

with polyethylene bags benefited by high humidity and high PAR (100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) during 3 weeks hardening and showed about 70% survival (97 out of 140). Hardened plants produced new leaves and an adequate root system for further establishment in soil. High PAR hastened the hardening process by accelerating the development of new leaves and the photosynthetic ability of rooted shoots (GROUT and MILLAM, 1985). All the hardened plants, so far, transplanted into 20 cm pots containing a mixture (1:1) of soil and sand were successfully established and showed apparently uniform growth and true-to-type morphology (Fig. 3).

To the best of our knowledge, no data on micropropagation of *S. alternifolium* using mature nodal segments were published, the results presented in this paper represent the first report on clonal multiplication procedure for this rare Indian medicinal tree. The *in vitro* technique has the advantage of establishing long-term proliferating shoot cultures from the 10-year-old experimental tree with a high multiplication co-efficient (2.3 at 6 week interval). Therefore, it offers a high potential for mass propagation and conservation of this species. It seems likely that this protocol, possibly with modifications, can be used for clonal multiplication of other species of the genus *Syzygium* using mature nodal segments.

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Short Note: Identification of Natural Hybrids *Juglans x intermedia* CARR. Using Isoenzyme Gene Markers

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Summary

Isoenzyme polymorphism in bud tissues from *Juglans nigra* L., *Juglans regia* L., and their natural hybrids *Juglans x intermedia* CARR. was analysed. The results reveal that isoenzyme variants of the enzyme systems Aspartat aminotransferase (AAT) and Phosphoglucomutase (PGM) are suitable to identify hybrids of known as well as unknown origin genetically. The use of this method is suggested for forest nursery practice to distinguish hybrids at an early ontogenetic stage.

Key words: *Juglans regia*, *Juglans nigra*, *Juglans x intermedia*, isoenzymes, hybrids, varietal test.

FDC: 165.71; 165.3; 176.1 *Juglans nigra*; 176.1 *Juglans regia*; 176.1 *Juglans intermedia*.

Introduction

Juglans x intermedia CARR. is a natural hybrid between *Juglans nigra* L. and *Juglans regia* L.. When compared to the parent species most of hybrids show an increased vegetative vigour, distinct disease resistance characteristics and good wood quality (SCHEEDER, 1990; SAUTER *et al.*, 1994). Especially in southwest Germany, there is a great demand in plants of *J. x intermedia* for forest utilisation. However, because of temporal separation between flower maturity of both parent species depending mostly on weather conditions and apparent incompatibility mechanism between some individuals of each species (SARTORIUS, 1990), controlled crosses remained yet unsuccessfully (SCHEEDER, 1990). Thus, the production of

Table 1. – Survey of enzyme systems, E. C. No., electrophoretic buffer systems, and enzyme coding gene loci in buds of *Juglans* species.

Enzyme system	E.C.Nr.	Buffer system ¹⁾	Gene locus
Aspartat aminotransferase (AAT)	2.6.1.1	Tris-citric acid pH 8.5	AAT-A, -B, -C ²⁾
Phosphoglucumutase (PGM)	2.7.5.1	Tris-histidin pH 7.5	PGM-A, -B ³⁾

¹⁾ according to recipes given by HUSSENDÖRFER *et al.* (1995)

²⁾ for inheritance analysis see ARULSEKAR *et al.* (1985)

³⁾ ARULSEKAR *et al.* (1986)

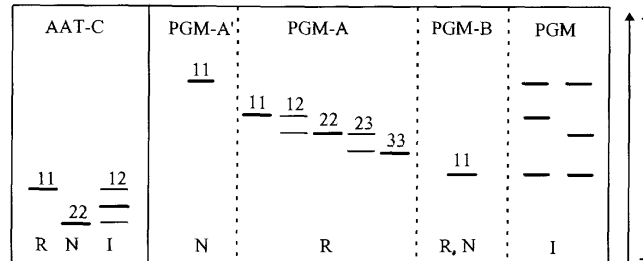


Figure 1. – Schem of isoenzymes patterns at gene loci AAT-C, PGM-A', PGM-A and PGM-B for *Juglans* species (R = *J. regia* L., N = *J. nigra* L., I = *J. x intermedia* CARR.).

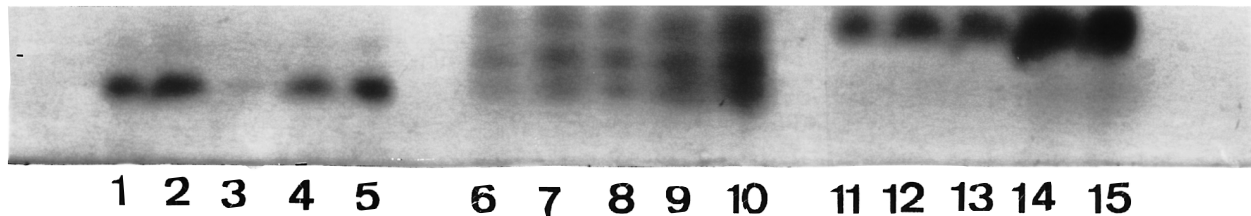


Figure 2. – Photo of a gel showing the patterns for the enzyme gene locus AAT-C as obtained from different single trees of *J. nigra* L. (Nos. 1 to 5), *Juglans x intermedia* CARR. (Nos. 6 to 10), and *J. regia* L. (Nos. 11 to 15). Bottom of the gel: origin; migration of bands: from the bottom (cathode) to the top (anode).

hybrid plants depends mostly on successful natural hybridisation. In practice, forest nurseries commonly collect seeds of *J. nigra* which are supposed to be pollinated by *J. regia*. After one or two years of cultivation hybrids are distinguished by phenotypic characteristics, for example by means of leaf characteristics or bud forms (METTENDORF, 1991). However, the natural variation of phenotypic traits sometimes leads to the problem of miss-identification of hybrids. In particular, if the identification is performed during winter by the characteristic "height growth" only, outstandingly large individuals of *J. nigra* might be certificated as hybrids.

For *Juglans* species isoenzyme gene markers are well established and the mode of inheritance has been investigated for several enzymes (ARULSEKAR *et al.*, 1985, 1986). For *J. regia* genetic data exist due to investigations on mating system (MALVOLI *et al.*, 1995) and geographic variation patterns (MALVOLI *et al.*, 1993, 1994). Furthermore, some studies were performed in order to distinguish between species and hybrids of *Juglans* species (e.g. ARULSEKAR *et al.*, 1985; GERMAIN *et al.*, 1993). In particular, the study of GERMAIN *et al.* (1993) provided that isoenzyme gene markers will be suitable for identification of hybrids between *J. nigra* and *J. regia*. However, GERMAIN *et al.* (1993) noticed that due to the limited number of investigated clones per species yet, further studies have to be performed in order to confirm their assumptions. The present study aimed on the establishment of isoenzyme gene markers in order to facilitate the identification of *J. x intermedia* of known and unknown origin. For this purpose, two enzyme systems revealed to be best suitable (Aspartat aminotransferase and Phosphoglucumutase).

Material and Methods

For *J. regia*, 8 adult trees located in the Kaiserstuhl (southwest Germany) which were supposed to be male parents of hybrids, 28 clones of the European range (*Juglans* clone collection, FVA Baden-Württemberg), and 60 individuals originating from India and Pakistan (sampled by PETER ROTACH, chair of silviculture, ETH Zürich, Switzerland) were investigated. For *J. nigra*, 4 adult trees located in the Kaiserstuhl which were known to be female parents of hybrids, and 25 plants which were offspring's of a bulk seed collection of a black walnut stand were investigated. For *J. x intermedia* 12 adult individuals which were known to be offspring's of the above mentioned *J. nigra*, 4 adult individuals of unknown origin, 10 plants of known origin, and 14 plants of unknown origin were investigated.

From each tree one or two dormant buds were homogenised in 0.1 M Tris-HCL buffer pH 7.3 containing Na₂EDTA (0.12 g/100 ml), soluble PVP-40 (4.0 g/100 ml), and 2-Mercaptoethanol (1.0 ml/100 ml). The separation of enzymes was performed by starch gel electrophoresis (starch concentration 12%), a voltage of 15 Vcm⁻¹ to 30 Vcm⁻¹, and a running distance of approximately 10 cm. Table 1 gives the number of investigated enzyme systems and information about electrode and gel buffers (for details see HUSSENDÖRFER *et al.*, 1995). The staining of enzymes was performed according to recipes given by MÜLLER-STARCK (1993) for *Fagus sylvatica* L.

Results and Discussion

Aspartat aminotransferase (AAT)

For all investigated species, three distinct zones were observed on gels stained for AAT, as is described by ARULSEKAR

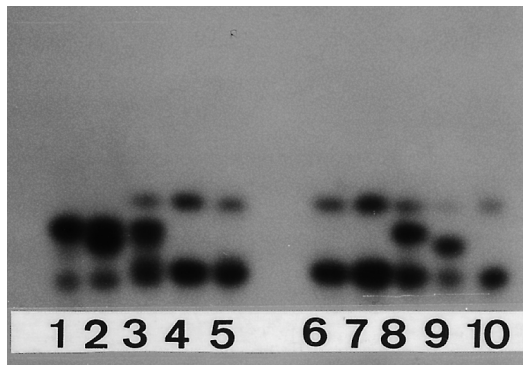


Figure 3. – Photo of a gel showing the patterns for the enzyme PGM as obtained from supposed male parent trees *J. regia* L. (Nos. 1 to 2), known female parent trees *J. nigra* L. (Nos. 4, 5, 6, 7, 10), and their hybrids *Juglans x intermedia* CARR. (Nos. 3, 8, 9). Bottom of the gel: origin; migration of bands: from the bottom (cathode) to the top (anode).

et al. (1985) and GERMAIN *et al.* (1993). The two fastest migrating zones were found to be invariable for all individuals, and between species no migration differences were obvious. The zone closest to the cathode – AAT-C – showed single bands for all individuals of *J. regia* and *J. nigra*. Between both species these bands differed clearly in migration (variants AAT-C “11” and “22” in figures 1 and 2). The distinct differences in migration between these two species at gene-locus AAT-C and the invariability for both species are also reported by GERMAIN *et al.* (1993). For all hybrids at this gene locus triple-banded variants were found showing the slowest and fastest bands stained less intensively, and the middle band stained more intensively (variant “12” in figures 1 and 2). Concerning a dimeric structure of his enzyme system (ARULSEKAR *et al.*, 1985), the observed triple-banded variants in hybrids indicate to be encoded by a heterogeneous genotype resulting from mating between both parent species.

Phosphoglucosmutase (PGM)

For *J. regia* and *J. nigra* two zones were found in gels stained for PGM. For *J. regia* ARULSEKAR *et al.* (1986) also observed two zones of activity which are encoded by two isoenzyme gene loci. As was described by ARULSEKAR *et al.* (1986), in the present study the slowest migration zone (gene locus PGM-B) was found to be invariable for *J. regia*. Additionally, at this gene locus no variation was found for *J. nigra*, and between both species at this gene locus no migration differences were obvious (see figures 1 and 3). At gene locus PGM-A however, between both species differences in number and migration of bands were observed. For *J. regia* at the PGM-A gene locus five different single- or double-banded variants were found (see figures 1 and 3) which are known to represent genotypes encoded by three alleles (ARULSEKAR *et al.*, 1986). For all individuals of *J. nigra* at this gene locus only one single band was found (variant “11” at gene locus PGM-A’ in figures 1 and 3) which migrated distinctly faster when compared to *J. regia*.

All individuals of *J. x intermedia* showed three zones of activity when stained for PGM (see figure 1 and 3). For all individuals in the slowest migrating zone one single-band was found which migrated identically when compared to the single-

band observed at gene locus PGM-B of the parent species. In the fastest migrating zone also one single-band occurred only, for which the migration was identical when compared to the band observed at gene locus PGM-A’ of *J. nigra*. In the middle migrating zone two different single-bands were observed for which the migration was identical when compared to bands encoded by the alleles PGM-A1 and PGM-A2 of *J. regia* (ARULSEKAR *et al.*, 1986). Thus, it is postulated that for hybrids both bands in the middle migrating zone are encoded by two alleles at this gene locus too. For *J. nigra* no data on genetic control of the PGM system exist, and thus isoenzyme variants must be regarded to be putative. However, due to the stable and reproducible banding patterns observed in all investigated *J. nigra* and hybrids, it is postulated that variants of this enzyme system are suitable to distinguish between species and to identify hybrids genetically.

Conclusions

The results of the present study confirm the hypothesis given by GERMAIN *et al.* (1993) who assumed that at gene locus AAT-C the species *J. nigra*, *J. regia*, and *J. x intermedia* can be distinguished by means of isoenzyme gene markers. Furthermore, the present results indicate that distinguishing between species is also possible using the enzyme system PGM.

For forest nurseries, the use of these gene markers will help to distinguish clearly between hybrids and parent species in early life stage. According to GERMAIN *et al.* (1993), enzyme extract can also be taken out of leaf tissues. This enables to identify and select hybrids immediately after germination, and thus reduce the costs of nursery production. Furthermore, forest nurseries are enabled to offer genetically certificated plants, and foresters are enabled to proof the regularity of hybrid plants.

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