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Identification of Potential Cytogenetical and Biochemical Markers in Bioindication of Common Oak Forests

By

V. BESENDORFER[1)], V. ZOLDOŠ[1)], T. PEŠKAN[1)], M. KRSNIK-RASOL[1)], T. LITTVAY[2)] & D. PAPEŠ[1)]

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Summary

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Cytogenetical and biochemical investigations of common oak (*Quercus robur* L.) were applied in order to find suitable markers in bioindication of oak populations. *Quercus robur* karyotype from investigated populations consisted of 24 chromosomes including a satellite pair. One or two small spherical chromatin fragments, "B-chromosomes" were present in some prophase or metaphase cells. Giemsa C-banding, chromomycine A_3/DAPI reverse staining and silver impregnation showed the same signal on spherical fragments as on the NORs of satellite chromosomes indicating that "B-chromosomes" were broken NORs. They were entirely heterochromatic, G-C rich, showing $AgNO_3$ positive reaction and they could be used as markers in bioindication of oak populations.

Protein bands of about 60 and 95 kDa were distinct in the leaves of heavily damaged trees. Due to its stability isoperoxidases pattern of dormant buds and young leaves might be convenient tissue for biochemical bioindication.

Introduction

Forest decline and decay have been monitored in Croatia for a few decades. The attention has been focused on the determination of trees and stands vitality (LITTVAY & al. 1993). Environmental pollution can cause damage of

[1)] University of Zagreb, Faculty of Science, Department of Molecular Biology, Rooseveltov trg 6, HR-10000 Zagreb, Croatia

[2)] Forest Research Institute Jastrebarsko, Streljačka 1, HR-10000 Zagreb, Croatia

genetic material which could be detected earlier than any phenotypic change (DRUŠKOVIČ 1988a). In early diagnosis of forest decline cytogenetic analyses could be useful. The relationship between environmental pollution, forest reproduction and cytogenetic damages has been successfully carried out in Slovenia (DRUŠKOVIČ 1988a,b, BAVCON & al. 1993) and in Austria (MÜLLER & al. 1991) on the Norway spruce (*Picea abies* L.). Concerning the fact that the oak species are economically and ecologically very important, but also very endangered woody species in Croatia, the *Quercus robur* was chosen as a bioindicator plant. There are only a few works on this subject (BUTORINA & al. 1983, OHRI & AHUJA 1990, BATIČ & al. 1994, ZOLDOŠ & al. 1994a,b). Peroxidase and its isozymes have often been used as biochemical markers in plant stress physiology (CASTILLO & al. 1987, VARSHNEY & VARSHNEY 1985, SIEGEL 1993).

Our research was carried out in order to establish genotype characteristics of the common oak and to define cytogenetic and biochemical markers which could help to detect plant damage before the visible symptoms appear.

Materials and Methods

The common oak (*Quercus robur* L.) from different natural populations of nothern Croatia was the object of our research. Material was collected from experimental plots of the Croatian Forester's Houses Bjelovar-Vrbovec, and Koprivnica. Four trees with different degree of damage were selected from each experimental plot. Acorns were germinated on the wet cotton in glass pots. Root tips of seedlings and leaf bud meristems of mature trees were used for analyses of mitotic activity and chromosomal aberrations. For karyological analysis root tips were pretreated in 2 mM 8-hydroxyquinoline at 16°C for 4-5 h and fixed in ethanol-acetic acid (3:1). Giemsa C-banding technique (OHRI & AHUJA 1990), chromomycine A3 (CMA)/DAPI fluorescent double staining (SCHWEIZER 1976, MALUSZYNSKA & SCHWEIZER 1989) and silver impregnation (MORENO & al. 1989) were applied to study oak karyotype. Root tips for mitotic index and chromosome aberration analysis were fixed and slides were prepared using Feulgen-Giemsa double staining technique (HOMMO & SARKILAHTI 1985).

Soluble proteins were extracted from cotyledones of dormant and germinating acorns, young leaves, dormant buds and leaves from pollution damaged trees. The extracts were prepared by grinding fresh tissue in a cold 0.1 M Tris/HCl buffer (pH 8.0) containing 0.5 M sucrose, 0.6 M ascorbic acid, 0.6 M cystein and 2.5% polyvinylpyrolidone. The homogenate was centrifuged at 40000 g for 1 hour at 4°C. The supernatant was used for protein and peroxidase analyses. Denatured proteins were separated in 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (LAEMMLI 1970). The isoperoxidases were separated by the means of isoelectric focusing (IEF) and visualized in the test solution prepared according to SIEGEL & GALSTON 1967.

Results and Discussion

The investigated oak plants had chromosome complement of 2n = 24 metacentric and submetacentric chromosomes and (Fig. 1a) one chromosome pair with a satellite (Fig. 1c). Secondary constrictions were often extended on one (Fig. 2a) or both satellite chromosomes. One or two small spherical fragments were found in some prometaphase or metaphase cells (Fig. 1b, 2a, b). OHRI & AHUJA

1990 also found 2n = 24 standard A-chromosomes and one extra fragment identified by them as a B-chromosome in the karyotypes of *Q. robur*, *Q. petraea* and *Q. rubra*. Extra chromosomes were smaller than the smallest A-chromosome. In the karyotype with two fragments they were often of different size. Giemsa C-banding revealed centromeric and telomeric heterochromatin which were stained with different intensity (Fig. 1a). Centromeric C-bands of different chromosome pairs also stained differently. C-bands on homologous chromosomes were heterogeneous i.e. the centromeric C-band was visible only on one chromosome of the same pair. Several chromosome pairs had heterochromatin dispersed along the chromosome arms. Two chromosome pairs seemed to be entirely heterochromatic while the other pair did not show any C-bands. Heteromorphism revealed in stainings after the same techniques applied and heterogenity of C-bands of homologous chromosomes are the common phenomena in plant and animal heterochromatin (SUMNER 1990).

Fluorescent staining with CMA revealed two spherical signals in interphase cells. In prometaphase and metaphase, signals were located on one chromosome pair (Fig. 1c). In the cells with one or two spherical fragments fluorescent signals were located on a satellite chromosome pair (Fig. 1c) and one independent fragment (Fig. 2a) or on the both fragments (Fig. 2b). One metacentric pair showed strong centromeric fluorescence while centromeric CMA bands on other two chromosome pairs were faintly fluorescent (Fig. 1c, 2a). After restaining with DAPI, negative bands appeared while DAPI fluorescent bands were not observed. There were no signals on fragments stained with DAPI (Fig. 2c). Silver impregnation showed two dark dots usually near nucleolus. The same technique revealed two dark dots (NORs) on one chromosome pair or on the fragments (Fig. 1b). Interphase nuclei had one large or rarely two nucleolus different in size (Fig. 1d).

Results of CMA/DAPI fluorescent double staining and silver impregnation indicated that nucleolar organizer regions (NORs) were probably located on a satellite chromosome pair or on fragments and they were GC rich. Many studies concerning NOR have revealed this area as very changeable region of eukaryotic chromosomes (KONVIČKA & LEVAN 1972, SCHUBERT & al. 1983, MALUSZYNSKA & SCHWEIZER 1989, PUIZINA & PAPEŠ 1995). There is a high possibility that position of NORs varied between individuals or even between cells in *Quercus robur* populations. This variations have been attributed to mechanism of unequal exchanges among r-RNA genes on homologous and non-homologous A- and B-chromosomes (GARRIDO-RAMOS & al. 1995). SCHUBERT 1984 and SCHUBERT & WOBUS 1985 have proved that active NORs in the genus *Allium* are movable. The other explanation could be that independent fragments were deleted satellites. On the basis of the karyologycal studies we consider that CMA signals could be used as ploidy markers. The number of fluorescence signals which is constant in diploid cells could increase during polyploidization as observed in premeiotic cells of *Q. robur*.

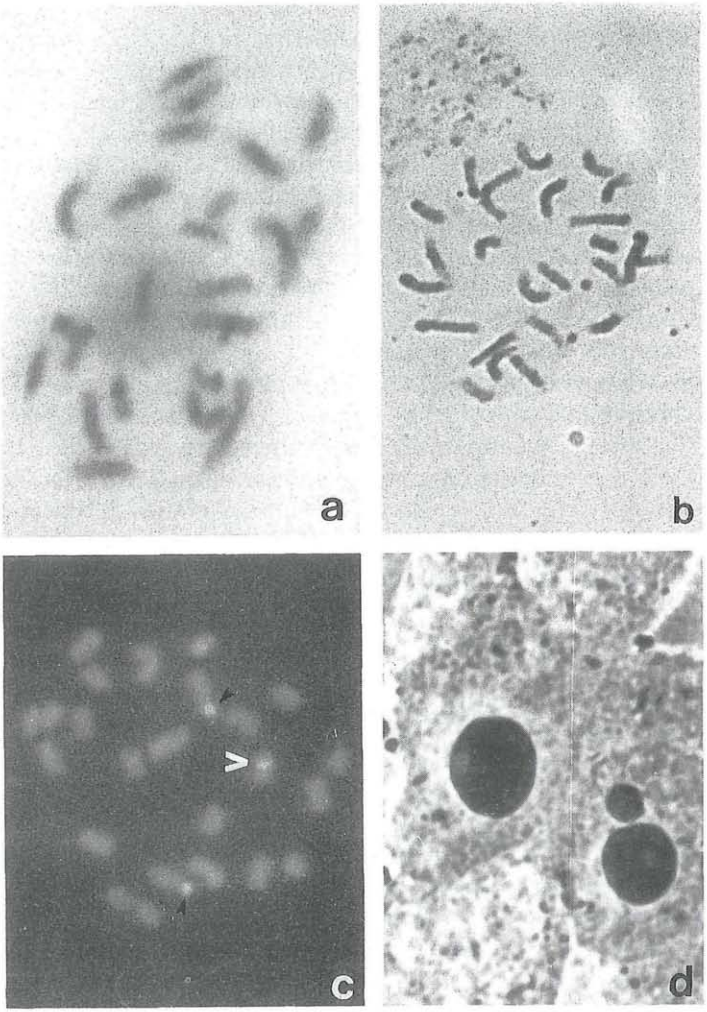

Fig. 1. Mitotic chromosomes (2n=24) and interphase of common oak after different staining techniques. a) Giemsa C-banding reveals centromeric and telomeric heterochromatin with different intensity. b) Silver impregnation of two dark small spherical fragments ("Bs") indicates that nucleolar organizer regions are located on them. c) Fluorescent staining with CMA reveals two signals located on a satellite chromosome pair (arrowed), one strong signal on the metacentric chromosom (arrowed) and faint CMA centromeric bands on the other chromosomes. d) Interphase nuclei with one and two nucleoluis different in size after a silver impregnation.

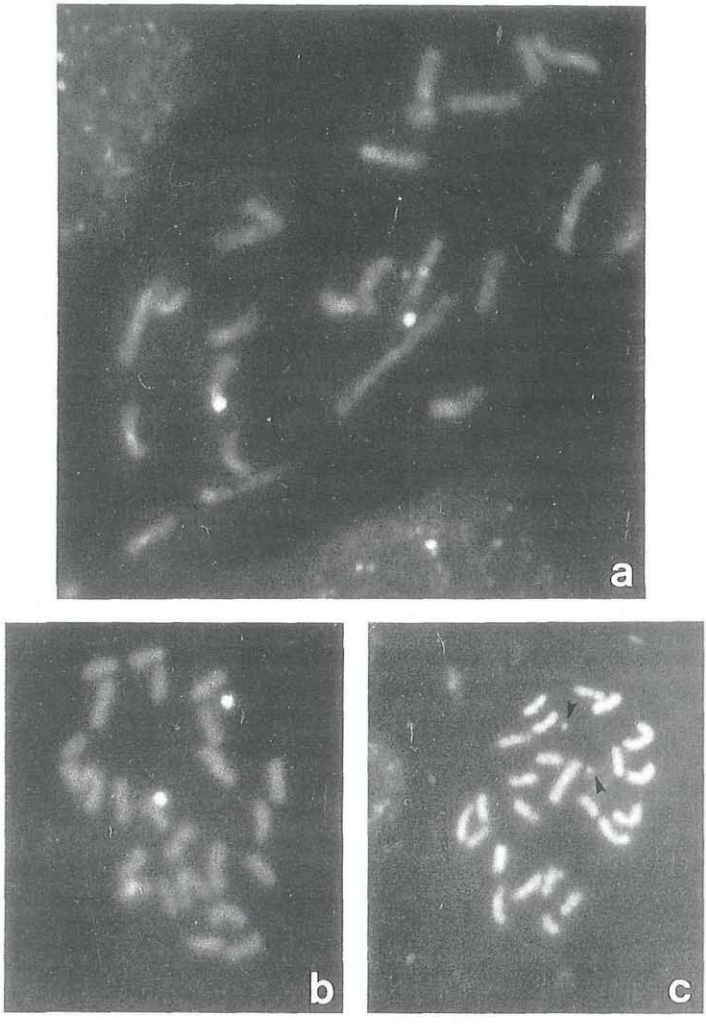

Fig. 2. Fluorescent stainings of common oak mitotic chromosomes. a) Prometaphase chromosomes with two CMA signals, one on a satellite chromosome and the other on an independent spherical fragment; one metacentric pair with strong centromeric and the other chromosomes with faintly fluorescent CMA bands. b) CMA reveals two signals on independent spherical fragments. c) Metaphase after restaining with DAPI: negative bands on chromosomes and no signals on two spherical fragments (arrowed).

Mitotic index (6.25-11.5%) showed similar mitotic activity in root-tip cells of seedlings and leaf meristems from mature trees. Therefore, the both tissues could be used for cytogenetic analysis. Chromosomal aberrations such as anaphase or telophase bridges, laggard chromosomes in metaphase and anaphase and micronuclei probably formed of laggards were recorded. As the frequency of chromosomal aberrations was very low (< 1.0%) we could not be sure that they were caused by the changes in ecosystem or were they common for this species. A certain mitotic irregularity could be connected with late dividing manner of B-chromosomes in *Quercus* genotype (OHRI & AHUJA 1990). In her bioindication method, DRUŠKOVIČ 1988a suggested collecting the root-tips of adult trees in the field. Unfortunately, it is almost impossible to collect oak root-tips in the field because its longitudinal roots are woody.

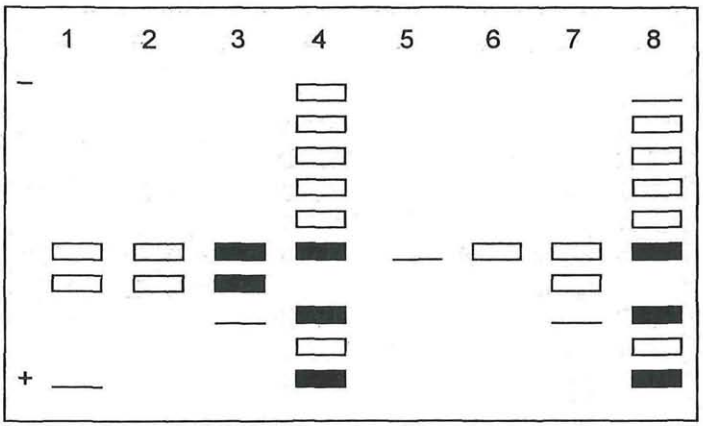

Fig. 3. IEF of oak peroxidases: 1, 5 - cotyledones of dormant acorns, 2, 6 - cotyledones of germinating acorns, 3, 7 - young leaves, 4, 8 - dormant buds.

band intensity:

strong
medium
low

Protein pattern of oak leaves collected from trees with different degree of damage was rather smeared with coloured gel background caused most likely by high content of phenolics. However, protein bands of about 60 kDa and 95 kDa were quantitatively distinct in the leaf extracts of heavily damaged trees. It remains to test if the two proteins had any relation to air or soil pollutants. Isoperoxidase patterns were developmentally specific (Fig. 3). Only acidic isozymes were observed in the examined tissue. Dormant buds showed up to nine isozymes, while in young leaves only three isozymes were observed. Isozymes of cotyledons were

rather variable and we could not establish an acorn marker isozyme. It could be a consequence of genetic differences or physiological and developmental changes in acorns. Isoperoxidases pattern of oak dormant buds and young leaves were stable among different plants suggesting that those tissues might be convenient for biochemical bioindication. The usefulness of buds and nuts, due to their isozymes stability, was reported also for beech (THIEBAUT & al. 1982).

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