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Random Amplified Polymorphic DNA (RAPD) Analysis of Long-term Cultured Hybrid Hazelnut

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***Abstract*. RAPD and phenotypic analysis were conducted to assess clonal stability of hazelnuts generated from axillary buds cultured in vitro for long-term. The nuts produced on in vitro-propagated plants were indistinguishable from those of donor plants. With the exception of rare horizontal (plagiotropic) growth, all in vitro-propagated plants exhibited phenotypes similar to those of donor plants. RAPD analysis did not reveal any somaclonal variation between donor plants from which in vitro cultures were initiated and micropropagated plants (6-year cultures), and no somaclonal variation was detected among in vitro-propagated plants. However, polymorphism (15.6%) was detected between the parent plant and its in vitro-propagated progenies (from seedlings). These results show a good discriminatory power of RAPD to detect polymorphism between samples where it is expected, and it can be effectively used for genetic assessment of micropropagated hazelnut. No evidence of genetic or epigenetic changes was observed in long-term cultured hazelnut, and thus long-term in vitro culture of hazelnut does not seem to limit its clonal propagation.**

Micropropagation of woody species is relatively successful when juvenile tissues, such as seedling tissues, are used. However, juvenile tissues are often not elite lines and they may have no commercial value. Mature tissues are preferred for micropropagation because maturation affects several tree features that are not possible to select during the juvenile phase of tree growth. Unfortunately, by the time trees are old enough to display their potential in the field, their tissues become recalcitrant for tissue culture. In order to minimize the recalcitrance problem, the most juvenile tissues (e.g., basal sprouts) from adult trees are used and source plant materials are subjected to rejuvenation treatments (Ballester et al., 1989; Bhojwani and Razdan, 1996; Diaz-Sala et al., 1994; Franclet et al., 1987; Read and Yang, 1987).

The most effective and common rejuvenation treatment is repeated subculturing of explants in vitro. Repeated subculturing of explants may be a requisite for explants to become fully adjusted to culture and no longer display episodic growth patterns. Adjustment of cultures to the in vitro environment may take several subcultures to several years depending on the species, the age and source of explants, and the culture environment (Diaz-Sala et al., 1994; McCown and McCown, 1987; Nas and Read, 2004).

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Hazelnut is one of the most important nut crops (Mehlenbacher, 1994) and micropropagation has been considered as an alternative to traditional hazelnut propagation techniques (Diaz-Sala et al., 1994; Nas and Read, 2001; Yu and Reed, 1995). Successful micropropagation of hazelnut with mature tissues is influenced by the number of subcultures (Diaz-Sala et al., 1994) and the use of an optimized culture medium (Nas and Read, 2001). Optimization of the culture medium is usually time-consuming and requires long-term in vitro culturing of explants (McCown and McCown, 1987). Using axillary buds of mature hazelnut cultured on high cytokinin-containing medium for a lengthy period (≈6 years), we have developed a culture medium and protocol that may allow commercial micropropagation of hazelnut (Nas and Read, 2004). However, postculture behavior of plants from long-term in vitro cultures, especially generation of off-type plants (somaclonal variants), is a serious concern (Bhojwani and Razdan, 1996; Jain, 1993; Read and Hosier, 1986; Skirvin et al., 2000). Therefore, the objective of this study was to assess the genetic stability of long-term in vitro cultured hazelnut plants compared to donor plants. Our hypothesis was that preexisting meristem-derived plants will retain their genetic make up and somaclonal variation will be absent or very low. To the best of our knowledge, the genetic stability of micropropagated hazelnut has not been analyzed by molecular markers. Here we report the genetic integrity of micropropagated hazelnut by using RAPD markers.

Materials and Methods

In vitro cultures were initiated in 1996 (Nas and Read, 2001) using forced-outgrowth (Read and Yang, 1987) axillary buds of mature hybrid hazelnut genotypes E-093-S, E-295-S, G-029-N and SS´-182, the last one being a seedling of S´-182. Starting from the initiation of cultures until 2002, axillary buds of in vitro grown shoots were subcultured every 4 to 6 weeks on a variety of culture media [MS (Murashige and Skoog, 1962); NN (Nitsch and Nitsch, 1969); WPM (Lloyd and McCown, 1980); DKW (Driver and Kuniyuki, 1984); Nas and Read, 2004) containing 17.74 to 22.2 μM 6-benzyladenine and 0.049 μM indole-3-butyric acid and gelled with 6 $g \cdot L^{-1}$ agar (Sigma, A-1296).

DNA extraction and RAPD analysis. Two in vitro-grown shoots (3–4 cm) of each genotype and partially expanded leaves of donor plants (E-093-S, E-295-S, and G-029-N) were separately ground using a mortar and pestle. Since the donor plant SS´-182 from which in vitro cultures were initiated was lost, the DNA of its parent plant, S´-182, was used as the control. Total genomic DNA was extracted using a modified method (Skroch and Nienhuis, 1995). Thirty arbitrary decamer oligonucleotides (Operon Technology, Alameda, Calif.) were used and DNA amplification was performed on a RapidCycler (Idaho Technology, Salt Lake City) in thin-walled glass capillary tubes as described by Skroch and Nienhuis (1995). The reactions (in 10-μL glass capillary tubes) consisted of 2.5 μL 10× buffer (3-tris (hydroxyl methyl) methylamine propane sulphonic acid, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% gelatin), 100 mM each of dNTPs, 0.2 μM of primer, 0.5 U *taq* polymerase, and the glass capillary tubes were sealed by melting their ends.

Following the initial denaturation at 91 °C for 4 min, the amplification program was 40 cycles: 2 cycles of 1 min at 91 °C, 7 s at 42 °C, 70 s at 72 °C, and 38 cycles of 1 s at 91 °C, 7 s at 42 °C and 70 s at 72 °C, followed by holding at 72 °C for 4 min to complete all extension that may have been incomplete, and then cooling the reaction products to 4 °C.

After amplification, DNA fragments were separated in 1% (w/v) agarose gel in 1× TAE buffer, and bands were stained with ethidium bromide and visualized under ultraviolet light. A 100-base pair ladder (Gibco BRL) was used to estimate sizes of RAPD bands to nearest 50 base pairs. Amplifications were performed using 30 primer sets, and only primers (A02, A03, A04, A05, A09, A10, A12, C05, C06, C07, C08, C09, C10, C11, C12, E03, E04, P06, P07, P08, P09, P10, P11, P12, P13) that produced repeatable bands were analyzed. Identical bands produced from two independent amplifications were considered repeatable. Polymorphic, reproducible amplification products were scored as present (1) or absent (0), and ambiguous bands were excluded.

Results and Discussion

About 3000 micropropagated hazelnut plants, from 5- to 6-year in vitro cultures,

have been transplanted into the field. Micropropagated plants retained their physiological maturity during long-term in vitro culture or regained it soon after they were removed from culture. Female flowers were set in their first growth year and bore nuts in the second growth year (Nas et al., 2003). Based on visual observation, the nuts produced on in vitro-propagated plants were indistinguishable from those of donor plants (Fig. 1A). All in vitro-propagated plants of G-029-N and SS´-182 had phenotypes similar to those of parent plants and displayed upright (orthotropic) growth. Micropropagated plants of E-093-S and E-295-S had similar phenotype to those of donor plants, but some plants of these two genotypes displayed horizontal (plagiotropic) growth (Fig. 1B). A function of the mature form, in some species cuttings (explants) taken from lateral shoots will produce shoots that grow horizontally (Ahuja and Libby, 1993). Genetic and epigenetic changes cannot be excluded; the plagiotropic growth in micropropagated hazelnut is probably a result of the effect of the position of the initial explant source on vegetative growth.

RAPD analysis revealed varying numbers of bands from different genotypes. With most of the primers the numbers of bands were greatest for genotypes E-093-S and E-295-S. In vitro grown shoots do not display horizontal growth, microshoots of these two genotypes that were used for DNA extraction could be horizontal and/ or upright growing. In both E-093-S and E-295-S, no clear polymorphism was detected between micropropagated plants and source plants or between micropropagated plants. However, with most of the primers because of a great number of bands produced from such a small amount of reaction product (10 µL), it was difficult to unambiguously judge if a band was present or absent.

Based on RAPD analysis alone, the clonal stability of genotypes E-093-S and E-295-S could not conclusively be assessed. Hazelnut naturally grows in a multistemmed form. When horizontal growing shoots were analyzed, it became evident that the plagiotropic growth was probably a result of the effect of the position of the initial explant source on vegetative growth (Ahuja and Libby, 1993). New shoots (branches) forming from axillary bud of micropropagated horizontal growing plants were plagiotropic but adventitious shoots (root suckers) were orthotropic (Fig. 1C).

With genotype G-029-N, of 30 primers that were used, 25 (83%) gave repeatable banding patterns and a total of 139 consistent bands (5.6 bands per primer) were generated. No polymorphism was detected between the donor G-029-N plant and the in vitro-propagated plants derived from axillary bud cultures, and banding patterns of in vitro-produced G-029-N plants were identical (Fig. 2).

With genotype SS´-182, of 30 primers that were used, 24 (80%) gave a consistent banding pattern and a total of 164 consistent bands (6.8 bands per primer) were generated. As expected, polymorphism was detected between in vitro-propagated SS´-182 (seedling) plants and S´-182 (parent of donor plant SS´-182). Of 24 primers, 13 produced polymorphic banding patterns. Twenty-six of 164 (15.9%) bands generated with SS´-182 and S´182 were polymorphic. Seventeen bands that were present in S´-182 were absent in SS´-182 while nine bands that were present in SS´-182 were absent in S´-182. The banding patterns of in vitro-produced SS´-182 plants, however, were identical, indicating no detectable somaclonal variation among in vitro propagated plants (Fig. 3). These results show a good discriminatory power of RAPD to detect polymorphism between samples where it is expected, and it can be used effectively for genetic assessment of micropropagated hazelnut.

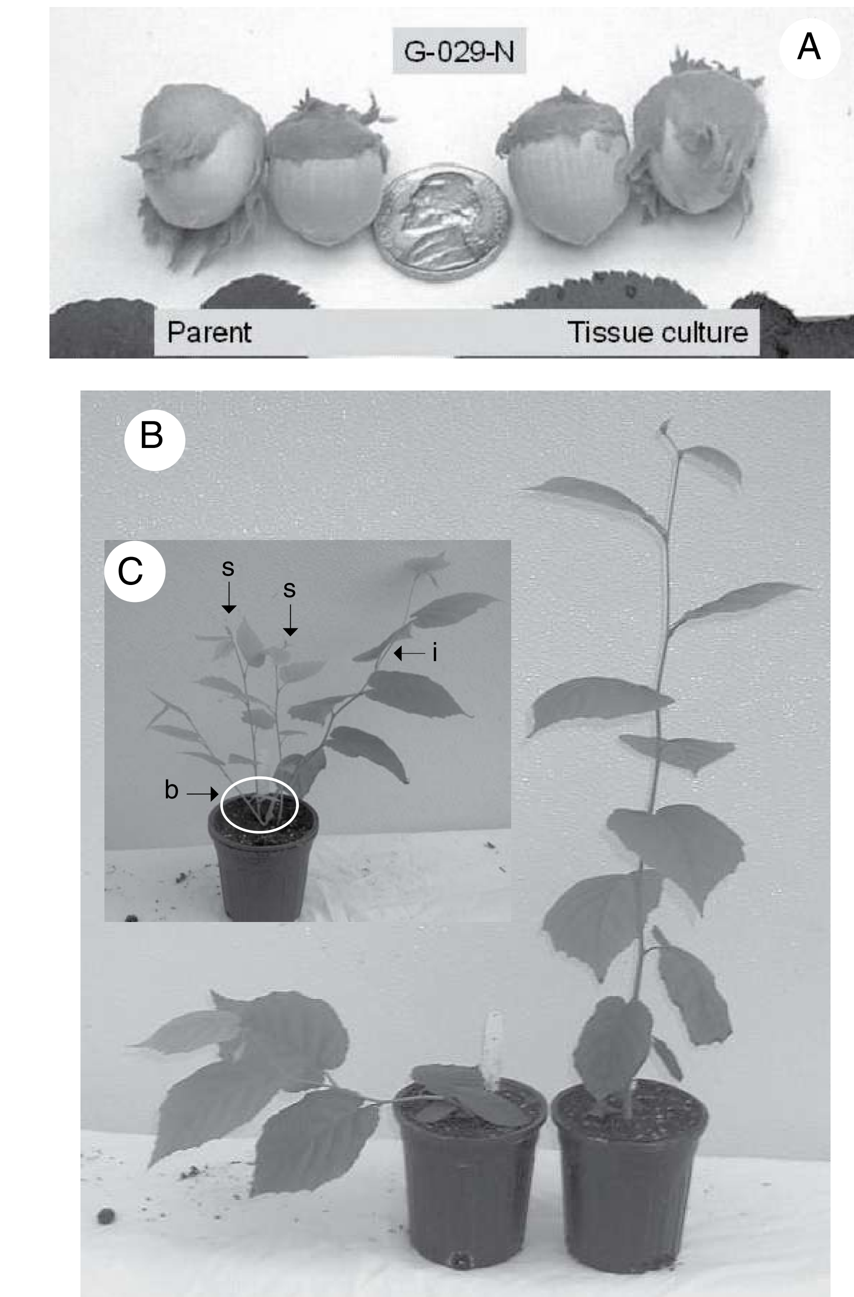

Fig. 1. (**A**) Nuts produced on parent (genotype G-029-N) and in vitro-generated plants. (**B**) Horizontal (plagiotropic) and upright (orthotropic) growth of in vitro-propagated hazelnut from axillary buds of genotype E-093-S. (**C**) Orthotropic adventitious shoots [root suckers (**s**)] and plagiotropic branches (**b**) forming from axillary bud of horizontal growing in vitro-propagated (**i**) plant of genotype E-093-S.

The source of the explant, mode of culture (organogenesis, somatic embryogenesis or axillary bud proliferation), medium content, and culture conditions are all reported to play an important role in the presence or absence of variation (Damasco et al., 1996; Ostry et al., 1994; Skirvin et al., 1994). Although it may not be the technique that gives the greatest multiplication rates, the use of pre-existing meristems (shoot-tip and axillary bud proliferation) is preferred for clonal propagation because it gives higher genetic stability than adventitious shoot regeneration or embryogenesis (Ahmed et al., 2002; Debergh and Read, 1991). Shoot generation in micropropagated mature hazelnut is primarily a function of axillary budbreak which may ensure the genetic stability of plants (Nas and Read, 2001). There is no evidence for morphological (except the plagiotropic growth),

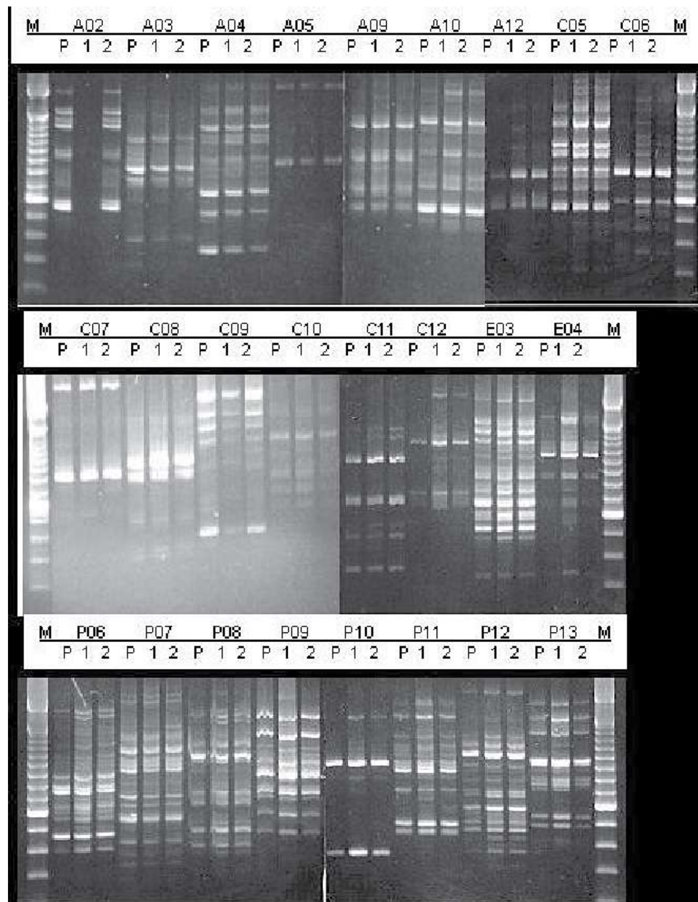

Fig. 2. Gel electrophoresis of RAPD fragments in micropropagated hybrid hazelnut (genotype G-029-N). Upper lanes: marker (**M**) and primers. Lower lanes: (**P**) donor plant G-029-N from which in vitro cultures were initiated; lanes (1) and (2) G-029-N plants generated from long-term (≈6 years) in vitro-cultured axillary buds. Note the lack of polymorphism between donor plant and in vitro propagated plants, and between two in vitro propagated plants. (Due to an accident, lane (1) with primer A02 did not contain any reaction product).

genetic or epigenetic changes observed in long-term cultured hazelnut. It can be concluded that long-term in vitro culture of hazelnut does not limit its clonal propagation. There have not been any long-term field trials of micropropagated hazelnut in sufficient numbers to determine if any genetic or epigenetic changes may have taken place that could affect the clonal performance. Currently ca. 3000 in vitro propagated hazelnut plants are undergoing field testing. Although no variants have been detected, it is necessary that these plants be monitored for several years to draw a firm conclusion about their growth in the field.

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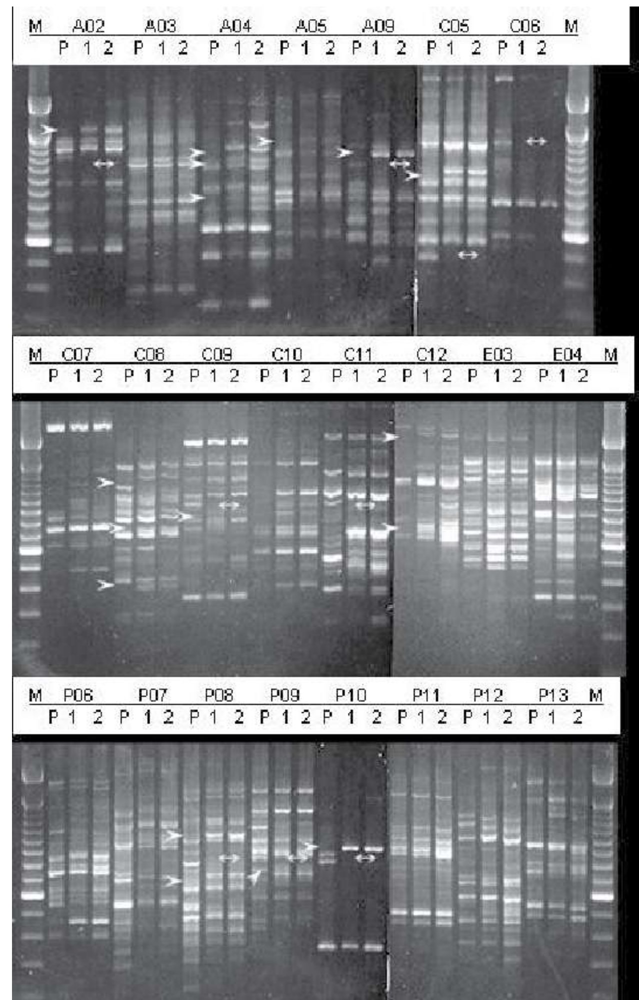

Fig. 3. Gel electrophoresis of RAPD fragments in micropropagated hybrid hazelnut (genotype SS´-182). Upper lanes: marker (**M**) and primers. Lower lanes: (**P**) parent (S´-182) of donor plant SS´-182 from which in vitro cultures were initiated; lanes (1) and (2) SS´-182 plants generated from long-term (≈6 years) in vitro-cultured axillary buds. Arrow indicates fragments that are present in in vitro-propagated progenies but missing in parent plant, and double arrow indicates fragments that are present in parent plant but missing in its in vitro-propagated progenies.

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