PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B, Vol. 66 (2012), No. 4/5 (679/680), pp. 200–209.
DOI: 10.2478/v10046-012-0010-3

REACTION OF FLAX (*Linum usitatissimum* L.) CALLI CULTURE TO SUPPLEMENT OF MEDIUM BY CARBON NANOPARTICLES

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Frequency of callusogenesis, type and capacity of regeneration, cell ploidy, DNA methylation level, histological features, autofluorescence and photobleaching effects were studied in flax calli grown on medium supplemented with different concentrations of carbon (C) nanoparticles. The presence of carbon nanoparticles on medium decreased the percentage calli formation. Detailed histological study led to better understanding of changes in calli caused by nanoparticles. Ploidy variation in calli cells significantly depended on carbon nanoparticle concentration in the medium. Different types of calli regeneration associated with different C nanoparticles concentration in medium were observed: somatic embryogenesis (direct or indirect) or organogenesis. C nanoparticle dose-dependent DNA hypermethylation in flax calli cells were found. Increased repair ability during laser irradiation was found in calli grown on medium with carbon nanoparticles.

Key words: *flax, calli, cell ploidy, DNA methylation, somatic embryo, histology, autofluorescence, photobleaching.*

INTRODUCTION

Linum usitatissimum L. (flax/linseed) is an important crop as a source of oil and fibre in Europe and North America (Millam *et al*., 1992; Bartošová, 2005). Flax has been widely used in biotechnology studies, including plant cell, tissue and organ culture, as it has a relatively short life-cycle, high regeneration capacity and small genome size (Bennet and Smith, 1976). Flax tissue culture has been used in studies of *in vitro* morphogenesis, embryo cultures, growth regulator influence on explant culture systems, environmental and genotypic effects on regeneration, stress tolerance lines obtaining and in many other fundamental studies (McHughen and Swartz, 1984; Pretova, 1990; O'Connor *et al*., 1991; Jain and Rashid, 2001; Bonell and Lassaga, 2002; Obert *et al*., 2004, Millam *et al*., 2005).

The cultivation of tissue and cells *in vitro* causes high variation, called somaclonal variation. Somaclonal variation has been described for many plant species (Larkin and Scowcroft, 1981; Leike, 1985; Kaeppler *et al*., 2000, Rutkowska-Krause *et al*., 2003), including flax. This variation depends on the duration of cultivation and usually occurs during the callus stage (D'Amato, 1995). Somaclonal variation has been used as a source of genetic variability in breeding programmes for many crops (Rutkowska-Krause *et al*., 2003). Due to somaclonal variation, new flax genotypes have been derived with resistance to biotic and abiotic stress, plant height, a number of seeds in a vessel, a number of seeds (Mc Hughen and Swartz, 1984, Poliakov, 2000; Rutkowska-Krause *et al*., 2003) and new oil flax varieties have been produced (Rowland *et al*., 2002). Therefore, this method is useful for obtaining source material for flax breeding (Grauda *et al*., 2006). The problem in use of this method in breeding programmes is the high genotype dependence of calli regeneration capacity and unclear causes of somaclonal variation.

There are many possible mechanisms of somaclonal variation, including changes in chromosome number, chromosome breakage and rearrangement, DNA amplification, point mutations, activation of transposons, changes in DNA

of organelles and changes in DNA methylation (Grandbastien, 1998; Kalendar *et al*., 2000; Cassells and Curry, 2001). Poliploidy, aneuploidy and chromosome aberrations in somatic plant and animal cells cultivated *in vitro* have been observed (Sacristán, 1971). Ploidy variations have been reported for different plants, including both regenerated and transgenic tomato (Ellul *et al*., 2003), *in vitro* regenerated Arachis hybrids (Singsit and Ozias-Akins, 1992), somatic hybrids and *in vitro* propagated potato (Uijtewaal, 1987; Chaput *et al*., 1990), sugar cane, tobacco (Larkin and Scowcroft, 1981), rice (Chen, 1977), *Brassica* (Keller *et al*., 1975), pear (Kadota and Niimi, 2002), strawberry (Hao *et al*., 2002) and many others. The use of flow cytometry for the measurement of nuclear DNA content, which is based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence intensity of stained nuclei (Doležel, 1991), has been considered as a fast and reliable method (Doležel *et al*., 1989). Using this method, nuclear DNA amounts can be analysed with high precision and representative numbers of nuclei can be measured in a short time (Kubaláková *et al*., 1996; Doležel *et al*., 2007).

Variation in DNA methylation is an additional factor of inherited changes in tissue culture and has both mutagenic and epigenetic effect (Matthes *et al*., 2001; Li *et al*., 2002). It is one of the mechanisms of rapid modifications of genome and can control gene expression (Cullis, 2005). The cultivation of tissue cultures can induce changes in methylation on a global level and in specific sites in regenerated plants and their progeny (Philips *et al*., 1994; Lei *et al*., 2006).

Nanoparticles are defined as particles with dimensions less than 100 nm and play a central role in many natural processes, for example, they serve as nanomagnets in bacteria for reception of the earth magnetic field and have a role in transport of radionuclides in groundwater (Kan and Tomson, 1990; Matsunaga *et al*., 2000). In engineered nanoparticles, different features can be optimised and created according to the expecting result, which is why engineered nanoparticles are widely used in many materials (Colvin, 2003). Modern nanomaterials have new optical, mechanical and electrical properties, which are vastly different from their conventional-sized counterparts. New features of these new materials can lead to unpredictable outcomes when they interact with biological tissues (Wang *et al*., 2010). Very few studies have describe the biotransformation of nanoparticles in plant species, and the possible biomagnification of nanoparticles in the food chain is unknown (Rico *et al*., 2011). For this reason, investigations on effect of nanoparticles on live cells, including those of plants, are necessary.

Autofluorescence examination is considered as a standard for screening and diagnostic of neoplastic changes, and for screening of early stages of infection in plants and changes in reinitiated calli. In addition, fluorescence can be used to identify apoptotic cells (Vigneswaran *et al*., 2009; Martinez *et al*., 2010). There are many causes for autofluorescence of tissue. Some types can be found in mammalian tissue only, such as the fluorescent pigment lipofuscin, while others are specific to plants or are independent of the species (Neumann and Gabel, 2002). Cells have autofluorescence due to naturally fluorescent products within the cell. Combination of photochemical destruction of fluorophores and changes in the quantum efficiency of fluorophores cause the effect of photobleaching (Rost, 1991). In photobleaching experiments, a high intensity laser is used and fluorescence recovery after photobleaching can be observed, and furthermore, the photobleaching can afffect plant cell dynamics (Sparkes *et al*., 2011). Fluorescence recovery after photobleaching (FRAP) has received increasing attention ever since it was first introduced into cell biological research (Houtsmuller, 2005). For example, FRAP revealed the mobility of many nuclear proteins including histones (Kimura, 2005). To our knowledge there is no papers published so far on autophluorescence and photobleaching effects in flax calli cultures.

The objectives of our study was to determine the effect of different C nanoparticle concentrations on morphological parameters, type and frequency of regeneration, change of ploidy level, features of histology, degree of genome methylation alteration, autophluorescence and photobleaching in flax calli.

MATERIALS AND METHODS

Calli cultures. Latvian origin flax (*Linum usitatissimum* L.) accession 'Blue di Riga' was used for calli formation. Seeds were washed in a soap solution for 3 min, rinsed with deionised water and soaked in 0.007% potassium permanganate for 30 min, rinsed with deionised water and sterlised in a 3% sodium hypochlorite for 20 min, and then rinsed at least five times in deionised autoclave-sterilised (120 ^{o}C, 1 atm) water. Seeds for germination were placed on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% sucrose and, 0.7% agar, pH 5.8. Donor plants were grown in growth chambers at 24 ^{o}C, 2 Lx, 16/8 h (day/night) photoperiod and 80% humidity Stem segments (5 mm) from seedlings (3–5 leaves stage) were used as explants for calli formation. For calli formation, explants were cultivated for 3 to 5 weeks on the basal MS medium and medium supplemented with 1 mg/l of 2.4D (2.4-Dichlorophenoxyacetic acid) and 1 mg/l of BAP (6-Benzyl-aminopurine). For further cultivation, calli were transferred on the MS medium without nanoparticles (control) and MS medium supplemented with different C nanoparticles (carbon nanoparticles corresponded to multiwalled carbon nanotubes with the following parameters: purity more than 85wt%, diameter 5–20 nm, length ~10 μm, manufactured by Nanohub Co. Ltd, South Korea) concentrations: 10^{-3} (g/l), 2 x 10^{-3} (g/l) and 3 × 10^{-3} (g/l).

Calli cultivation on each medium treatment was repeated three times. Calli were maintained in growth chambers at 24 ^{o}C, 2 Lx, 16/8 h (day/night) photoperiod and 80% humidity.

After four weeks of cultivation all calli were placed onto regeneration medium (MS medium with 1 mg/l of BAP). Af-

ter two months of calli cultivation, regeneration zones and regeneration type were recorded.

Morphological features of calli were examined using a microscope Nikon 90i and stereomicroscope Nikon AZ 100. Images were processed by NisElement AR and NisElement BR software. For examination of specific features a laser scanning microscope Zeiss LSM 5 PASCAL was used.

Histology. Control calli, as well as calli grown on medium with different concentrations of C nanoparticles, were fixed by immersion in 10% neutral buffered formalin approximately for 48 h. Fixed calli were dehydrated in alcohol and xylene, embedded in paraffin wax, sectioned at 5 μm, stained with hematoxylin and eosin and observed with a AxioLab light microscope (Carl Zeiss, Jena), camera AxioCam MRc5. Images were processed by AxioVision Rel.4.7.1 software.

Detection of cell ploidy. The ploidy level was tested in somatic calli after four months of cultivation. For determination of ploidy, approximately 50 mg of calli tissue were placed onto a plastic Petri plate, 0.5 ml of CyStain UV Ploidy (Partec, Germany) was added, and the specimen was chopped with a sharp razor to release the nuclei from the cells. Additionally, 5 ml of CyStain UV Ploidy were add and tissues were incubated at room temperature for five minutes. Then, calli were filtered through a Partec 50 μm CellTrics disposable filter and analysed on Partec CyFlow® space Cytometer using UV excitation and measure blue emission. Each measurement was made three times and a minimum of 5 000 nuclei per sample were analysed. As a diploid control, young leaves of the laboratory-grown diploid plant from flax accession 'Blue di Riga' were used.

DNA extraction. Genomic DNA was extracted from calli cultivated on medium with and without nanoparticles. Before DNA isolation, calli were dried in silica gel for 3–4 days. DNA isolation was made using NucliSens® easyMAG™ (bioMérieux, France) extractor and the Versatile and Flexible DNA/RNA extraction kit (bioMérieux, France) following the protocol of the manufacturer.

Methylation analysis. For determination of DNA methylation in calli cells of *Linum usitatissimum* L., genes rich with CpG sites were chosen: complete sequence of 26S ribosomal RNA gene, 26S-18S ribosomal RNA intergenic spacer, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 S ribosomal RNA gene, and internal transcribed spacer 2 (Gene bank accession number EU307117.1). PCR primers and the sequencing primer were designed using PyroMark® Assay Design SW 2.0 from Qiagen (See Table 1).

Table 1

PCR PRIMER AND SEQUENCING PRIMER SEQUENCES FOR PCR AND PYROSEQUENCING

Primer	Sequence (5'-3')
Forward PCR primer	AGGAGGGGTATTGTTTTTTTTTTTGGATT
Reverse PCR primer (biotinylated)	CAACCAAACTCCCCACCTAA
Sequencing Primer	GGTATTGTTTTTTTTTTTGGATTAA

The PCR product was 246 bp

The first step for methylation analysis was sodium bisulfite conversion of unmethylated cytosines in DNA according to the EpiTect® Bisulfite protocol (Qiagen, Hilden, Germany) by using a EpiTect Bisulfite Kit® (Qiagen, Germany). The bisulfite-treated DNA was amplified by PCR technique. Amplification reaction was carried out on a Veriti Thermal Cycler GeneAmp© PCR System (Applied Biosystems, CA, USA) following the protocol of the PyroMark PCR Master Mix (Qiagen, Hilden, Germany). Two modifications of the protocol were used: with and without Q solution (Qiagen, Hilden, Germany) in the reaction mix. Each 25 μl PCR reaction without Q-solution contained 9.5 μl Milli-Q water, 12.5 μl 2 × PCR buffer, 0.5 μl 25 mM Mg^{2+} solution, 0.5 μl 10 μM mix of F and R primers (F primer was biotinylated) and 2 μl of DNA. Each 25 μl PCR reaction with Q-solution contained 4.5 μl Milli-Q water, 12.5 μl 2x PCR buffer, 0.5 μl 25 mM Mg^{2+} solution, 0.5 μl 10 μM mix of F and R primers (F primer was biotinylated), 5 μl of 5x Q-solution and 2 μl DNA. The reaction was performed using the following protocol: 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s with the final extinction at 72 °C for 10 min.

The sequence created by PyroMark® Assay Design SW was used for creation of the assay setup with PyroMark Q24 Application Software. Pyrosequencing was conducted using Pyromark Q24 and data analysis was performed using PyroMark Q24 Software (Qiagen, Hilden, Germany). The methylation level of each CpG site was calculated as a percentage using the software as the peak height of cytosine, divided by the sum of cytosine and thymine peak heights multiplied by 100. The Mann-Whitney test was used to detect significant ($P < 0.05$) differences between different calli and between calli and control.

Detection of autofluorescence and photobleaching effect of calli. The autofluorescence spectrum was measured using a confocal microscope LEICA TCS SP5. Calli were irradiated with an argon laser under 458 nm wavelength. The fluorescence spectrum was observed in the range 480 nm to 800 nm with step 3 nm. The laser star was focused till 2 μm, power was 0.3 mWt and scanning square was 24x24 μm. Autofluorescence intensity was detected in three spectral ranges: 530, 690, and 750 nm. For detection of photobleaching effect of calli, the excitation source was a 458 nm laser. Irradiation time was (i) 5 min under continuous excitation and (ii) 5 min under excitation source repeated with interval 1 minute.

RESULTS

Calli formation. Calli induction frequencies were high and varied from 65 to 98% (Table 2). Among the control and calli grown on different concentrations of C nanoparticles (10^{-3} mg/l, 2×10^{-3} mg/l and 3×10^{-3} mg/l) callusogenesis

Table 2

MEAN DIAMETER, WEIGHT AND TYPE OF REGENERATION OF FLAX (*LINUM USITATISSIMUM* L.) CALLI

Variant	Calli induction frequency,%	Number of measured calli	Calli diameter (mm) after 4 weeks cultivation	Calli weight (g) after 4 weeks cultivation	Direct embryogenesis, %	Indirect embryogenesis, %	Organogenesis, %
Control medium	98	40	8.5 ± 0.1	0.6 ± 0.1	10.0	40.0	0
Medium with $1x10^{-3}$ g/l C nanoparticles	83	40	8.5 ± 0.3	0.5 ± 0.1	0	25.0	0
Medium with $2x10^{-3}$ g/l C nanoparticles	79	40	6.6 ± 0.1	0.5 ± 0.4	0	25.0	0
Medium with $3x10^{-3}$ g/l C nanoparticles	65	40	5.0 ± 0.4	0.2 ± 0.4	0	0	10.0

frequency differed considerably: control explants placed on MS medium exhibited the highest (98%) percentage of calli formation. Explants grown on the medium with highest concentration (3×10^{-3} mg/l) of C nanoparticles had the lowest level of callusogenesis (65%). The range of callus diameter after four weeks of cultivation varied from 5.0 mm (calli on medium supplemented with 3×10^{-3} g/l C nanoparticles) to 8.5 mm (control). A similar tendency was observed in mean weight of calli, which varied from 0.2 g (calli on medium supplemented with 3×10^{-3} g/l C nanoparticles) to 0.6 g (control).

Cross sections of calli developed on MS medium with and without nanoparticles after six weeks of cultivation are shown in Figure 1. Somatic embryos with concentric tracheary elements were easily discernible in control calli Somatic embryos were visible in calli developed on MS medium with 10^{-3} g/l C nanoparticles,. Aggregates of vacuolated cells and embryo-like structures were only distinguished in calli developed on MS medium with 2×10^{-3} g/l C nanoparticles. Calli on MS medium with 3×10^{-3} g/l C nanoparticles were composed of large cells with dense cytoplasm, which formed vast and disorganised cell aggregates. Embryo-like structures were not visible; however on the outer side of calli a tuber-like structure was observed. Reticular-like network in intracellular spaces of calli tissue developed on MS medium with 10^{-3} g/l and 2×10^{-3} g/l C nanoparticles (Fig. 2).

Ploidy level in calli culture. Flax calli grown both on medium with and without C nanoparticles were tested by flow cytometry (Fig. 3). In the control 90% of calli were diploid. In calli with single and double concentration of nanoparticles, the proportion of diploid cells decreased by 25%, and by 10% in calli grown on triple concentration of nanoparticles. Tetraploid cells were found in all examined groups of calli, and their proportional occurrence varied from 10% in the control to 75% in calli grown on medium with 10^{-3} mg/l nanoparticles. In calli cells grown on MS with 2×10^{-3} mg/l and 3×10^{-3} mg/l C nanoparticles, 4n calli were detected in 60% of cases. Mixoploid calli (2n+4n) also were observed in calli grown on medium with double and triple concentrations of nanoparticles at relative frequencies of 15% and 30%, respectively.

Methylation analysis. Analysis of methylation level in the six CpG sites in the 26S ribosomal RNA gene, 26S-18S ribosomal RNA intergenic spacer, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8 S ribosomal RNA gene, and internal transcribed spacer 2 in calli revealed significant differences between control calli and calli grown on medium supplemented with C nanoparticles. The results of methylation analysis are presented in Figure 4. The lowest level of methylation was detected in control calli, for which the methylation level varied from 36% in position 1 to 76% in position 5. The highest level of methylation in all sites investigated was detected in calli grown on medium with 3×10^{-3} mg/l C nanoparticles: range 61% in position 1 to 100% in position 5. Significant differences between control and calli grown on medium with different concentrations of nanoparticles were found. In calli grown on medium with nanoparticles the methylation level was significantly higher than in control calli ($P < 0.05$). Increased concentration of nanoparticles caused increased methylation level in all CpG sites, excepting in position 4 for which the methylation level in control calli and in calli grown on medium with 10^{-3} mg/l C nanoparticles was the same.

Autofluorescence and photobleaching effect. Optical spectrums of flax calli in the control group and in calli grown on medium with 3×10^{-3} mg/l C nanoparticles are presented in Figure 5. Differences between groups were detected in peaks 530 nm and 750 nm, which were degraded within 2–5% in the case of presence of C nanoparticles in medium.

Intensity of photobleaching during continuous laser irradiation 458 nm of calli grown on medium with 3×10^{-3} mg/l C nanoparticles and for the control calli group during continuous laser irradiation 458 nm is presented in Figure 6. Calli demonstrated an exponential decrease of fluorescence intensity during irradiation, but control calli differed in fluorescence amplitude.

DISCUSSION

In our experiment the earlier elaborated modification of the calli induction and cultivation method (Grauda *et al*., 2006) resulted in high calli induction frequencies in all variants: range 65–98% (Table 2). Morphological and histological analysis of induced flax calli showed that there were differences in development of calli structures, such as somatic embryos, tracheary elements and reticular network, caused

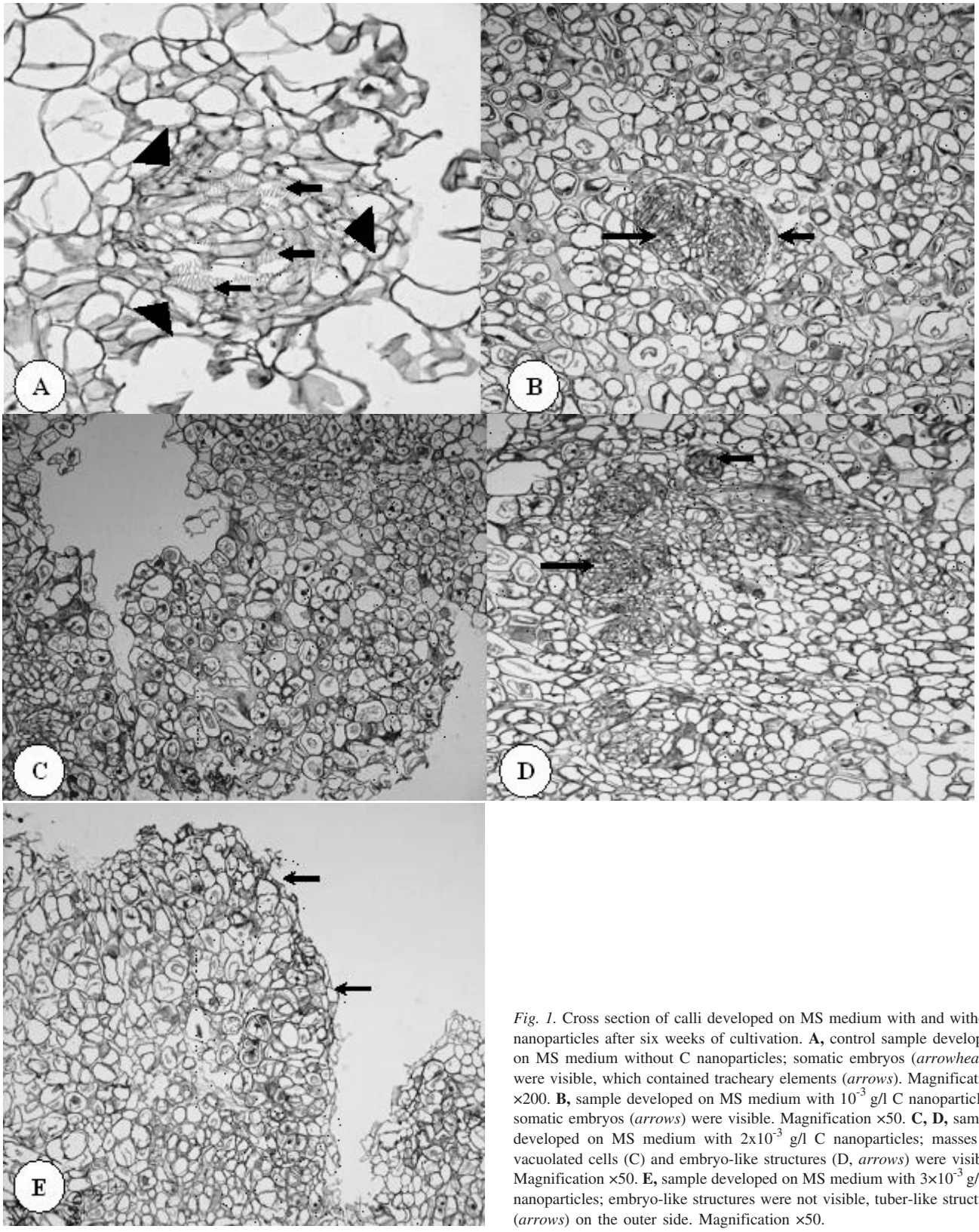

Fig. 1. Cross section of calli developed on MS medium with and without nanoparticles after six weeks of cultivation. **A,** control sample developed on MS medium without C nanoparticles; somatic embryos (*arrowheads*) were visible, which contained tracheary elements (*arrows*). Magnification ×200. **B,** sample developed on MS medium with 10^{-3} g/l C nanoparticles; somatic embryos (*arrows*) were visible. Magnification ×50. **C, D,** sample developed on MS medium with $2x10^{-3}$ g/l C nanoparticles; masses of vacuolated cells (C) and embryo-like structures (D, *arrows*) were visible. Magnification ×50. **E,** sample developed on MS medium with $3×10^{-3}$ g/l C nanoparticles; embryo-like structures were not visible, tuber-like structure (*arrows*) on the outer side. Magnification ×50.

by different concentrations of C nanoparticles in the cultivation medium. Several authors have shown that a reticular-like network mediates intercellular contacts among cells, and that a dense reticular network filling large intercellular "caves" forms during somatic embryogenesis (Ovečka and Bobak, 1999; Popielarska *et al*., 2006). The formation of the extracellular matrix surface network can be associated with a stress response in explants imposed by specific *in vitro* conditions (Bobįk *et al*., 2004). We suppose that addition of C nanoparticles to cultivation medium influences intercellular interaction which, in turn, modifies differentiation of tissues and organs in calli cultures.

Calli grown both on medium with and without C nanoparticles were tested by flow cytometry to determine possible

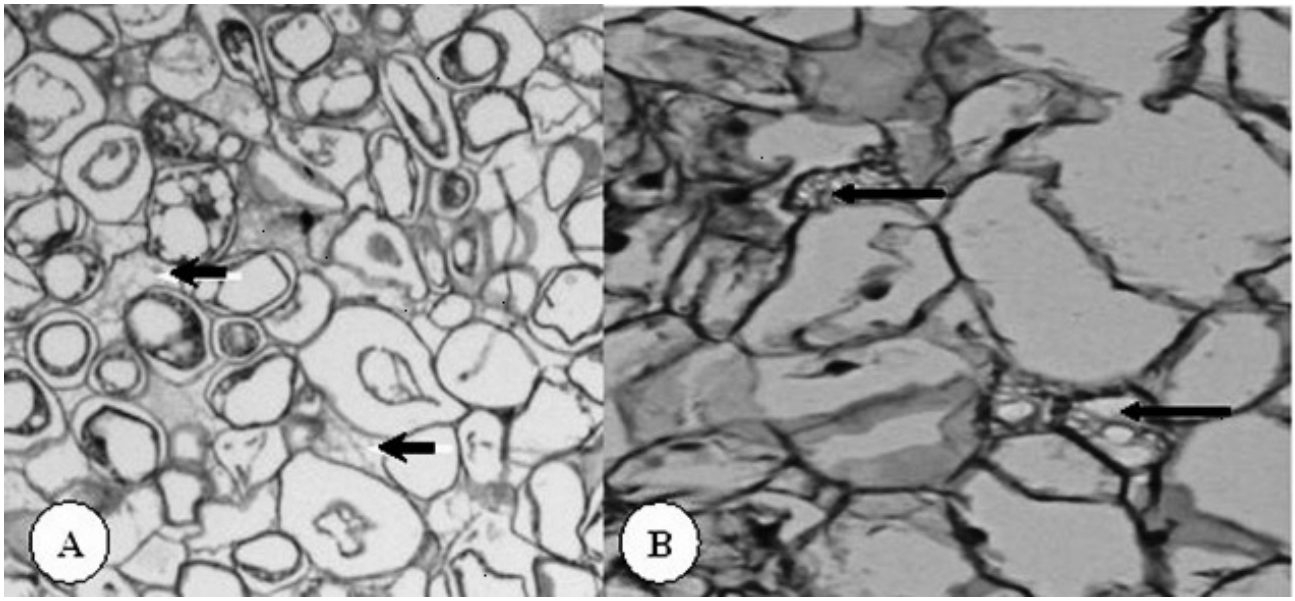

Fig. 2. Intercellular spaces within callus tissue. Reticular structures (*arrows*) in calli developed on MS medium with 10^{-3} g/l C nanoparticles C nanoparticles (A) and in calli developed on MS medium with 2×10^{-3} g/l C nanoparticles (B).

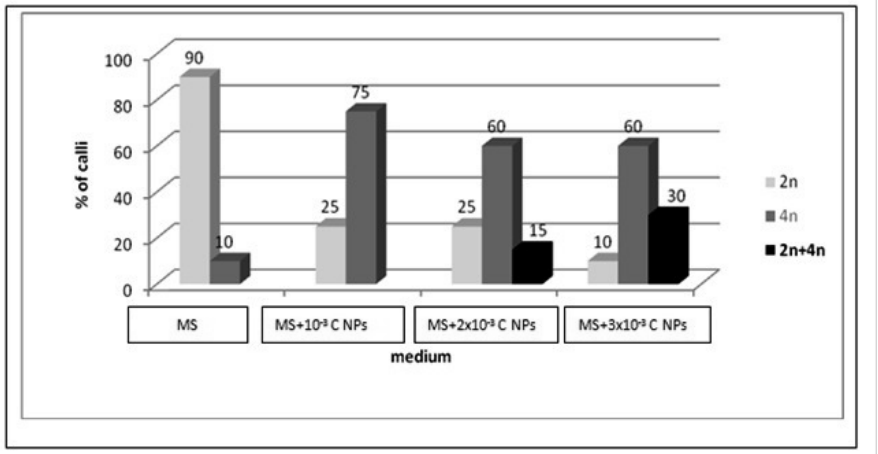

Fig. 3. Ploidy level after four months of calli cultivation.

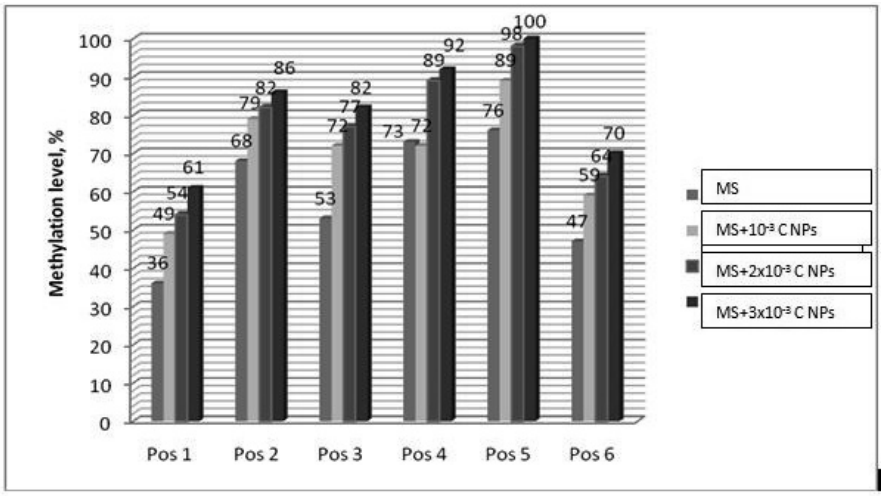

Fig. 4. Methylation levels (%) of six CpG sites in the 26S ribosomal RNA gene (Pos1), 26S-18S ribosomal RNA intergenic spacer (Pos2), 18S ribosomal RNA gene (Pos3), internal transcribed spacer 1(Pos4), 5.8 S ribosomal RNA gene (Pos5), and internal transcribed spacer 2 (Pos6) in flax calli.

cause of differences in regeneration capacity of calli. Changes of ploidy are considered as one of the results of somaclonal changes (Millam, 2005). Many studies that nanoparticles can cause a decrease of mitotic index, cell activity, and chromosomal stickiness in metaphase and anaphase stages (Li *et al*., 2003; Kumari *et al*., 2009; Kumari *et al*., 2011)**.** Our study showed that the ploidy variation in calli significantly depended from C nanoparticle concentration in medium. With increasing concentration of C nanoparticles, the numbers of cells containing a mixture

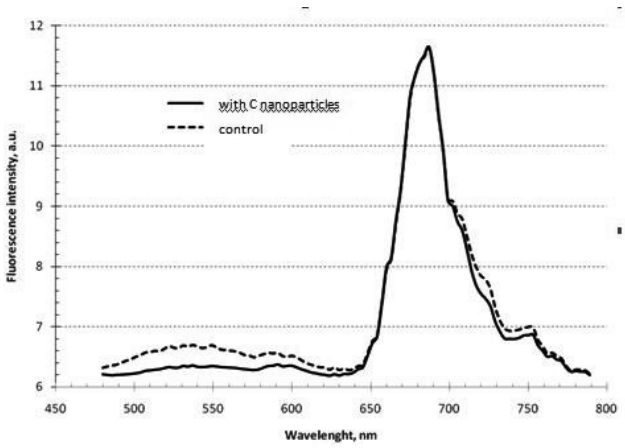

Fig. 5. Autofluorescence spectrum of calli grown on medium with 3×10^{-3} g/l C nanoparticles and of calli grown on medium without nanoparticles (control) (excitation source 458 nm).

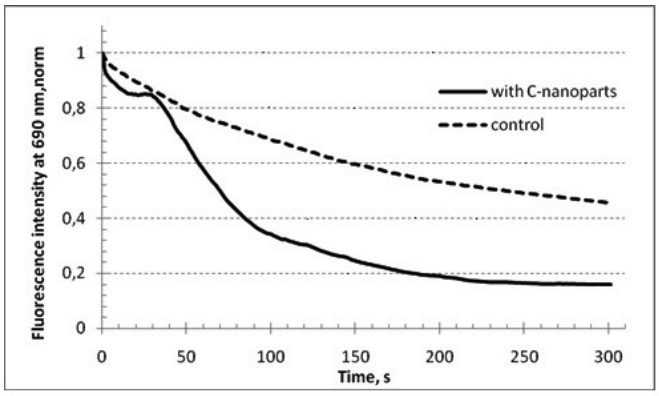

Fig. 6. Intensity of photobleaching of calli grown on medium with 3×10^{-3} g/l C nanoparticles and of calli grown on medium without nanoparticles (control) under continuous excitation source 458 nm.

of ploidy levels increased. We observed two *in vitro* regeneration systems for flax: somatic embryogenesis (direct or indirect) and organogenesis. The results showed that calli ploidy was related with the frequency and the type of regeneration. Regeneration occurred only in diploid calli. The observed yellowish-white friable calli were nonembryogenic, while embryogenic callus was green, round and compact.

A lack of direct embryogenesis was observed in all calli grown on medium with C nanoparticles. Regeneration ability was the highest in control calli: 50% embryogenesis and capacity was reached 50%, including 10% direct embryogenesis of calli, which produced more embryos per explant. Calli grown on medium with 10^{-3} mg/l and 2×10^{-3} mg/l of C nanoparticles were embryogenic as well, but the relative frequency of embryogenesis was twice lower than in the control. Regeneration through rhyzogenesis (percentage of regeneration was 10%) was observed only in calli grown on triple concentration of nanoparticles (Table 2). Calli growing on medium without C nanoparticles formed embryos with better quality: higher number of normal embryos in globular stages were detected (Salaj *et al*., 2005; Vidoz *et al*., 2006; Chakravarthi *et al*., 2010). Calli grown on medium with C nanoparticles, formed numerous abnormal embryos with different variations like embryous fusion. There are no previous studies on the influence of nanoparticles on ploidy level of calli culture. However, optimal culture conditions, medium, type and level of plant growth regulators to induce somatic embryogenesis in different species, have been widely discussed (Komamine *et al*., 1992; Jimenez, 2001; Jimenez *et al*., 2005; Park *et al*., 2011). Obtaining all the developmental stages similar to those of zygotic embryous are exclusive (Hofmann *et al*., 2004; Korbes and Droste, 2005; Santos *et al*., 2006; Chitra Devi and Narmathabai, 2011).

Our data showed C nanoparticle concentration-dependent hypermethylation in flax calli cells. DNA methylation, similar to other epigenetic mechanisms, can be considered as a key factor in the regulation of cell growth and differentiation, and thereby, the nuclear reprogramming necessary for dedifferentiation and calli formation (Berdasco *et al*., 2008). Hypomethylation of different organisms (for example, white clover *Trifolium repens,* industrial hemp *Cannabis sativa*, polar bear *Ursus maritima* etc.) in different cells and tissues due to chemicals such as benzopyrene, mercury, cooper, zinc, cadmium, and 5-azacytidine have been observed (Shugart, 1990; Pilsner *et al*., 2009; 2010; Vandegehuchte *et al*., 2010; Vandegehuchte and Janssen, 2011). Recent evidences have shown significant increase of locus- specific DNA methylation in goldfish (*Carassius auratus*) in presence of cooper, zinc, lead, cadmium and mixtures of these metals and in three-spine stickleback (*Gasterosteus aculeatus*) in the presence of 17β-oestradiol, HBCD and 5-AdC (Zhou *et al*., 2001; Aniagu *et al*., 2008). Regarding calli cultures, methylation rates in genomic DNA from embryogenic callus have been observed to be significantly lower in comparison with those in non-embryogenic calli for different plants (Chakrabarty *et al*., 2003). Our data also demonstrated decreased somatic embryogenesis in calli (indicated by hypermethylation) grown on medium with nanoparticles, compared to that in the control (Table 2). Variation in DNA methylation reflects gene expression during development and differentiation (Finnegan *et al*., 2000).

Significant differences in photobleaching after laser irradiation 458 nm repeated with interval 1 min were found between control and calli grown on C nanoparticles (Fig. 7). Fluorescence intensity exponential decreased during irradiation. However, in the control group, after 1 min of relaxation between irradiations, autofluorescence intensity returned to close to the initial level (lower only by 2–3% only). In calli grown on the medium with C nanoparticles autofluorescence after relaxation had returned to only 16–17% of the initial level. Thus, C nanoparticles decreased the effect of photobleaching of calli. Untreated calli were found to have decreased repair ability during laser irradiation, but it is possible that the laser pulses caused more severe damage to calli grown on medium with C nanoparticles. It is known that changes in autofluorescent pattern in

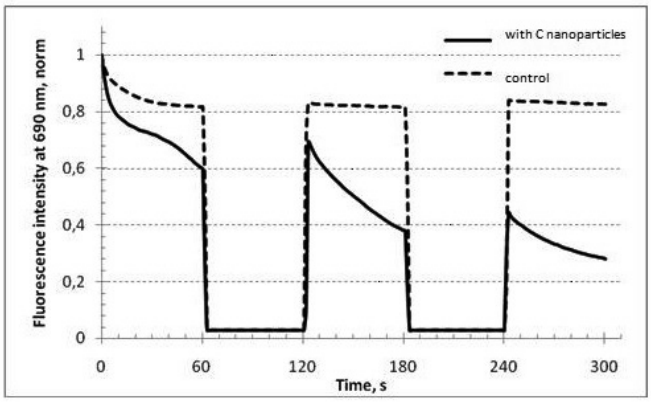

Fig. 7. Intensity of photobleaching of calli grown on medium with 3×10^{-3} g/l C nanoparticles and of calli grown on medium without nanoparticles (control). Excitation source 458 nm repeated with interval 1 min.

plants occurs in early stages of infection, in calli reinitiated from cell suspension cultures, and in intact secretory cell of plants (Kwa *et al*., 1997; McEwan, 2000; Roshchina, 2003). It is possible that the autofluorescence changes found in our study were related to peroxidation of fluorescing products under C nanoparticle-nduced stress conditions. Our data suggested that calli autofluorescence could be a sensitive parameter for the analysis of nanoparticle-cell interaction and that spectral characteristics of calli cells measured *in vitro* could be a significant tool for identification of nanoparticles and for diagnostics of cellular damage. However, more detailed studies are necessary to determine the precise mechanisms of the observed effects.

This is the first study to report on the response of flax calli culture to medium supplemented with carbon nanoparticles. The results showed considerable differences in callusogenesis frequency between the treatments. Somatic embryos with concentric tracheary elements were only detected in calli grown on medium without C nanoparticles. Reticular-like network in intracellular spaces were typical for callus tissue developed on MS medium with 10^{-3} g/l and 2×10^{-3} g/l carbon nanoparticles. Ploidy variation in calli significantly depended on the carbon nanoparticle concentration in medium. Two *in vitro* regeneration systems were observed for flax calli (direct and indirect somatic embryogenesis and organogenesis), although only diploid calli were capable of regeneration. The level of DNA methylation in calli grown on medium with C nanoparticles was significantly higher than in control calli. Somatic embryogenesis was significantly lower in calli grown on medium with C nanoparticles compared to calli on control medium, estimated by hypermethylation in calli cell DNA. Decreasing effect of photobleaching was observed in calli grown on medium supplemented with carbon nanoparticles. To clarify mechanisms of interactions between cells and nanoparticles *in vitro* it is necessary to conduct a more detailed study on various plants with different nanoparticles in various doses. It is of interest to investigate this phenomenon in more detail and to determine whether nanoparticles could be used to increase somaclonal variation. The study was a good example of interdisciplinary research where bioscience, biotechnology and nanoscience can be combined successfully.

ACKNOWLEDGEMENT

This study is supported by the Project of the European Regional Development Fund No. 2010/0321/2DP/2.1.1.1.0/10/APIA/VIAA/144.

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Received 23 March 2012

LINU (*Linum usitatissimum* L.) KALLUSU KULTŪRU REAKCIJA UZ BAROTNES PAPILDINĀŠANU AR OGLEKĻA NANODAĻIŅĀM

Linu kallusu kultūrās uz barotnēm, kuras tika papildinātās ar oglekļa (C) nanodaļiņām, tika pētīta kallusu veidošanas frekvence un spēja reģenerēties, kallusu histoloģiskās īpatnības, hromosomu skaits šūnās, DNS metilācijas pakāpe, autofluorescence un fotoizbālēšanas efekts. Nanodaļiņu klātbūtnē kallusu veidošanas frekvence pazeminājās. Hromosomu skaits kallusu šūnās variēja atkarībā no oglekļa nanodaļiņu koncentrācijas barotnē. Nanodaļiņu klātbūtne ietekmēja kallusu reģenerācijas tipu: tiešā vai netiešā embrioģenēze, vai organoģenēze. Kallusu šūnās novērota DNS hipermetilācija atkarībā no nanodaļiņu koncentrācijas. Kallusiem, kuri audzēti uz barotnes ar nanodaļiņām, novērotas šūnu atjaunošanās spēju pieaugums pēc apstarošanas ar lāzera starojumu.