ACTA
PHYSIOLOGIAE
PLANTARUM
Vol. 21. No. 1. 1999: 37-43

Nuclear DNA content of *Solanum* species grown *in vitro*, as determined by flow cytometry

Urszula Maciejewska[1], *Janusz S. Skierski*[2] *and Anna Szczerbakowa* [1]

[1]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Pawińskiego 5a, Poland
[2]Flow Cytometry Lab., Drug Institute, Warsaw, Chełmska 30/34, Poland

Key words: cell suspension, DNA content, flow cytometry, mesophyll, protoplast, *Solanum* species.

Abstract

The DNA relative content in nuclei from several *Solanum* species, which were used as partners for somatic hybridization, were determined using a flow cytometry method. The nuclei were isolated mechanically or *via* protoplasts from leaves of *in vitro* grown plants. In the case of *S. nigrum* as well as *S. tuberosum cv.* Bzura and dihaploid clone H8105, the nuclei were also obtained from suspension cultured cells by lysis of protoplasts. The source and the method of nuclei isolation affected the pattern of nuclear DNA in the genotypes studied. The mesophyll nuclei showed two distinct peaks on the DNA histograms, whereas the DNA peaks produced by cell suspension nuclei were broad and less distinct. The DNA content in the nuclei, calculated from the DNA histograms of the samples and a DNA standard historgam (Trout Red Blood Cells, having DNA content of 5.05 pg per nucleus), were much lower in mesophyll nuclei than in those obtained from the cell suspension for the same genotypes. The results are discussed in respect of the genetic instability of *Solanum* genotypes. The usefulness of a flow cytometry approach in somatic hybridization research is also discussed.

Introduction

The improvement of the cultivated potato by the incorporation of desirable traits, such as pathogen resistance, is of major interest in breeding programs. An important source of these polygenically encoded traits are wild *Solanum* species. However, the transfer of the genes from the wild species to the cultivated potato by conventional crossing is often hampered by sexual incompatibility. The genetic-engineering approach, as it is still limited to the transfer of one or a few genes, is also not suitable. A promising method to overcome these barriers is somatic hybridization *via* fusion between protoplasts from the wild and cultivated species. In such research, determination of nuclear DNA content in the parental material is an important basis for selection and molecular analysis of the somatic hybrids.

We aimed to obtain potato somatic hybrids with increased resistance to the fungal pathogen *Phytophthora infestans*, which causes a potato late blight disease. As fusion recipients, we have chosen susceptible potato cultivars: tetraploid Bzura, diploid Zel 1136, and dihaploid clone H8105; and as donors, we used wild species completely resistant to this pathogen: hexaploid *S. nigrum* and diploid *S. bulbocastanum.* The fused protoplasts were isolated both from leaf mesophyll of the *in vitro* grown plants and cell suspensions established from plant leaves. As genetic instability in the *in vitro* cultured *Solanum* species was frequently reported (Ramulu *et al.* 1985, Ramulu and Dijkhuis 1986), the present

study was undertaken with the aim of examining the nuclear DNA content in the material currently being fused. In addition the influence of two nuclei isolation procedures (mechanical and *via* protoplasts) on the nuclear DNA content was investigated.

To determine the nuclear DNA content we used flow cytometry, which offers a rapid method for the analysis of the DNA content in thousands of plant nuclei (Doležal 1991, Nicoloso *et al.* 1994, Valkonen 1994). The flow cytometry (FCM) analysis of nuclear DNA content consists in measuring the fluorescence associated with each nucleus after stoichiometric staining with DNA specific fluorochrome and comparing the results with a reference DNA standard. Standards may be internal, mixed together with the nuclei examined, and measured simultaneously, or external, checked before and/or after each sample.

In this work, results on the nuclear DNA content in given *Solanum* genotypes with different nuclei sources and isolation methods are reported.

Material and methods

Axenic shoot cultures of *S. bulbocastanum* PI 243512 (*S. blb*), *S. tuberosum* (*S. tbr*) *cv.* Zel 1136 and clone H8105 were kindly supplied to us by prof. J. Jakubiec (Agriculture Academy, Warsaw), *S. nigrum var. gigantea, S. tbr. cv.* Bzura were obtained from Młochów and Bonin Research Centers (IHAR, Poland). The plant shoots were grown *in vitro* in a MS medium (Murashige and Skoog 1962) containing 2 % sucrose and solidified with 0.6 % agar. They were placed under controlled conditions: photoperiod 16 h, the illumination of daylight fluorescent lamps 20-30 $W \cdot m^{-2}$ (according to plant requirements), day/night temperature 22/18 °C. The plants were subcultured at 4-6 week intervals.

The cell suspension cultures established from the leaves of *S. nigrum, S. tbr. cv.* Bzura and clone H8105 were cultivated as described previously (Avan *et al.* 1997). The cells were subcultured every 7 days.

Isolation of protoplasts

The leaves of 4-5 week old shoots, after preconditioning according to the procedure of Haberlach *et al.* (1985), were cut into small pieces. The cell suspensions 5-6 days after subculturing (at the end of the exponential growth phase) were used in the experiments. To isolate protoplasts either the leaf material or the suspension cells were incubated overnight in a K4 solution (Nagy and Maliga 1976) containing 0.6-1.6 % w/v cellulase "Onozuka" R-10 and 0.3-0.6 % macerozyme R-10, in the dark at 26-28 °C. Cell suspensions required higher enzyme concentrations and temperatures. After the digestion period, protoplasts were filtered through steel sieves (74 or 100 μm mesh) and subsequently centrifuged (80 g for 5 min) in a gradient, made up from K4 and W5 salt solution (Menczel *et al.* 1981). The protoplasts floated at the K4/W5 interphase were transferred into a W5 solution and centrifuged again. The pelleted protoplasts were resuspended in the same solution at a final concentration of 10^6 protoplasts per ml.

Measurement of DNA content by flow cytometry

The FCM measurements were carried out on nuclei released either by lysis of protoplasts or mechanically by chopping of leaf tissues. Samples of protoplast suspensions were mixed with the DAPI (4',6-diamidino-2-phenylindole, Molecular Probes D-1306) solution (10 mM PIPES-Na_2, 2 mM $MgCl_2$, 0.1 % Trition X-100, 1 $mg \cdot L^{-1}$ DAPI, 20 $mg \cdot L^{-1}$ sulforhodamine 101) and incubated on ice for 10 min. Alternatively, young leaves from *in vitro* grown shoots were chopped with a razor blade in a droplet of the DAPI solution and incubated at room temperature for 10 min (Menke *et al.* 1996). After the incubation period, both suspensions were filtered through a 35 μm nylon filter and analyzed within one hour.

In some experiments, the protoplast nuclei were fixed with ethanol-acetic acid 3:1 v/v (Nicoloso *et al.* 1994) prior to staining, or prepared and stained using citric acid/Tween20 solution, according to the procedure of Ulrich and Ulrich (1991).

FCM measurements were carried out using a FACS-Vantage (Becton-Dickinson) flow cytometer, equipped with a dual-wavelength argon ion la-

ser (Innova Enterprise 621) emitting UV (351 nm) and blue (488 nm) light. DAPI fluorescence was analyzed with 424±44 nm band-pass filter, and sulforhodamine 101 with 630±22 nm band-pass filter. As an external standard, trout erythrocytes, TRBC (PARTEC, Germany, Cat. No Ox-5-7302), with a DNA content of 5.05±0.18 pg per nucleus, were used. Data acquisition was performed using CellQuest software (Becton-Dickinson, USA). At least 10,000 nuclei were counted for each sample.

Results

In the FCM analysis, the DNA histograms reflect the frequency of nuclei with different DNA content. The first main peak represents the nuclei in a G_0/G_1 phase (2C complement of DNA). The next peak corresponds to the nuclei in a G_2/M, post -DNA synthesis, phase (4C complement of DNA) or doublets of nuclei; multiple peaks show higher levels of ploidy. Signals occurring in the lower channel-number region (sub-G_0/G_1) result from disrupted nuclei and/or non-specific fluorescence of other cell constituents.

Some representative examples of DNA histograms from clone H8105 nuclei isolated mechanically or *via* protoplasts from leaves, and those from cell suspension by lysis of protoplasts are given in Fig.1A, B and C, respectively. We used TRBC nuclei as an external standard, checking before and after each sample their peak position, which was set at channel 200. This peak is shown in Fig. 1A, B, and C by a dashed line. A variance in fluorescence of each sample was estimated by the coefficient of variation (cv) values, defined as a standard deviation at a half height of peak, expressed as percentage of the mean channel. The DNA content in leaf nuclei of *S. tbr.* clone H8105, isolated either mechanically (Fig. 1A) or by lysis of protoplasts (Fig.1B), was distributed predominantly in one main peak (marked as M1) corresponding to non-dividing cells, and in a small peak (marked as M2) reflecting cells in post-DNA synthesis phase or nuclei doublets. The cv values of the relative DNA content of both nuclei groups ranged from 2 % to 5 %. However, it should be mentioned that the DNA peak position of the mesophyll nuclei released *via* protoplasts was lower than those isolated mechanically (compare Fig. 1A and B). In contrast, the nuclei derived from the cell suspension (Fig.1C) showed variations in their relative DNA content as reflected by broader, less distinct, and multiple peaks, in comparison with those of the leaf nuclei (compare Fig. 1B and C). The cv values of the peaks were approx. equal to 8 %.To improve resolution of the DNA peaks we tried several methods of preparation and fixation of the nuclei, but these procedures did not result in better DNA histograms.

The nuclei obtained from the leaves of other species examined produced the DNA histograms similar to those presented above. In the case of nuclei derived from

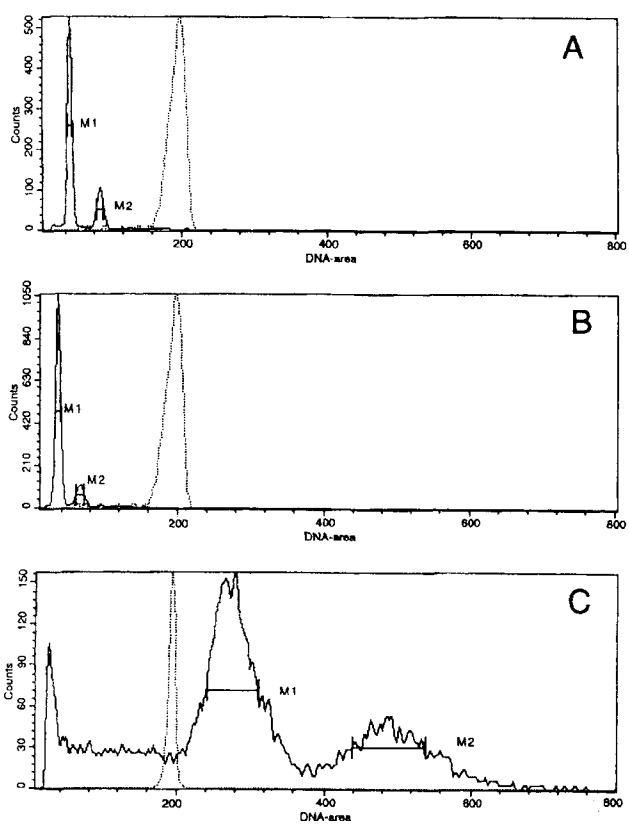

Fig. 1. Histograms of DNA from *S. tuberosum* clone H8105. Dotted line: DNA standard (TRBC). Mesophyll nuclei - A: isolated mechanically (by chopping the leaves); B: obtained *via* protoplasts; C: nuclei derived from cell suspension.

the corresponding cell suspensions, the resolution of the DNA peaks was better than that shown for clone H8105 (Fig. 1C).

In the case of clone H8105, microscopic observations revealed that leaf protoplasts were about 20-30 μm in diameter (Fig. 2A), whereas those from the cell suspension amounted to 80 μm in diameter (Fig. 2D). The leaf protoplasts stained with DAPI disintegrated easily (Fig. 2B). They usually contained single nuclei (Fig. 2C). The protoplasts derived from the cell suspension were rather stable in the DAPI solution and they appeared to be predominantly double or triple nuclear (Fig. 2E).

The position of the first peak for the nuclei investigated, relative to that of the standard TRBC nuclei, was used to calculate the average DNA content (in pg) of plant nuclei (Table). The genotypes with higher ploidy showed higher nuclear DNA content. The estimates of DNA content in leaf nuclei isolated mechanically were somewhat higher than in those obtained *via* protoplasts. The nuclei derived from cell suspensions contained 1.3 to 6.5 times as much DNA as the corresponding leaf nuclei. The shift from lower to higher DNA content was pronounced mostly in the case of genotypes resulting from genetic manipulations (clone H8105).

Table. The DNA content in nuclei isolated from leaves and cell suspensions of different *Solanum* species.

Species	Ploidy	DNA content in nuclei isolated from different *Solanum* species (pg)		
		Mesophyll		Cell suspension
		Leaf	Protoplast	
S. nigrum var. gigantea	6x	4.31±0.23	3.69±0.13	4.91±0.79
S. bulbocastanum	2x	1.14±0.21	0.97	NT*
S. tuberosum cv. Bzura	4x	2.73±0.27	2.89±0.13	4.08±0.95
S. tuberosum cv. Zel 1136	2x	1.38±0.18	1.06±0.04	NT
S. tuberosum clone H8105	2x	1.35±0.15	0.99±0.06	6.40±0.73

*NT - not tested

Discussion

The results of the present study show that the patterns of nuclear DNA content of leaf nuclei, irrespectively of the isolation methods, were almost identical (Fig.1 A and B). The DNA peaks had good resolution, and cv values of the main peak were rather low as compared with the data reported by other authors (Sgorbati *et al.*1986, Nicoloso *et al.*1994). A vast majority of nuclear DNA was distributed in the first peak, which corresponds to non-dividing cells. This is in accordance with the report of Hanish ten Cate *et al.* (1987) for *in vitro* grown potato cv. Bintje plants. Since the phase S of cell cycle is not distinctly visible, we presume that the second, lower peaks in the DNA histograms represent nuclei doublets rather than nuclei in G_2/M phase.

The DNA contents calculated for leaf nuclei of the genotypes studied (Table) were in the range of those published by other authors (Valkonen 1994, Jakuczun *et al.* 1997) for different species of *Solanum* with comparable levels of ploidy.

The present estimates of DNA content in leaf nuclei isolated mechanically showed some correlation with the ploidy level of the genotype. Previously, Valkonen *et al.* (1994) and Valkonen (1994) have found a high correlation between nuclear (2C) DNA content and the ploidy level within stable, standard *Solanum* genotypes, but for *S. tbr.* at tetraploid level, the correlation was low. It should be noted that within the same genotype the estimates of DNA content in leaf nuclei obtained *via* protoplast were lower than those in the nuclei isolated mechanically. This observation is in agreement with the data of Doležel et al (1989) obtained for alfalfa (*Medicago sativa*) tissue, although for cell suspension, he obtained similar results for both methods of nuclei isolation. In our study it may be conceivable that the decrease in the DNA content in protoplast-derived nuclei was due to the degradation of the DNA during the whole procedure of leaf protoplast isolation (Galun 1981).

The DNA histograms of cell suspension nuclei showed less distinct and broader peaks positioned in higher channels in comparison with those obtained for mesophyll nuclei of the same genotypes. The features of DNA histograms of cell suspension nuclei may reflect a shift to a higher levels of ploidy, large variations in nuclear DNA content and/or may be due to irregular division or clustering

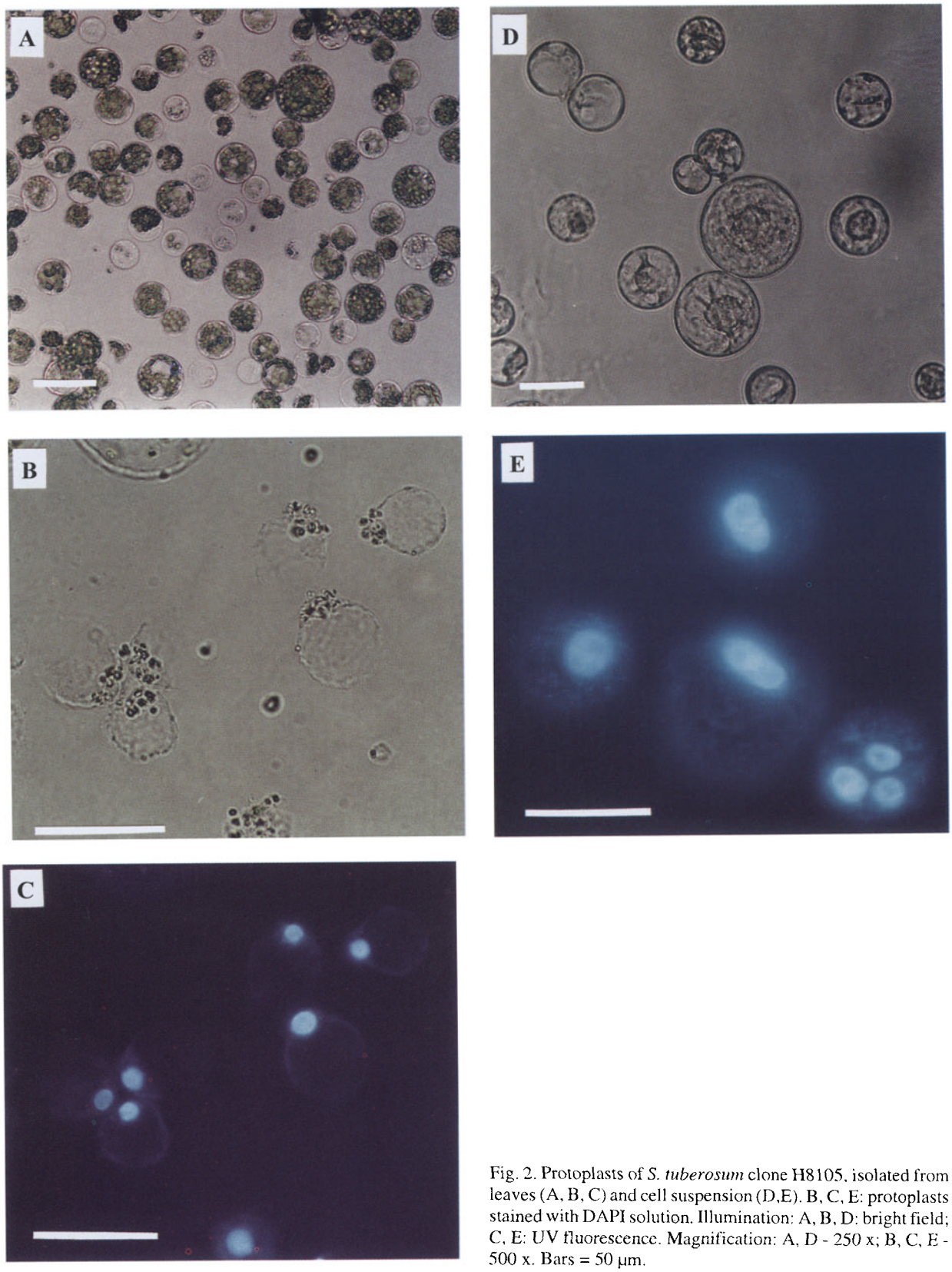

Fig. 2. Protoplasts of *S. tuberosum* clone H8105, isolated from leaves (A, B, C) and cell suspension (D,E). B, C, E: protoplasts stained with DAPI solution. Illumination: A, B, D: bright field; C, E: UV fluorescence. Magnification: A, D - 250 x; B, C, E - 500 x. Bars = 50 μm.

of nuclei. The fact that the cell suspensions used for the DNA measurements were in an exponential growth phase, suggests irregularity in cell division. The differences in the DNA histograms between the cell suspension and mesophyll nuclei were pronounced mostly in the case of *S. tbr.* dihaploid clone H8105. The microscopic observations that revealed double and trinuclear H8105 protoplasts speak in favour of the variations in the nuclear DNA content. However, it cannot be excluded that the increase in relative DNA content was due to clustering of nuclei. According to Nicoloso *et al.* (1994), what is important for DNA analysis in flow cytometry is to obtain single nuclei surrounded by little if any cytoplasm. As shown in Fig. 2E, the clone H8105 protoplasts derived from cell suspension disintegrated with difficulty in the DAPI solution. The results obtained are in line with reports on polyploidization, which occurred predominantly in mono- and dihaploid/diploid potato genotypes, during the early stages of protoplast cultures (Ramulu *et al.* 1989).

The present results show that the sources and methods of nuclei isolation affect the pattern and content of nuclear DNA in *Solanum* genotypes. In conclusion, the nuclei isolated mechanically from the leaves of young regenerated plants will constitute the best material for the DNA analysis by a flow cytometry method. This approach might be used for the preliminary selection of somatic hybrids at early stages of regeneration only in the case of both mesophyll fusion partners. When one protoplast fusion partner is derived from cell suspension, identification of somatic hybrids using a flow cytometry method is very ambiguous.

Acknowledgements

The authors thank M.Sc. Maria Borkowska for the propagation of the *in vitro* grown plants. We also express our gratitude to Professor K. Kleczkowski for his encouragement and valuable comments on the manuscript.
This research was supported by the State Committee for Scientific Research (KBN), project no 6 PO4B 021 12 .

References

Avan M.F., Maciejewska U., Kleczkowski K., and Wielgat B. 1997. Differential responses of potato cell suspensions to a culture filtrate of *Phytophthora infestans*. Z. Naturforsch. 52c: 333-338.

Doležel J., Binarova P., and Lucretti S. 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. Biol. Plant. 31: 113-120

Doležel J. 1991. Flow cytometry analysis of nuclear DNA content in higher plants. Phytochem. Anal. 2: 143-154

Galun E. 1981. Plant protoplasts as physiological tools. Ann. Rev. Plant. Physiol. 32: 237-266

Haberlach G.T., Cohen B.A., Reichert N.A., Baer M.A.,Towill L.E. and Helgeson J.P. 1985. Isolation, culture and regeneration of protoplasts from potato and several related *Solanum* species. Plant Science 39: 67-74.

Hanisch ten Cate Ch.H., and Ramulu K.S. 1987. Callus growth, tumor development and polyploidization in the tetraploid potato cultivar Bintje. Plant Sci. 49: 209-216.

Jakuczun H., Strzelczyk-Żyta D., Narkiewicz M. 1997. Zastosowanie pośrednich metod oznaczania poziomu ploidalności ziemniaka. Biul. Inst. Ziemniaka 48: 91-98.

Menczel L., Nagy F., Kiss Zs.R., and Maliga P. 1981. Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: Correlation of resistance to *N. tabacum* plastids. Theor. Appl. Genet. 59: 191-195.

Menke U., Schilde-Rentschler L., Ruoss B., Zanke C., Hemleben V., Ninnemann H. 1996. Somatic hybrids between the cultivated potato *Solanum tuberosum* L. and the 1EBN wild species *Solanum pinnatisectum* Dun.: morphological and molecular characterization. Theor. Appl. Genet., 92: 617-626.

Murashige T., and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 15: 473-497.

Nagy J.I., and Maliga P. 1976. Callus induction and plant regeneration from mesophyll protoplasts of *Nicotiana sylvestris*. Z. Pflanzenphysiol. 78: 453-455.2

Nicoloso F.T., Val J., van der Keur M., van Iren F., and Kijne J.W. 1994. Flow-cytometric cell counting and DNA estimation for the study of plant cell population dynamics. Plant Cell, Tissue and Organ Cult. 39: 251-259.

Ramulu K.S., Dijkhuis P., Hanisch ten Cate Ch.H., and Groot B. De. 1985. Patterns of DNA and chromosome variation during *in vitro* growth in various genotypes of potato. Plant Sci. 41: 69-78

Ramulu K.S., Dijkhuis P. 1986. Flow cytometric analysis of polysomaty and *in vitro* genetic instability in potato. Plant Cell Rep. 3: 234-237

Ramulu K.S., Dijkhuis P., and Roest S. 1989. Patterns of phenotypic and chromosome variation in plants derived from protoplast cultures of monohaploid, dihaploid and diploid genotypes and in somatic hybrids of potato. Plant Sci. 60: 101-110

Sgorbati S., Levi M., Sparvoli E., Trezzi F. and Lucchini G. 1986. Cytometry and flow cytometry of 4' , 6-diamidino-2-phenylindole (DAPI) - stained suspensions of nuclei released from fresh and fixed tissues of plants. Physiol.Plant. 68: 471-476.

Ulrich I. and Ulrich W. 1991. High-resolution flow cytometry of nuclear DNA in higher plants. Protoplasma 165: 212-215.

Valkonen J.P.T. 1994. Nuclear DNA content of the *Solanum spp.* in the series Etuberosa as determined by laser flow cytometry. Ann. Appl. Biol. 125: 589-600.

Valkonen J.P.T., Watanabe K.N. and Pehu E. 1994. Analysis of correlation between nuclear diploid *Solanum brevidens* and (di)haploid *S. tuberosum*. Jpn J. Genet. 69: 525-536.

Received July 02, 1998; accepted December 21, 1998