produced in 7 wk of multiplication for *M. oleifera* and *M. stenopetala* is shown in Fig. 3. Very wide ranges of multiplication rates were revealed within each species, some clones hardly multiplied while others were very prolific. The span between slowest and fastest *M. oleifera* clones was above one order of magnitude, but was much narrower in *M. stenopetala* (Fig. 3). In addition, a small scale experiment of shoot multiplication by virtue of single-node culture conducted with *M. peregrina* yielded results similar to those obtained with *M. oleifera* node culture (data not shown).

Shoots with at least three nodes were transferred to rooting medium. Rooting percentage was  $95 \pm 4$  and  $92 \pm 7$  for *M. oleifera* and *M. peregrina*, respectively, but only  $19 \pm 7$  for *M. stenopetala*. *In vitro* roots appeared similar in the three species: they were straight, not branched and brittle (Fig. 4a). Nevertheless, more than 95% of rooted shoots in all species survived transfer to soil (Fig. 4c,d), and all these plants resumed visible growth 4–5 wk later, a period during which *in vitro* roots degenerated and were substituted by new soil-adapted roots (Fig. 4d). Plants that gained two new fully expanded leaves in the growth room were transferred to a greenhouse without any loss.

## Discussion

The three *Moringa* species revealed competence for multiple shoot generation from cotyledonary node tissue of decapitated seedlings, elicited by repeated shoots excisions. Manifestation of this ability was accomplished without stimulation by exogenous PGR. Induction of multiple adventitious shoot regeneration by repeated harvests in plants cultured on a medium without PGR has recently been shown in a number of *Lycopersicon* species (Steinitz et al. 2006), and is now demonstrated for the first time in cotyledon-stage seedlings of *Moringa* trees.

As a rule, the origin of shoots regenerated in decapitated seedlings may be either from axillary meristems found in the cotyledon axil or, in case of total loss of all pre-formed shoot meristems, by differentiation of a *de novo* apical shoot meristem. There are, however, exceptions to this rule where seedlings develop accessory shoot meristems. For example, not a single axillary meristem in each cotyledon axil but additional axillary meristems formed in the node tissues, as has been found in *Eucalyptus cinerea* (Graham et al., 1998), *Arabidopsis thaliana* branchy mutant (Grbić and Bleecker 2000), *Banksia* (Mibus and Sedgley 2000), *Quercus* (Pascual et al. 2002), and *Lotus japonicus* (Alvarez et al. 2006). *Moringa* seedlings produced shoots after removal of the cotyledons' axillary meristem but, without a histology study, we can not determine whether the new shoots emerged from pre-existing accessory axillary meristems or by induction of adventitious meristems, and therefore we termed them "supplementary" shoots. Within a given species, differences in extent of response to repeated excisions (Fig. 2) probably reflect seedling-dependent genotypic variation in supplementary shoot generation ability.

Excised main, axillary, and supplementary shoots could be multiplied by single-node segment culture, and clones of tens of propagules per productive source plant were established in a period of several months. Biodiversity amongst the wild germplasm utilized in our program could be sustained, given that multiplication by axillary bud formation was possible from seedlings unable to generate supplementary shoots. The search for a PGR regime suitable for organogenesis induction as one of the first steps in the development of a micro-cloning method became redundant, and no compromise had to be made by excluding biotypes that would be non-regenerating on a medium supplemented by exogenous regulators. Our work suggests, for the first time, that a PGR-independent *in vitro* cloning scheme can assist efforts to conserve biodiversity in cases where cloning of endangered (*Moringa* or other) plants commences from seeds. The scheme ideally suits species, like those of the Moringaceae, with high ability for producing supplementary shoots, and without the need to add plant growth regulators, including the rooting stage. Clones can subsequently be enlarged either asexually with a

PGR-dependent protocols adapted per clone, or by seed propagation.

Onset of vegetative cloning was based on the seedlings' competence to replace a lost shoot by a new one, which is a trait fundamental to plant survival conserved in many species. Therefore, this method could probably be applicable for taxonomic groups beyond the Moringaceae. The low rooting rate encountered in *M. stenopetala* could be due to one or more of two reasons: (a) low PGR-independent adventitious rooting capacity as a genetic trait; (b) bacterial contamination of the cut edge at the shoot base that interfered with rooting.

In conclusion, our work uncovered competence for multiple shoot regeneration from the cotyledonary node tissue of decapitated *Moringa* spp. tree seedlings, and realization of this competence without support of exogenous PGRs. Based on these properties, two technical advances were demonstrated, namely establishment of vegetative clones while circumventing laborious and time-consuming definition of PGR culture regimes at the beginning of a micropropagation program of wild plants, and cloning that secured biodiversity of a seed population. The model suits also establishment of clones of selected elite plants in frame of tree germplasm enhancement programs.

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