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THE INFLUENCE OF COHERENT IRRADIATION ON PLOIDY IN FLAX (*LINUM USITATISSIMUM* L.) CALLI

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Latvian origin flax (*Linum usitatissimum* L.) accession 'Blue di Riga' was used for calli formation. Cytometric analysis and confocal microscopy were used for detection of ploidy changes in cells of flax calli exposed to weak coherent radiation with a wavelength of 457 nm. The analysis of the optical transmittance of the calli tissue by using the optical spectroscopy was done to chooce the irradiation wavelength. The number of diploid and tetraploid nuclei in control samples of calli was 94–96 % and 4–6 %, respectively. Significant increasing of the number of tetraploid nuclei (more than 40 %) in irradiated samples was detected. We demonstrated also the dependency of the number of tetraploid nuclei on the irradiation intensity and duration of the exposure.

Key words: calli culture, flax, laser irradiation, ploidy changes.

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INTRODUCTION

Flax is one of the most globally important commercial oil seed crops, and is also a good food resource for animals (Behar et al. 2011, Štefúnová et al. 2013). Flax oil is a source of good quality essential fatty acids, which are necessary for proper functioning of cell membranes, as well as of the brain and nervous system (Harper & Jacobson 2001, Davis & Kris-Etherton 2003, Khadake et al. 2011, Ganorkar & Jain 2013). New highly adaptable varieties of flax are necessary for the stable yield and high quality production of fibre under different growing conditions (Razukas et al. 2009). Currently, the use of biotechnology methods, for example plant cells and tissue cultures, are favourable for plant breeding. After cultivation, regenerated plants usually have a high level of somaclonal variation. There is known somaclonal variation in flax that confers resistance to biotic and abiotic stress, as well as influencing plant height, number of seeds in a vessel and total number of seeds (Kaeppler et al. 2000, Grauda & Rashal 2005, Chen et al. 2009).

It is known that the cells of calli tissue are usually of marked genetic heterogeneity, which manifests itself primarily in the different ploidy levels, i.e. calli cells differ by number of

chromosomes (Chen et al. 1998).

In callus crops, it is possible to encounter cells that have a diploid set of chromosomes that are characteristic of the primary plant, and polyploid cells containing three, four, five and more sets of chromosomes. As well as polyploidy in callus culture, it is often possible to observe aneuploidy (an increase or decrease in the set of chromosomes shown by several chromosomes). The longer callus cells are cultivated, the more they differ in their ploidy. Obviously, ploidy changes are influenced by the cultivation conditions, and primarily by the substances consituting the nutrient medium. Moreover, in a number of plants, differentiated tissues are characterized by the presence of cells of different ploidy, and only those tissues that proliferate actively during ontogeny of the tissue, such as the apical meristems, cambium, etc., are always diploid. Another possible reason might be prolonged passaging of tissue and cell cultures, leading to the accumulation of genetic changes, including irregular ploidy changes (Rutkowska-Krause et al. 2003, Haoa et al. 2004). Violation of correlative links when isolating sections of plant tissue and placing them on a nutrient medium also leads to genetic instability of cells. Results such as these may also be related to the impact of phytohormones, which comprise a proportion of nutrient substances, on the genetic apparatus of the cell. The nutrient medium for the development of obligatory calli contains hormones such as as auxins and cytokinins. The mutagenic activity of these substances has been described in a number of studies. The most active mutagen substance is 2.4-dichlorophenoxyacetic acid, which comprises the majority of the medium. Cytokinins, and kinetin in particular, facilitate the polyploidization of cells. However, the causes of changes in ploidy are stated as facts, without considering the process mechanisms. It is therefore necessary to undertake research to clarify the preconditions and determine the exact parameters of the influencing factors affecting ploidy changes, and which will therefore enable for the mechanism underlying the process to be determined. Moreover, increases in ploidy without the use of harmful substances, such as colchicine (Huang & Hu 2005), which is used to increase the ploidy of clover, rye, buckwheat, etc., may be used in agriculture (Mba 2013).

In this context, studying the effect of laser irradiation on undifferentiated callus tissue is a convenient approach. It is known that weak coherent irradiation affects the development of callus cells and the outcome of cell differentiation in particular. It has been demonstrated that, when undifferentiated tissues are exposed to irradiation transmitted through a leaf of a similar plant, the differentiation process of plant tissues is stimulated by spatially modulated laser irradiation (Malov 1993, Malov et al. 1996, Budagovsky 2005).

The objective of this study was to determine the regularities of ploidy changes in flax callus cells when they are exposed to a low-intensity coherent irradiation.

MATERIALS AND METHODS

Methodology of growing flax calli

Latvian origin flax (*Linum usitatissimum* L.) accession 'Blue di Riga' was used for calli formation. The sterilization of flax seeds and acquisition of primary explants were performed according to Gomes da Cunha and Ferreira (1996). For calli formation, stem segments were cultivated for three to five weeks on a Murashige and Skoog basal medium supplemented with 1 mg l^{-1} of 2.4-D (2,4-dichlorophenoxyacetic acid) (Alfa Aesar, USA) and 1 mg l^{-1} of BAP (6-benzyl-aminopurine) (SERVA Feinbiochemica, Germany) (Kokina et al. 2012).

Cytometric analysis

After four weeks of cultivation, calli were used for ploidy detection. The ploidy level was analysed on a Partec CyFlow® space Cytometer (Partec GmbH, Germany) according to Kokina et al. (2012).

Methods of exposure to coherent irradiation

A laser beam (1) Melles Griot 85BLS 601 (Melles Griot Laser Group, USA) with a wavelength of 457 nm and a coherence length of up to 5 m, was directed through an optical filter (3) and lens (4) to obtain a parallel homogeneous light output. A mirror (5) was used to direct the low intensity (0.1–1 mW cm^{-2}) laser irradiation to the callus (6), which was placed in a Petri dish containing the nutrient medium. The upper cover of the Petri dish was removed prior to exposure. The exposure time was set by the electronic mechanical valve (2) in the 5–60 seconds range (Fig. 1). The intensity of irradiation before every exposure was controlled by an Ophir NOVA II power meter with a photodiode detector (Ophir Optronics Solutions Ltd., Israel).

Confocal microscopy

An inverted microscope Nikon ECLIPSE Ti-E (Nikon Instruments Inc., Japan) was used to measure the size of cells and nuclei and to obtain the fluorescence spectra. The wavelength of the excitation irradiation was 405 nm. A layer of the callus tissue was excised, or the callus tissue was shred, and then filled for a minute with the staining solution *CyStain UV Ploidy* (Partec, Germany), the molecules of which conjugated with the DNA cells of the callus. The callus tissue was then scanned using 405 nm irradiation. A microscope was used to fix the fluorescence of the dye molecules.

Optical spectroscopy

The spectra of the optical transmission and absorption were recorded using a SHIMADZU MCP-2200 spectrophotometer (Shimadzu Corporation, Japan). The calli tissue sections used in the experiments were 0.5 mm thick. More than 20 samples were measured. The measurement error was less than 2%.

RESULTS AND DISCUSSION

Flax calli up to 1 cm^3 in size, grown in a Petri dish, were placed in a homogeneous light field of the laser beam (Fig. 1) at a wavelength of 457 nm. The choice of irradiation wavelength was made on the basis of an analysis of the optical transmittance of the callus tissue. As shown in

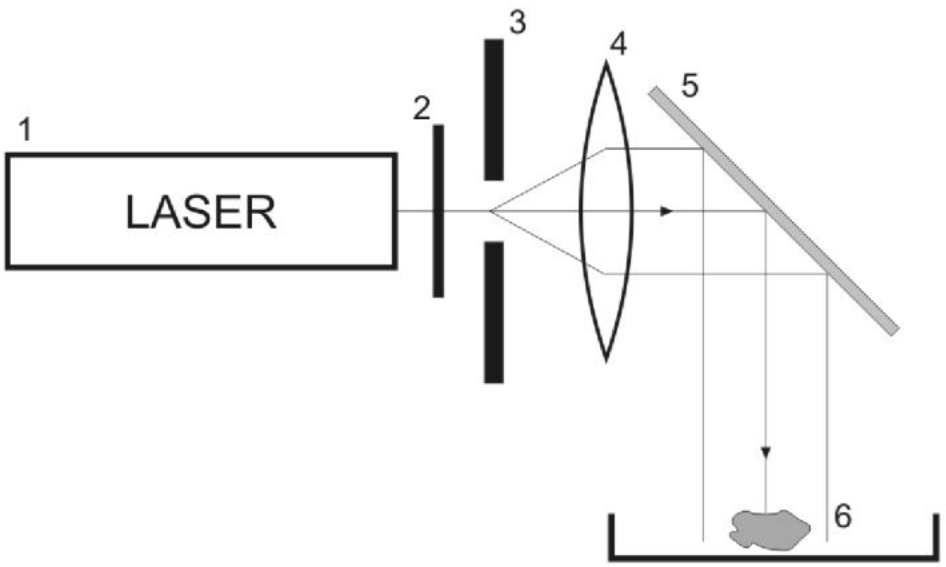

Fig.1. Optical scheme of the setup for the irradiation of callus tissue by coherent laser irradiation: 1 – laser, 2 – electromechanical valve, 3 – pinhole, 4 – lens, 5 – mirror, 6 – the callus tissue.

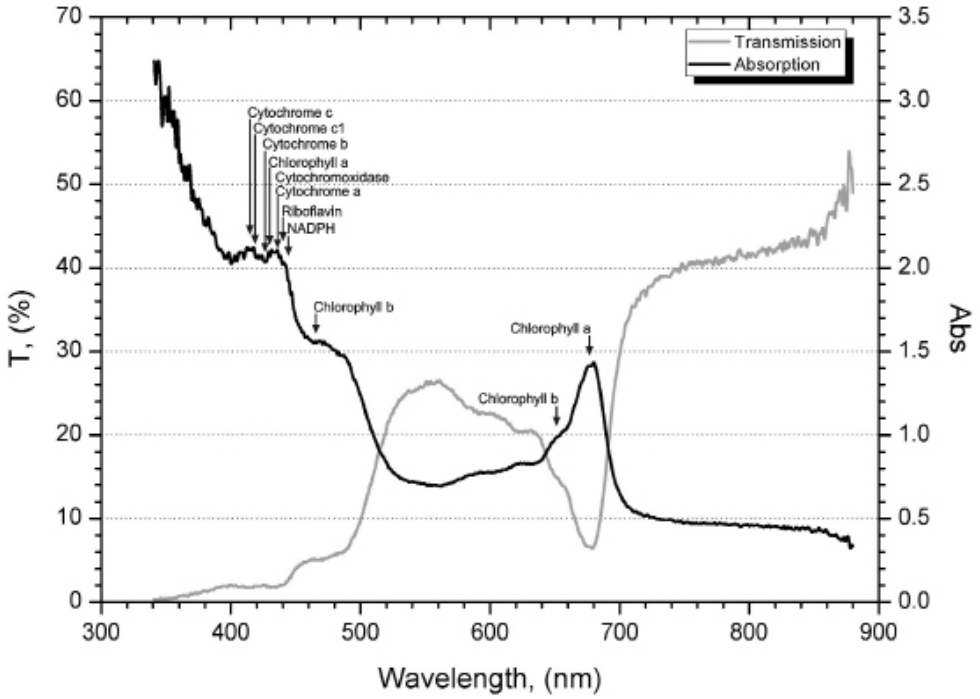

Fig.2. Transmission and absorption spectra of flax calli sections with a layer thickness of 0.5 mm.

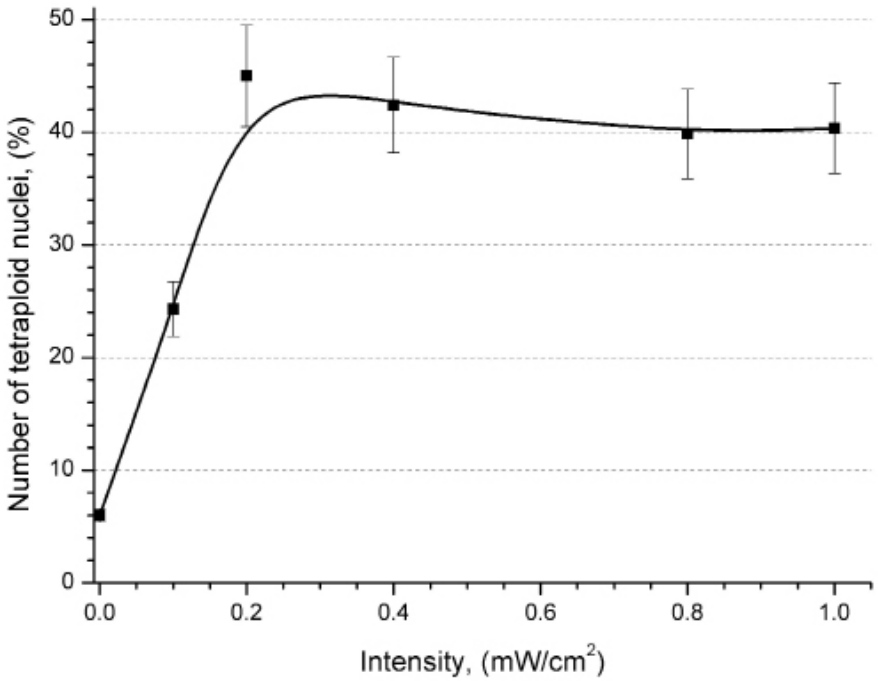

Fig.3. The influence of the number of tetraploid nuclei in a flax callus on the intensity of laser irradiation (λ = 457 nm).

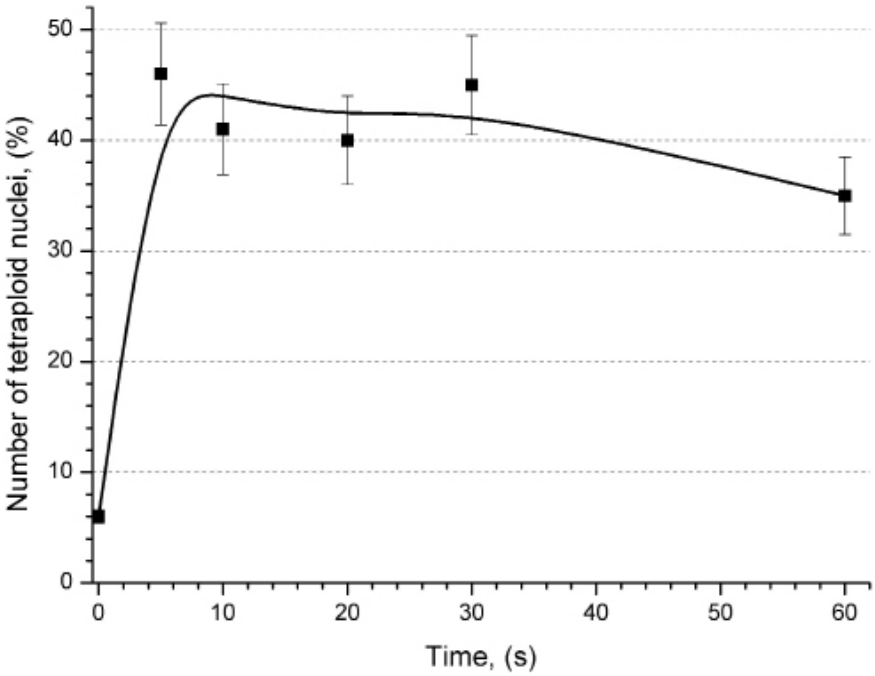

Fig.4. The dependence of the number of tetraploid nuclei in a flax calli on the duration of irradiation (λ = 457 nm).

Fig. 2, the absorption spectrum had a marked peak at 690 nm, which corresponds to the absorption of chlorophyll. A small absorption peak was observed in the wavelength range of 400–480 nm, which may be caused by riboflavin (440 nm), NADPH (445 nm), the restored forms of cytochrome a (439 nm), b (429 nm), c (415 nm), c1 (418 nm) and cytochromoxidase (439 nm). The presence of certain biological acceptors determined the choice of irradiation wavelength (Banerjee & Batschauer 2005).

After exposure in a Petri dish with the upper cover removed, calli were covered and placed in a thermostat for twenty four hours, after which a cytometric analysis of the callus tissue was performed. An analysis of the control samples demonstrated that cells with diploid (2n) set of chromosomes constituted 94–96% of the total quantity of cells. Accordingly, 4–6% of the cells had a tetraploid (4n) set of chromosomes. To achieve statistical plausibility, we studied several tens of calluses, and determined the ploidy of 10,000 nuclei in each experiment. It is known (Budagovsky 2005, Golovatskaya 2005, Głowacka et al. 2007, Hernandez et al. 2010) that the coherent irradiation of callus tissues at a low intensity of 0.2–2 mW cm^{-2} leads to a number of changes in cell development. We therefore selected an irradiation intensity interval of 0.1–1 mW cm^{-2} in order to determine the effect of irradiation on cell ploidy. Fig.3 demonstrates the dependency of the number of tetraploid nuclei on the irradiation intensity. The maximum value of the selected range of intensities corresponds to 45% of the cells at an intensity of 0.2 mW cm^{-2} and an irradiation duration of 30 seconds. A change in intensity from 0.2 to 1 mW cm^{-2} did not reduce the number of tetraploid nuclei.

The dependence of the number of tetraploid nuclei on the duration of the exposure is illustrated in Fig. 4. The sharp increase in the number of tetraploid nuclei from 6% in unexposed cells to 46% at an exposure of five seconds duration testifies to the high sensitivity of cells to laser irradiation. It is characteristic that there was a decrease in the appearance of

tetraploid cells when the duration of the exposure was increased to 60 seconds. We should note that attempts to change the ploidy of callus cells under irradiation in a closed Petri dish did not yield any results; all the nuclei, except for 4–6%, remained diploid. It is therefore clear that the organic substance of the cover changes the irradiation parameters.

As asserted by the authors (Budagovsky 2005), biological cells are able to respond to highly coherent irradiation if the size of a cell is smaller than the coherence volume. In our experiments, the coherence length of the laser was ~5 m and the size of the callus cells was 30–50 μm; the conditions created therefore satisfied the ability of cells to respond to coherent irradiation. It is considered (Banerjee & Batschauer 2005, Golovatskaya 2005) that the primary acceptors of photons are chromoproteids, which in our case were responsible for absorption in the blue region of the spectrum. Their excitation by coherent irradiation led to a change in the regulatory functions, therefore allowing the use of weak luminous flux for commutative aims. The propagation of the signal into the depth of callus tissues from cells exposed to laser irradiation at the surface confirmed the fact that only a small number of cells, approximately 10% of all cells of the callus, were exposed to irradiation. Laser irradiation, passing through a 0.5 mm layer of callus, has an absorption of 1.7 (Fig. 2), i.e. the radiation does not in practice penetrate deeper into the cells. Despite this, about 50% of all callus cells changed their ploidy when irradiated. Consequently, the irradiated cells signal the factor of laser irradiation to their neighbours. This is also supported by the following experiment. We irradiated half the area of the callus at an intensity of 0.2 mW cm^{-2} for 30 seconds. Subsequently, after twenty-four hours, we cut the callus and performed a

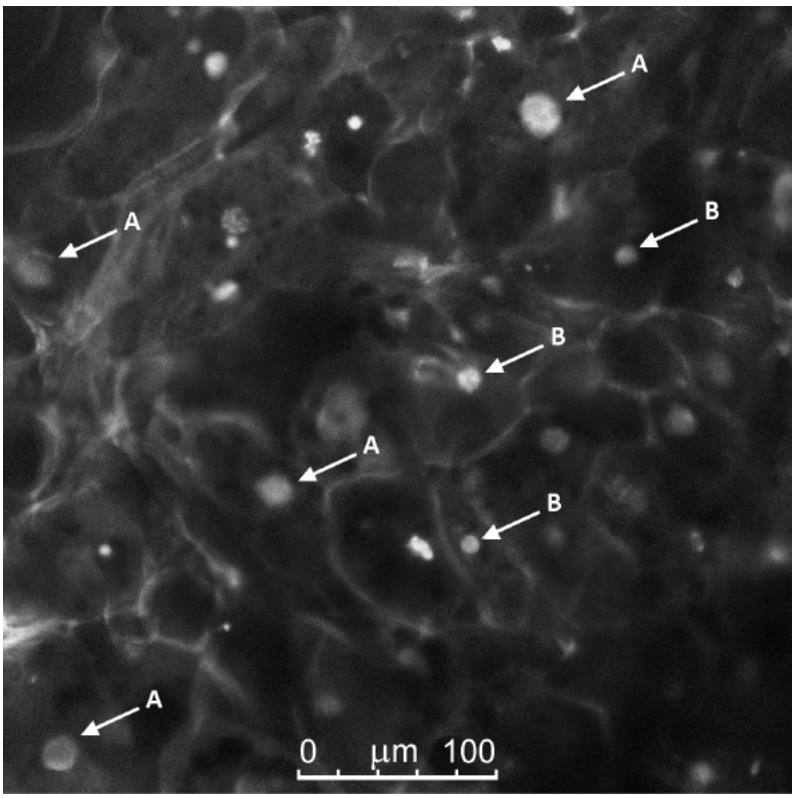

Fig.5. Nuclei of flax calli tissue after irradiation: A – tetraploid and B – diploid nuclei.

ploidy analysis of both the irradiated and non-irradiated areas. The number of 4n cells was 55% in the irraditated half, and 16% in the non-irradiated part. It is important to remember that a ploidy of 4n was present in no more than 6% of cells in the control sample. The increase in ploidy after irradiation led to an increase in the size of the nuclei. Thus the size of the nuclei of non-irradiated callus was 6–7 μm, but after irradiation it was 12–18 μm (Fig. 5). As shown in Fig. 5, large nuclei with a ploidy of 4n and small nuclei with a ploidy of 2n were observed in the irradiated part of the callus.

CONCLUSIONS

1. It was established that, when exposed to weak coherent irradiation (from 0.05 mW cm^{-2}), the callus cells of flax with a 2n ploidy increase their ploidy to 4n. The size of tetraploid nuclei increased by approximately 2–2.5 times.

2. The number of cells that increase their ploidy depends on the intensity of laser irradiation, which influences 50% of all the callus cells.

3. Coherent irradiation (on the totality of callus cells) affects about 10% of cells, mostly those that are on the surface. Ploidy change is observed in 50% of all callus cells, which is indicative of a strong commutative interaction between the callus cells as a whole (single) body.

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