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BRIEF COMMUNICATION

Genome size stability in six medicinal plant species propagated *in vitro*

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Abstract

DNA content was estimated by flow cytometry in seedlings and *in vitro* clones of six species: *Oenothera paradoxa*, *Inula verbascifolia* ssp. *aschersoniana*, *Rubus chamaemorus*, *Solidago virgaurea*, *S. graminifolia* and *Pueraria lobata*. With the exception of *P. lobata*, there was no difference in genome sizes between seedlings and *in vitro* cultured plants from any species, indicative that they maintain their genetic stability during *in vitro* culture. This confirms the usefulness of tissue culture for production of certified plant material to obtain herbal medicines.

Additional key words: DNA content, flow cytometry, genetic fidelity, micropropagation.

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In vitro culture techniques can be used to propagate medicinal plants in controlled conditions for cultivation on a commercial scale. Nevertheless, the use of *in vitro* cultures has to be customized to avoid changes in a plant genome. Generally, multiplication *via* buds (existing meristems) is the preferred strategy to maintain genetic stability (Bajaj *et al*. 1988). However, outgrowths from *de novo* meristems, which are formed adventitiously from *in vitro* propagated shoots, may show genetic variability. Confirmation of genetic stability is of particular importance in medicinal plants when considering them as a source of metabolites with biological activity; the presence and the composition of these secondary metabolites should remain unchanged after micropropagation.

Tissue culture often promotes genetic disturbances, which result in somaclonal variation. This can be the result of chromosomal aberrations, changes in the number of individual chromosomes, or in the ploidy level in response to the physical agents and chemical composition of the culture medium and the presence of growth regulators (Sliwinska *et al*. 2003, Canter *et al*. 2005). Therefore, control of genome stability of medicinal plants growing *in vitro* is desirable. Flow cytometry (FCM), a fast and accurate method for estimation of nuclear DNA content, has been successfully applied to study genome changes in micropropagated plants (Kubaláková *et al*. 1996, Sliwinska *et al*. 2003, Thiem and Sliwinska 2003, Loureiro *et al*. 2007).

The aim of this study was to establish by flow cytometry absolute DNA contents in seedlings and *in vitro* clones of six medicinal plant species which produce secondary metabolites, mainly phenolic compounds, used for the production of herbal medicines. Comparison of the values obtained from these two different kinds of plant material provided information on genome stability during micropropagation.

Seed-derived plants (5- to 6-week-old seedlings) and axillary shoots (micropropagated for over a year) of six species: *Oenothera paradoxa* Hudziok (*Onagraceae*), *Inula verbascifolia* (Willd.) Hausskn. ssp. *aschersoniana* (Janka) Tutin (*Asteraceae*), *Rubus chamaemorus* L. (*Rosaceae*), *Solidago virgaurea* L. (*Asteraceae*), *Solidago graminifolia* (L.) Elliott (*Asteraceae*), *Pueraria lobata* (Willd.) Ohwi (*Fabaceae*), grown *in vitro* as described previously (Skrzypczak *et al.* 1999, Thiem *et al*. 1999, 2003, Thiem 2001, 2003), constituted the experimental material. *Zea mays* CE-777 line (2C=5.43 pg; Lysák and Doležel 1998) was used as an internal standard.

For flow cytometric analysis, young leaves of the

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target species (from seedlings or plantlets growing *in vitro*) and of the internal standard were chopped simultaneously with a sharp razor blade in a plastic Petri dish with 1 cm^3 nucleus-isolation buffer [0.1 M Tris, 2.5 mM $MgCl_2 \cdot 6\ H_2O$, 85 mM NaCl, 0.1 % (v/v) Triton X-100, 1 % (v/v) PVP-10; pH 7.0], supplemented with propidium iodide (PI; 50 μg cm^{-3}) and ribonuclease A (50 μg cm^{-3}). After chopping, the suspension was passed through a 50 μm mesh nylon filter. For each sample, 5 000 - 10 000 nuclei were analysed directly after preparation using a *Partec CCA* (Münster, Germany) flow cytometer, equipped with an argon laser. Analyses were replicated 10 times for each species and plant material. Only two samples of the same plant material and species were analysed per day, to avoid errors due to random instrument drift. All analyses were performed on the same machine with the same operator. Histograms were analysed using a *DPAC v.2.2* computer program. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions target species*/Zea*, on a histogram of fluorescence intensities. The results were estimated using a single-factor analysis of variance and Duncan's test.

Flow cytometric analysis showed that despite of the presence of some phenolic compounds in the cytosol of all studied species (compounds known to inhibit PI staining of DNA) it was possible to obtain high resolution histograms when 1 % PVP was added to the standard isolation buffer (Fig. 1). The genome size of the six species under study ranged from 1.75 pg/2C (*S. graminifolia*) to 2.5 pg/2C (*R. chamaemorus* and *I. verbascifolia*; Table 1). To our knowledge this is the first report on the genome size of *O. paradoxa*, *S. virgaurea*, *S. graminifolia* and *I. verbascifolia*. The DNA content of *R. chamaemorus* derived from *in vitro* cultures, established in our previous studies (2.46 pg/2C; Thiem and Sliwinska 2003), was very similar to that obtained here. *P. lobata* was previously found to have 2.20 pg/2C (established using FCM/PI and *P. sativum* as an internal standard; Bennett *et al.* 2000) and in the present study we obtained a slightly lower value, close to 2.0 pg/2C, since a different internal standard was used here (*Zea mays*).

For all species but one, *P. lobata*, no statistical changes in absolute DNA content were found between the seedlings and micropropagated plantlets (Table 1). This suggests that even during one year *in vitro* culture, the genome size of these five species was stable. A significantly higher genome size, however, was present in seedlings of *P. lobata* as compared to *in vitro* clones.

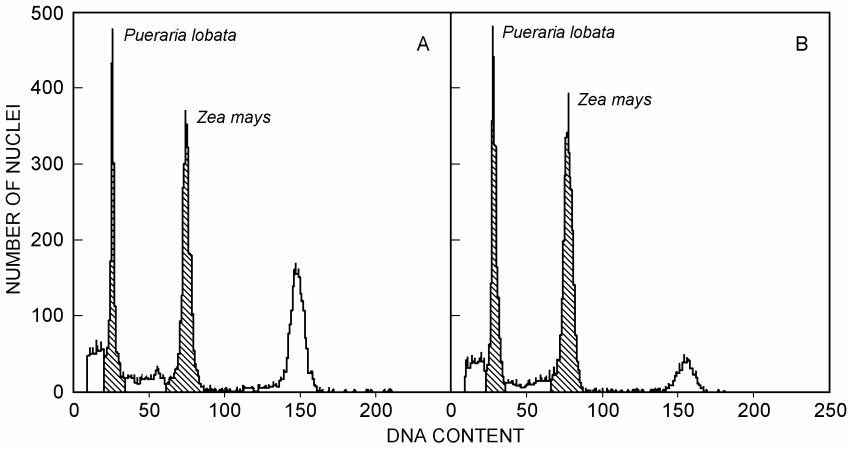

Fig. 1. DNA-histograms of nuclei isolated simultaneously from young leaves of *Zea mays* (internal standard) and *Pueraria lobata*: *A* - *in vitro* clone (*Pueraria/Zea* peak ratio 0.35); *B* - seedling (*Pueraria/Zea* peak ratio 0.38).

Table 1. Nuclear DNA content of some medicinal plant species obtained from seeds and in *in vitro* culture. Means ± SD, n = 10, Only values for particular species followed by different letters are significantly different at P = 0.05 (Duncan's test)

Species	*In vitro* clones pg/2C	CV [%]	Seedlings pg/2C	CV [%]
Oenothera paradoxa	2.22 ± 0.02	3.70	2.21 ± 0.02	3.83
Solidago virgaurea	2.14 ± 0.06	4.83	2.16 ± 0.03	4.50
Solidago graminifolia	1.74 ± 0.03	5.46	1.75 ± 0.04	5.56
Rubus chamaemorus	2.49 ± 0.07	4.99	2.50 ± 0.07	4.43
Inula verbascifolia	2.51 ± 0.06	4.10	2.50 ± 0.03	4.78
Pueraria lobata	1.94 ± 0.07b	4.56	2.01 ± 0.05a	5.48

Two reasons for this are possible. Firstly, some genetic changes may have taken place during *in vitro* culture. Secondly, the concentration of phenolic compounds is not the same in the cytosol of seedlings and micropropagated plants. Indeed, different content of isoflavonoids such as formononetin, daidzein and genistein was found in different tissues of *P. lobata* from *in vitro* culture, however, no seedling tissue was analyzed (Thiem 2003). It is possible that in the present study these compounds were not inactivated to the same extent in different plant material by the reductant (PVP) added to the buffer during preparation of the flow cytometric samples. Residual amounts could cause a stoichiometric error in genome size estimation by PI-staining inhibition. It has already been noted that in other species such reductants have no measurable effect on reducing the inhibition of fluorescence (Price *et al.* 2000, Sliwinska *et al*. 2005). Additional analyses, of the *P. lobata* karyotype and/or biochemical composition of its cytosol, are necessary to verify which of these two hypotheses is correct.

In conclusion, flow cytometric data provide evidence that *in vitro* derived plants maintain the same diploid genomic DNA content as seedlings. This confirms that the *in vitro* technique (a regeneration of multiple shoots without a callus phase from axillary buds) can be used for production of genetically-stable medicinal plants. Each species, however, has to be checked individually for genome stability.

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