Genetic stability, *ex vitro* rooting and gene expression studies in *Hagenia abyssinica*

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Abstract

Randomly amplified polymorphic DNA (RAPD) markers were used to assess genetic stability of 80 micropropagated *Hagenia abyssinica* plants, 40 of axillary origin and 40 of adventitious origin. The shoots were isolated from the same mother tree and micropropagated for over two years. Among the 83 RAPD primers screened, 16 gave reproducible band patterns. These 16 primers produced 115 bands for each plant. One plant from axillary origin showed two unique bands with primer OPC-11. All other plants showed identical band patterns. Generally, there was no significant difference in the shoot multiplication rate between shoots of axillary and adventitious origin. Indole-3-acetic acid (IAA) resulted in better *ex vitro* rooting compared to indole-3-butyric acid (IBA) and α-naphthaleneacetic acid (NAA). Non-micropropagated plants that were grown in the greenhouse for about one year were better in *ex vitro* rooting compared to those of juvenile material and mature tree derived micropropagated plants of the same treatment. Adventitious rooting related oxygenase gene (*ARRO-1*) isolated from apple (*Malus domestica*) was not expressed in *H. abyssinica* using a complementary DNA representational difference analysis fragment (cDNA RDA14) as a probe.

Additional key words: adventitious shoots, *ARRO-1*, axillary shoots, RAPD bands, RDA14, shoot multiplication.

Introduction

Hagenia abyssinica (Bruce) J.F. Gmel. is a multipurpose tree belonging to the family *Rosaceae*. It is dioecious and the only species representing the genus *Hagenia*. The height reaches up to 20 m with a short trunk, thick branches and compound leaves. Recently, this species is one of the most endangered tree species due to the high demand for its timber. Regeneration of the species using seeds in what used to be its natural habitat is becoming almost impossible as a result of human interference and climatic changes that usually follow deforestation (Negash 1995). Therefore, micropropagation of this tree is an important alternative for propagation of elite genotypes.

Tree improvement by conventional breeding is a slow process because of the long juvenile period and high heterozygosity of trees (Singh *et al*. 2002). *In vitro* clonal propagation of trees is an attractive alternative for obtaining high number of elite genotypes (Bindiya and Kanwar 2003). However, somaclonal variation of the micropropagated elite genotypes can be a potential draw back. On the other hand, stable somaclonal variations of specific type may be advantageous for improvement of certain traits (Antonetti and Pinon 1993, Bindiya and Kanwar 2003). DNA markers are powerful tools for examining genetic similarity/variation among cultivars and species. Random amplified polymorphic DNA markers (RAPDs) have been used to characterize micropropagated forest trees (Isabel *et al*. 1993, Rani *et al*. 1995, Barrett *et al*. 1997, Rahman and Rajora 2001, Rout and Das 2002, Bindiya and Kanwar 2003). Recently, a micropropagation protocol from juvenile materials and sprouts of mature trees of *H. abyssinica*

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Abbreviations: 2-ODD - 2-oxoacid-dependent dioxygenase; ARRO-1 - adventitious rooting related oxygenase; BAP - benzyl-aminopurine; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog (1962); NAA - α-naphthalene-acetic acid; PCR - polymerase chain reaction; RAPD - randomly amplified polymorphic DNA; RDA - representational difference analysis.
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(Feyissa *et al*. 2005a) has been developed and can be used to propagate elite genotypes. Furthermore, a regeneration protocol from leaf explants of this species (Feyissa *et al*. 2005b) has been established for further genetic transformation experiments or possibly for mass propagation of this species. Therefore, studying the genetic stability of plants obtained from both axillary and adventitious origin is important.

It is difficult to propagate *H. abyssinica* by conventional cutting methods due to rooting problems. Therefore, developing efficient and cost-effective method of rooting is important to propagate elite genotypes of this species within relatively short time. Adventitious root formation is a key step in micropropagation (De Klerk *et al*. 1997). Microcuttings can be rooted in two ways, *ex vitro* or *in vitro* (George 1996). *Ex vitro* rooting is more advantageous than *in vitro* rooting in saving time, labour and cost. Although several studies have been carried out on *in vitro* and *ex vitro* rooting, gene expression during root meristem formation in woody plants is poorly understood (Butler and Gallagher 1999). A test system based on the induction of adventitious roots in stem discs of micropropagated apple (*Malus domestica* cv. Jork 9) has been used to investigate gene expression during adventitious root formation (Butler and Gallagher 1999). Using a complementary DNA representational difference analysis fragment (cDNA RDA), a 216 base pair (bp) cDNA fragment (designated RDA14) was isolated and found to be highly expressed in IBA-induced stem discs of *Malus domestica* cv. Jork 9 (Butler and Gallagher 1999). A RDA14 cDNA clone was isolated and designated adventitious rooting related oxygenase (*ARRO-1*). A strong hybridization of *ARRO-1* with several fragments *Pyrus communis* and *Prunus domestica* genomes using Southern blot analysis suggests that *ARRO-1* is relatively conserved within the *Rosaceae*, but not in the more divergent species such as *Betula pendula* (Butler and Gallagher 2000).

The objective of this study was to assess the genetic stability of micropropagated plants derived from axillary and adventitious shoots, to compare *ex vitro* rooting with *in vitro* rooting as well as to examine the expression of *ARRO-1* gene using RDA14 as a probe.

Materials and methods

Plants and cultivation: Shoots obtained from a female tree of *Hagenia abyssinica* (Bruce) J.F. Gmel. (designated genotype 3, G3) were taken from Intoto Mountain, about 10 km North of Addis Ababa, Ethiopia. The shoots were propagated *in vitro* by axillary bud proliferation on MS (Murashige and Skoog 1962) medium containing 4.4 μM benzylaminopurine (BAP) and 0.49 μM indole-3-butyric acid (IBA) for the first seven subcultures and then on 2.2 μM BAP and 0.25 μM IBA for subcultures 8 to 15. Some cultures produced shoots directly from leaves during the first three subcultures and were thus subcultured separately. The shoots were subcultured every six weeks, except at the 8th and 12th subcultures, where they were maintained for three months at 10 °C to reduce the rate of growth so that it could be stored for short term at this temperature. The multiplication rate of these shoots of axillary and adventitious shoot origin was evaluated at subcultures 3, 4, 5, 6 and 15. After 28 months in culture (at the 15th subculture), shoots were rooted in 1/3 MS medium containing 4.9 μM IBA following the procedure described by Feyissa *et al.* (2005a). After rooting, the plantlets were planted in pots containing soil and covered with plastic caps. After three weeks leaves were collected from a total of 80 plants derived from 40 axillary and 40 adventitious shoots and used for DNA extraction.

DNA extraction and PCR amplification: Total DNA was extracted from fresh leaf tissue using CTAB procedure (Wang *et al*. 1996) with some modifications. The leaf samples were ground to powder in liquid nitrogen. Powdered leaves (0.15 - 0.20 g) were collected in Eppendorf tubes and 0.75 cm^3 of extraction buffer (0.1 M Tris pH 7.5, 50 mM EDTA, 500 mM NaCl) and 0.1 cm^3 10 % sodium dodecyl sulfate (SDS) (m/v) was added. The mixture was incubated for 20 min at 65 °C and 0.25 cm^3 of 5 M potassium acetate was added and kept on ice at least for 1 h before centrifugation at 10 000 *g* for 15 min. The supernatant was precipitated with an equal volume of cold iso-propanol and centrifuged. The pellet was air dried and diluted in 0.25 cm^3 Tris-EDTA (TE) and 0.25 cm^3 CTAB buffer (0.2 M Tris HCl, pH 7.5, 50 mM EDTA, 2 M NaCl, 2 % CTAB), and incubated at 65 °C for 15 min. After extraction with equal volume of chloroform twice, the final aqueous-phase was precipitated with one volume of cold iso-propanol and centrifuged. The pellet was air-dried, diluted in 0.1 cm^3 TE, 0.0025 cm^3 RNase (1 mg cm^{-3}) was added and incubated at 37 °C for 30 min. The samples were stored at -20 °C for later use. The concentration was measured with *UV-240* spectrophotometer (*Shimadzu*, Tokyo, Japan) at 260 nm. Eighty-three RAPD primers (*Operon Biotechnologies GmbH*, Cologne, Germany) were screened for PCR amplification using five clones from axillary shoot origin. PCR reactions were carried out in reaction mixtures with a total volume of 0.02 cm^3, containing 0.6 U of Taq DNA polymerase (*ABgene*, Epsom, UK), 20 ng primer, 0.10 mM each dNTP (*Sigma-Aldrich*, St. Louis, USA), 3.5 mM $MgCl_2$ (*ABgene*), 25 ng of template DNA and final concentration of 1× reaction buffer (*ABgene*). Reactions took place in a *Thermal Cycler GeneAmp*® PCR *System 9700*, *version 3.01* (*Applied Biosystems*, Singapore): an initial denaturation step of 3 min at 94 °C, followed by

45 cycles of 1 min at 94 °C (denaturation), 1 min at 37 °C (annealing) and 2 min at 72 °C (extension). The last cycle was followed by a final extension step of 10 min at 72 °C. After amplification, samples were loaded and electrophoresed on 1.5 % agarose gels and stained with ethidium bromide. A 100-bp ladder (*Amersham Biosciences*, Buckinghamshire, UK) was used as size marker. Among the 83 primers screened, 16 showed reproducible patterns and were selected for further amplifications (Table 1). The amplifications using these primers were repeated twice. When a polymorphic fragment was detected, the whole experiment was repeated to confirm the results.

Table 1. Sequence of RAPD primers used for genetic stability analysis of micropropagated plants of *Hagenia abyssinica*, and results obtained.

Primer	Sequence (5'-3')	Number of scored bands	Fragment size ranges [bp]
OPA-05	-AGGGGTCTTG-	10	400-1700
OPA-11	-CAATCGCCGT-	5	1200-2500
OPC-01	-TTCGAGCCAG-	5	700-1800
OPC-02	-GTGAGGCGTC-	4	700-1500
OPC-03	-GGGGGTCTTT-	9	500-2100
OPC-05	-GATGACCGCC-	7	500-2100
OPC-06	-GAACGGACTC-	8	500-2500
OPC-08	-TGGACCGGTG-	11	500-2000
OPC-11	-AAAGCTGCGG-	5	500-1800
OPC-20	-ACTTCGCCAC-	8	300-2100
OPG-01	-CTACGGAGGA-	6	1000-2200
OPG-02	-GGCACTGAGG-	10	400-2500
OPG-03	-GAGCCCTCCA-	7	500-2100
OPG-11	-TGCCCGTCGT-	7	400-2100
OPG-14	-GGATGAGACC-	8	600-1900
OPG-16	-AGCGTCCTCC-	5	1100-2100

***Ex vitro* rooting:** *In vitro* propagated shoots of male tree origin (designated genotype 2, G2) were used in this experiment. Shoots (after 15 subcultures) were separated and the basal end was dipped in 4.9 mM IBA, 5.7 mM indole-3-acetic acid (IAA) or 5.3 mM α-naphthalene-acetic acid (NAA) for 1, 5 or 10 min and planted in 9.0 cm pots containing 2:1 (v/v) soil and *Perlite*. The shoots were covered with plastic pots and after 6 weeks the percentage of rooting, number of roots per shoot and length of roots were measured. In a separate experiment, cuttings from 10 to 12-month-old greenhouse grown (non-micropropagated) plants, and micropropagated juvenile and mature shoots origin that were grown in the greenhouse for one year were also used for *ex vitro* rooting by treating with 2.5 mM IBA for 5 min.

Evaluating the expression of *ARRO-1*: Shoots from 5-week-old *in vitro* growing G3 were placed on rooting medium containing 1/3 MS basal medium, with 4.9 μM IBA or without IBA. About 0.2 g of basal parts of the stems were cut from IBA-induced and non-induced shoots for total RNA extraction and frozen in liquid nitrogen after 0, 24, 48 and 72 h. The tissue was crushed in 1.5 cm^3 microcentrifuge tubes and RNA was extracted according to Small Scale RNA Isolation of *Concert™ Plant RNA Reagent* Protocol (*Invitrogen*, Foster City, USA). 15 μg of total RNA from each sample was separated on 1.4 % (m/v) agarose gel containing 4.8 % (v/v) formaldehyde and transferred to *Hybond* N^+ membrane. The membrane was hybridized with [^{32}P]dCTP-labelled DNA probe obtained from a PCR amplified 216 bp RDA14 fragment from *ARRO-1* gene. Hybridization was performed overnight at 64 °C in a mixture of 0.5 M $Na_2HPO_4 . 2 H_2O$ and $NaH_2PO_4 . H_2O$, pH 7.2, and 0.1 % SDS. The membrane was washed at 64 °C in 1 × SSC/ 0.1 % SDS and 0.5 × SSC/0.1 % SDS, sealed in plastic cover and was exposed to *Electronic* autoradiography (Canberra, Australia) for 41 h. The RDA14 probe was used as positive control. The whole experiment was repeated.

Statistical analysis: Statistical analysis of quantitative data for shoot multiplication and *ex vitro* rooting was carried out by using the *Statgraphics* statistical software (*Statgraphics®Plus 5*, *Manugistics, Inc*., Rockville, MD, USA). Differences among means were analysed by Duncan's multiple range test and a difference at probability level of $P \leq 0.05$ was considered significant for all analyses.

Results

Assessment of genetic stability using RAPD: RAPD analysis in 80 micropropagated plants of axillary and adventitious shoots of *H. abyssinica* was performed with a total of 115 amplified reproducible bands per plant produced from 16 random primers. The number of bands per primer ranged from 4 in OPC-02 to 11 bands in OPC-08. The size of amplified bands varied from 300 bp to 2500 bp (Table 1). The average number of bands per primer was 7.2. The total number of bands (number of micropropagated shoots × number of bands with the 16 primers) was 9200. The RAPD amplification profiles obtained using primers OPC-11, OPC-06, OPG-02 and OPG-14 (Fig. 1*A-D*) in 10 micropropagated axillary and adventitious shoot showed identical bands except one plant, which is from axillary origin. This plant showed two additional bands using primer OPC-11 (Fig 1*A*). These unique bands have a molecular size of approximately 450 bp and 800 bp.

Shoots of axillary origin showed significantly higher multiplication rate (4.7 ± 2.3) as compared to those from adventitious origin (3.7 ± 2.3) when the total number of shoots at all subcultures were considered. However, when

Table 2. Number of shoots produced by axillary and adventitious shoots origin at different subcultures. Means ± SD, 20 shoots were used per treatment. Means within each column followed by the same superscript, a or b, are not significantly different at 5 % probability level. Means within each row followed by the same superscript, 1 or 2, are not significantly different at 5 % probability level.

Subculture	Axillary	Adventitious
3	4.4 ± 2.8^{b1}	3.6 ± 1.9^{b1}
4	4.7 ± 1.2^{b1}	3.0 ± 1.0^{b2}
5	4.5 ± 2.6^{b1}	3.3 ± 2.1^{b1}
6	3.6 ± 1.0^{b1}	3.3 ± 1.2^{b1}
15	6.2 ± 2.4^{a1}	5.5 ± 1.6^{a1}

the shoot multiplication rate was evaluated at different subcultures, significant differences between axillary and adventitious shoot origin was observed only at the fourth subculture, with axillary origin producing 4.7 ± 1.2 and adventitious origin producing 3.0 ± 1.0 average number of shoots per explant (Table 2). At the 15th subculture, shoots both from axillary and adventitious origin showed significantly higher multiplication rate as compared to subcultures, 3, 4, 5 and 6.

Shoots from 15th subculture were kept on rooting medium (according to Feyissa *et al*. 2005) for four weeks and the percentage and number of roots per shoot was evaluated. Among 60 shoots of axillary origin 77 % rooted while among adventitious shoot origin 82 % rooted. However, the shoots that did not produce roots on rooting medium (14 shoots from axillary origin and 11 shoots from adventitious origin) were planted in pots and all rooted increasing the total percentage of rooting to 100 %. Number of roots per plantlet was 4.1 ± 3.2 for axillary shoot origin while it was 3.9 ± 2.6 for adventitious shoot origin. There was no significant difference in number of roots per plantlet between plantlets of axillary and adventitious shoot origin.

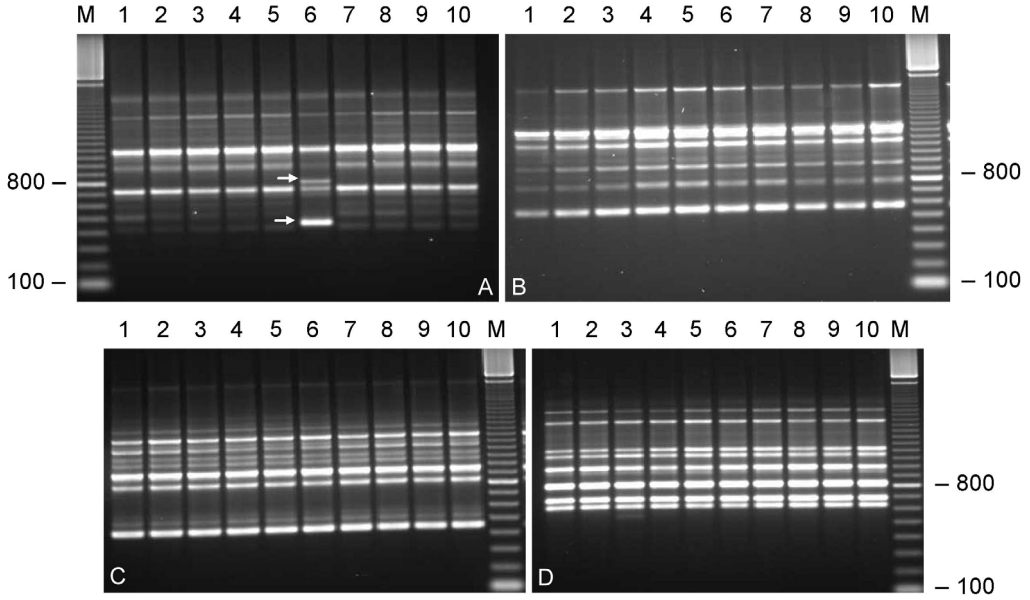

Fig. 1. Gel electrophoresis of RAPD fragments obtained with primers OPC-11 (*A*), OPC-06 (*B*), OPG-02 (*C*) and OPG-14 (*D*). In *A*, *B* and *C*, lanes 1 to 10 show RAPD bands of plants from axillary origin. Note the polymorphic bands indicated by *arrows* on lane number 6 in *A*. In *D*, lanes 1 to 10 show RAPD bands of plants from adventitious origin. The lane M represents the molecular size marker used (100 bp ladder, *Amersham Biosciences*).

***Ex vitro* rooting:** Among IBA, IAA and NAA, the highest *ex vitro* rooting percentage (40 %) and the longest roots per shoot (4.5 ± 1.3) were obtained from shoots treated with IAA for 5 min (Table 3). The control plants also produced the longest roots (4.5 ± 2.1). However, shoots treated with IBA for 5 min produced the highest number of roots per shoot (12 ± 4.2). IBA treatment for 1 min or NAA treatment for 10 min failed to produce any roots. In the control plants, all rooted and non-rooted shoots after six weeks stayed green while in all other treatments all non-rooted shoots died. The type of auxin and treatment time influenced the quality of the shoot and root. IAA treated shoots grew faster with good appearance followed by IBA treated shoots, and NAA treated shoots developed poorly. Among cuttings from micropropagated and non-micropropagated greenhouse grown plants that had been treated for *ex vitro* rooting, significant differences were observed both in percentage of rooting and root number per shoot. The highest percentage of rooting (45 %) and maximum root number

Table 3. Percent rooting, number of roots and length of roots produced by *ex vitro* planted micropropaged shoots treated with different auxins at different pulses. Means ± SD, 20 shoots were used per treatment. Means within each column followed by the same superscript, a-c, are not significantly different at 5 % probability level.

Auxin	Pulse [min]	Rooting [%]	Number of roots [shoot^{-1}]	Root length [cm]
0	0	10^{bc}	2.0 ± 1.4^{c}	4.5 ± 2.1^{a}
IBA	1	0^{c}	0^{c}	0^{b}
	5	10^{bc}	12.0 ± 4.2^{a}	4.3 ± 2.5^{a}
	10	20^{abc}	9.0 ± 5.4^{ab}	3.1 ± 1.3^{ab}
IAA	1	30^{ab}	3.2 ± 1.9^{bc}	3.8 ± 1.9^{ab}
	5	40^{a}	5.1 ± 2.2^{bc}	4.5 ± 1.3^{a}
	10	20^{abc}	4.5 ± 1.9^{bc}	4.0 ± 2.4^{a}
NAA	1	15^{bc}	4.3 ± 3.2^{bc}	1.8 ± 0.8^{ab}
	5	10^{bc}	2.5 ± 0.7^{c}	2.0 ± 0.7^{ab}
	10	0^{c}	0^{c}	0^{b}

Fig. 2. Micropropagated plants after two months in greenhouse. Normal plant (left) and the plant that showed RAPD band polymorphism (right). *Bar* = 4.5 cm.

per cutting (6.2 ± 4.2) was obtained from non-micropropagated plants that were grown in the greenhouse for 10 - 12 months. Cuttings from micropropagated plants of juvenile origin resulted in 25 % of rooting and 5.4 ± 4.3 roots per cutting while cuttings from non-micropropagated plants that were not treated with IBA resulted in 20 % rooting and 4.0 ± 1.4 roots per cutting. Cuttings from micropropagated mature origin, irrespective of IBA treatment, failed to produce any root.

Evaluating the expression of *ARRO-1*: *ARRO-1* was not expressed at any stages of IBA-induced and non-induced shoots of *H. abyssinica*. The positive control (RDA14 probe) was expressed within 30 min after exposure to *Electronic* autoradiography. The positive control part of the membrane was cut out to avoid blackening of other parts, and the rest samples were exposed to the *Electronic* autoradiography for 41 h and no expression was observed (Fig. 3).

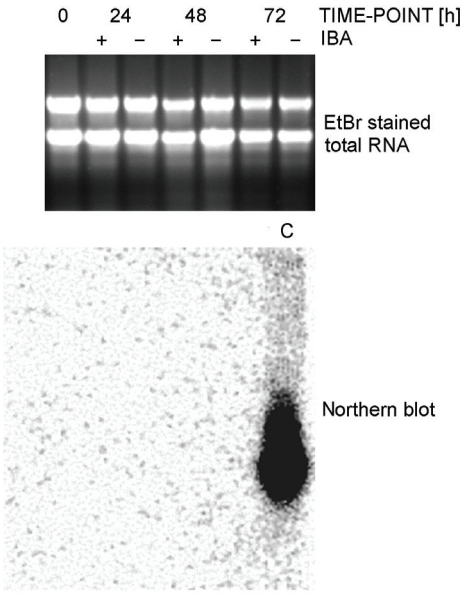

Fig. 3. Total RNA isolated from IBA-induced (+) and non-induced (-) stem bases of *H. abyssinica* at 0, 24, 48, and 72 h and Northern blot analysis. C represents positive control (RDA14 probe).

Discussion

Assessment of genetic stability using RAPD: In this study using 16 RAPD primers, one clone from the 40 plants of axillary shoots showed polymorphism with two unique bands while no polymorphism was detected among the other 40 adventitious shoots. The overall frequency of somaclonal polymorphisms among plants of axillary and adventitious shoots was 0.02 % whereas if plants of only axillary shoot are considered it was 0.04 %. Several reports showed that plants of adventitious shoots are more subjected to somaclonal variation than those of axillary shoots. However, our study showed the reverse. The polymorphism in amplified bands might result from changes in either the sequence of the primer binding site or change which alter the size or prevent successful amplification of target DNA. Using RAPD technique, several authors have reported the absence of somaclonal variation in long-term micropropagated trees like *Pinus thunbergii* (Goto *et al*. 1998) and *Festuca pratensis* (Vallés *et al*. 1993). On the other hand several investigators reported the presence of genetic variations in micropropagated plants. Bindiya *et al*. (2003) reported 32 % band polymorphism in *Robinia pseudoacacia* plants of axillary origin that had been on culture only up to sixth subculture. Rani *et al*. (1995) observed polymorphic

bands in six of 23 micropropagated plants of *Populus deltoides*. Munthali *et al*. (1996) and Hashmi *et al*. (1997), also reported polymorphisms in micropropagated plants of *Beta vulgaris* and *Prunus persica*, respectively. Similarly, Hofmann *et al*. (2004) reported RAPD band polymorphism in embryogenic cultures of soybean that were treated with ethyl methanesulfonate (EMS).

The present study showed that somaclonal variation arose in plants of axillary origin of *H. abyssinica*. Analysis of genetic variation among micropropagated shoots of three morphotypes (short, medium, and long needles) in *Pinus thunbergii* using RAPD did not show any variation (Goto *et al*. 1998). After carrying out RAPD analysis of *in vitro* propagated plants of *Castanea sativa* × *C. crenata*, Carvalho *et al*. (2004) could identify one different clone among four clones that were thought to be from the same mother plant. In the present study, polymorphism was observed only in one among eighty plants. It is therefore difficult to conclude that plants of axillary shoots are more subjected to somaclonal variation than plants of adventitious origin. It has been reported that plants micropropagated through meristem multiplication or by serial subculture of stem nodes are genetically stable (Potter and Jones 1991). However, the occurrence of natural mutations should not be overruled. In general, plants of axillary and adventitious shoot origin, showed the same pattern of multiplication and rooting.

***Ex vitro* rooting:** Since IAA is applied by a short dip of a cut end in IAA solution or in rooting powder for *ex vitro* rooting, plants take up IAA during a brief, initial period of treatment (De Klerk 1997). The plant tissues rapidly oxidize IAA (De Klerk 1997, Epstein and Ludwig-Müller 1993) and higher concentrations have to be applied as compared to other auxins such as IBA. Therefore, IBA is preferable. Similarly, Bhatia *et al*. (2002) reported 83 % *ex vitro* rooting of *Stackhousia tryonii* microshoots that were treated with 2 g dm^{-3} IBA for 15 s. However, our study shows that for *ex vitro* rooting of *H. abyssinica*, IAA is preferable. Higher rooting percentage and faster growth and better appearance of shoots were observed in IAA treated shoots as compared to those treated with IBA or NAA. Like *in vitro* rooting, *ex vitro* rooting is affected by the type and concentration of auxins and the explant source. There were strong differences between *in vitro* rooting and *ex vitro* rooting of *H. abyssinica*. *In vitro* rooting resulted in 100 % rooting after transfer to greenhouse as compared to 40 % for micropropagated shoots or 45 % non-micropropagated cuttings of *ex vitro* rooting. This suggests that *in vitro* rooting is the best treatment for micropropagated plants of *H. abyssinica*.

Evaluating the expression of *ARRO-1*: Using a 216 bp cDNA RDA14 clone, *ARRO-1* was highly expressed in IBA treated stem discs of *Malus domestica* cv. Jork 9 (Butler and Gallagher 1999). *ARRO-1* is represented in apple genome by a number of distinct gene copies. It is 1282 bp long and encodes a 2-oxoacid-dependent dioxygenase (2-ODD). It is specific to the root development process and the strong hybridization fragments of *Pyrus communis* and *Prunus domestica* genomes and absence of hybridization with *Betula pendula* indicates this gene is conserved within closely related species in Rosaceae family (Butler and Gallagher 2000). In our study, the absence of expression of *ARRO-1* in *H. abyssinica* using 216 bp RDA14 probe suggests that probably there is no homologous sequence of this probe in this species or the degree of homology is very low for the gene to be expressed. Since *H. abyssinica* is the only species in the genus *Hagenia* and confined to the mountains of East Africa, it is possible that this is a divergent species from other members within the *Rosaceae* family.

Conclusion: Our study showed that RAPD technique could be used to evaluate the genetic stability of micropropagated *H. abyssinica* plants. It is also important to evaluate the phenotypic variations during field growth of micropropagated plants. Although *ex vitro* rooting is more advantageous than *in vitro* rooting in saving time, labour and cost, the process appeared more difficult to achieve in *H. abyssinica* as compared to *in vitro* rooting. Further studies are needed to understand gene expression during root meristem formation in *H. abyssinica*.

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