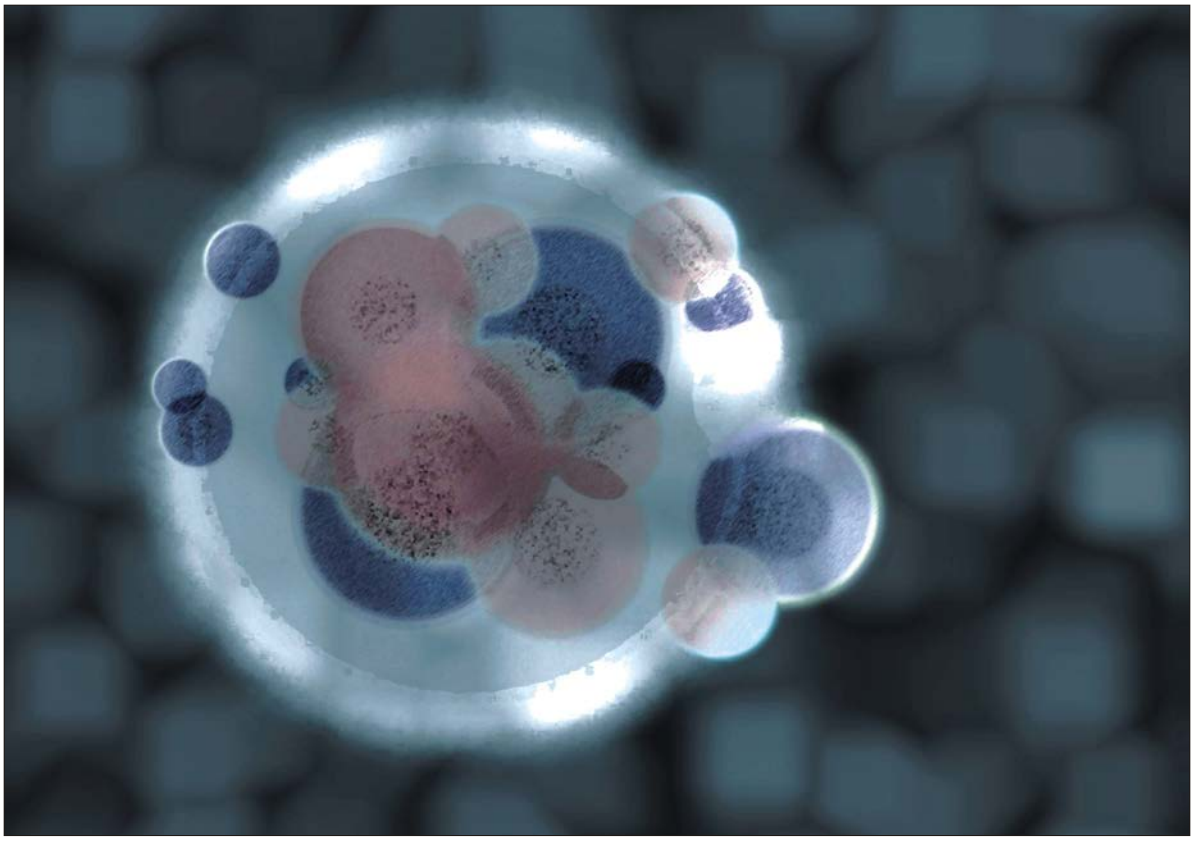

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Analysis of Cytological Activities in Sugar Beet Calluses

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Abstract: The present study focus on the results of cytological studies of embryogenic (E) and Non-Embryogenic (NE) calluses as the main tissues for cytological studies and the cells of primary root meristem derived from seedling or *in vitro* cloned plant, as reference tissues. The cellular parameters, such as Mitotic Index (MI), cell-doubling time (Cdt), cell and nuclear area and DNA C value of the different tissues, which may be involved with cell activity, have quantitatively been compared. Embryogenic callus of sugar beet, containing meristematic centers unlike the non-embryogenic one, the cytological behavior of their cells was very close to typical meristematic tissue i.e., primary root of seedling. In non-embryogenic cells, increased in the rate of cytoplasm/nuclear (C/N) area, endoreduplication and reduction of mitotic index followed by increasing of cell doubling times were observed.

Key words: *Beta vulgaris*, cell doubling time, cell cycle, embryogenic callus, image analysis

INTRODUCTION

Understanding of the cytological behavior of different tissues has a key role for the controlling of *in vitro* behavior of explants, particularly callus as a plant material for genetic manipulation of some recalcitrant crops such as sugar beet. In spite of some progresses, which have recently been achieved from production of whole plant through sugar beet callus (Tsai and Saunders, 1999; Ehsani-Moghaddam and Taha, 2005) or use of it for transformation (D'Halluin *et al*., 1992) more studies on the behavior of the callus cells are still needed to overcome obstacles which are against the use of it for direct transformation of sugar beet (Krens *et al*., 1996).

In very few reports that have so far been published about the cytological behavior of sugar beet callus, some specific characters have been discussed in two abnormal calluses i.e., habituated organogenic and non-organogenic (Crèvecur *et al*., 1992; Kevers *et al*., 1999a). Such studies, particularly on normal callus could illuminate the existence of any relations between regenerative capacity of the callus and cytological parameters of their cells, which may be used for better understanding of the environmental requirements of the cell or anticipation of callus totipotency. Present research is in fact the second part of an investigation on the cytology of embryogenic and non-embryogenic calluses of sugar beet. In the first part, which was entirely focused on ultrastructural of the cells, various organelles of the cells were qualitatively compared with special emphasis on the cytoskeleton (Ehsani-Moghaddam *et al*., 2000; Ehsani-Moghaddam and Taha, 2005). In this study, those parameters that may be dealing with nuclear behavior have quantitatively been compared and discussed in the same explants as well as two completely different tissues i.e., the primary root of seedling (as a standard explant for cytological studies) and the primary root of *in vitro* cloned plants (as an organogenic tissue).

MATERIALS AND METHODS

Plant material: The diploid sugar beet, line 7233 was used for all experiments. Our studies were performed on the cells of somatic embryogenic (E) and Non-Embryogenic (NE) calluses as the main tissues for studies and the cells of primary root meristem of seedling (PRS) and *in vitro* cloned plant (PRC), as references tissues.

The E and NE calluses (Fig. 1) were produced according to the previous report (Ehsani-Moghaddam and Taha, 2005). Briefly, seeds were sterilized according to Krens *et al*. (1990) and were cultured on half strength MS medium (Murashige and Skoog, 1962) containing 3 mg L^{-1} TIBA, 3% sucrose and 0.8% agar. Leaves of seedlings were cultured on MS medium supplemented with 1 mg L^{-1}

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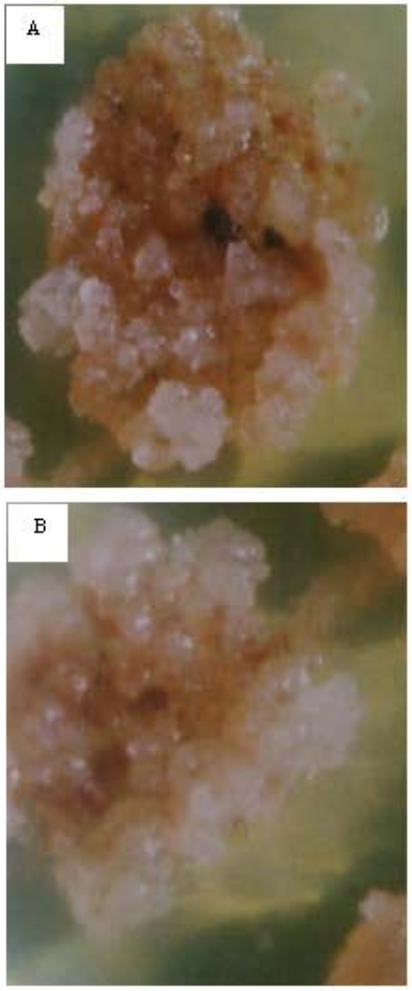

Fig. 1: (A) Embryogenic callus and (B) non-embryogenic callus from leaf disc culture of sugar beet on MS medium containing 1 mg L^{-1} BAP in the dark at 25±1°C

BAP, 3% sucrose and 0.35% phytagel in the dark at 25°C. After two months, light color callus was transferred onto embryo induction medium, i.e., MS medium containing 3% sucrose, 50 mM proline, 10 mg L^{-1} boric acid, 1 mg L^{-1} BAP, 0.5 mg L^{-1} TIBA and 0.35% phytagel. The cultures were maintained onto the medium under low light (16 h) for one month. Those cell colonies containing meristematic centers derived from the E callus were subcultured onto the fresh embryo induction medium. The cell suspension of the E callus was prepared using 2-3 g of the callus into 30 mL of the embryogenic medium, without growth regulators and phytagel. The cultures were maintained in the dark and shaken at 120 rpm. One week later, the cytological studies of the E cells were carried out before somatic embryo formation.

Alternatively, the leaves of *in vitro* seedlings were also used for the NE callus production. They were cultured on the same medium, which was used for the E production (without TIBA and proline) under the same conditions. The callus was subcultured for five times with one-month interval on the same medium and conditions. Similarly, the cell suspension was prepared from 2-3 g of the last subculture of callus into 30 mL MS liquid medium containing 3% sucrose and shaken at 120 rpm. Sampling of the cells for cytological studies was done after one week. In order to ensure that the NE cells have truly lost totipotency and their culture did not contain the E cells, the suspension of the NE cells of the same culture, which were used for sampling were subcultured onto embryogenic medium and observed under the embryogenesis conditions.

For the PRS production, the seeds were washed with tap water and cultured between two layers of clothes strip at room temperature. The primary roots of seedlings with length of 30 mm were harvested and used for studies.

The same method, which mentioned for the NE seedling preparation, was used for the PRC production. The young *in vitro* seedlings were harvested and their shoot apices were used for propagation. Multiplication and root formation of plants were accomplished through transfer of the shoot apices to the MS medium containing 0.1 mg L^{-1} IBA, 0.5 mg L^{-1} GA_3 and 1 mg L^{-1} BAP supplemented with 3% sucrose and 0.35% phytagel. After another month, the cloned plants with about 30 mm root length were carefully isolated from medium and their roots were used for cytological studies.

Slide preparation: Slides of all explants were prepared from squashed (for cell cycling studies and DNA measurements) and non-squashed cells (for cell/nuclei area measurement) using following methods.

The procedure included fixation of explants in 3:1 (v/v) absolute ethyl alcohol and glacial acetic acid for at least 24 h, washing in distilled water, hydrolysis in 1N HCl for 10 min at 60°C, washing in tap water and staining in Feulgen solution (Francis, 1981) at room temperature for 2 h. Permanent slides were prepared from stained tissues as follows: the cytoplasm of stained cells were rinsed twice in SO_2 water for 10 min, hydrolysis of specimens using different ethyl alcohol concentrations (10-100%) with 10 min interval, cleared in xylene for 10 min and eventually the specimens were covered with cover slips using one drop of DPX. Staining of cytoplasm for cell area measurement, was done on non-squashed samples using 0.2% fast green for 10 min before cleaning of the slides with xylene.

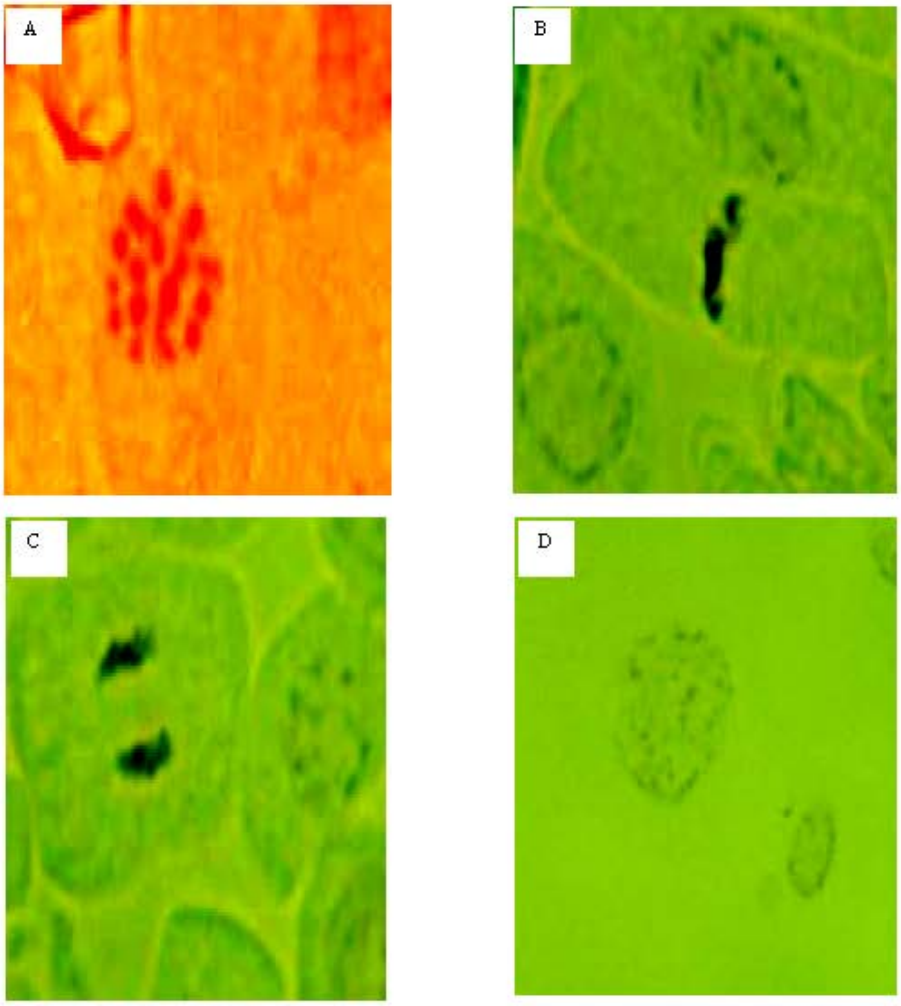

Fig. 2: Different cell phases of the primary root meristem of sugar beet: (A) prophase, (B) metaphase, (C) anaphase and (D) interphase

Cytometric parameters: The mitotic index (MI) i.e., the percentage of cells in a population undergoing mitosis, was estimated from five non-permanent slides of each sample, in a series of transects across the width of the coverslip by scoring 1000 squashed cells, according to the following formula:

$$MI = (\text{Mitotic cells}) \times 100 / \text{Total cells} \quad (1)$$

Cell doubling time (Cdt) and mitosis time (Mt): The duration of the cell cycle is the time interval between the formation of a cell and the subsequent division of that cell. Estimation of the Cdt for different tissues was based on nuclei arrested in metaphase, using Clowes (1961) method, which has been described for root meristem. Cell suspension of the E and NE calluses were treated with 0.0125% colchicine and shaken with 120 rpm for 6 h. Every hour, about 5 mL cell suspension were washed with tap water for three times and used for slide preparation. The percentage of cells in prophase, metaphase, anaphase and telophase (Fig. 2) in squashed thin layer of the cells, were counted in each sampling time, according to what has been mentioned before for MI estimation. For the PRS and PRC, they were immersed into 0.025% (w/v) colchicine solution for 6 h at room temperature. Every hour, 10 roots from each sample were washed with tap water for three times and their meristems were used for non-permanent slide preparation. In this method a quadratic equation including linear regression between sampling time and percentage of arrested cells in metaphase:

$$Y = mX + b \quad (2)$$

together with the following equations, are used for calculation of Cdt:

$$m = e^{\lambda S2} / 1-L \quad (3)$$

$$\%(metaph.+anaph.+teloph.) = e^{\lambda S2}-1/1-L \quad (4)$$

$$Cdt = \ln (2-L)/\lambda \quad (5)$$

where, L is the fraction of the cells which do not divide, s_2 is a constant marking the boundary between prophase and metaphase in untreated roots or in roots treated with colchicine, λ is also a constant, which may be calculated from the rate of accumulation of metaphases.

The time required for mitosis has been estimated from following formula (Webster and McLeod, 1980):

$$Mt = Cdt \times MI/\ln 2 \quad (6)$$

For measurement of DNA content and cell/nuclei area, at least five permanent slides were used. Using a light microscopy (Zeiss), which was connected to a Sony video camera, the images from single cells were captured and transferred to a host computer for image processing and data analysis. The system was supported by two special softwares. First, DNA VIDAS Program (from Germany) for calculation of C value was used (Taha and Francis, 1990). The 2C value was determined by calibrating with the Integrated Optical Density (IOD) of reference nuclei (telophase nuclei from sugar beet root meristem). A total of 500 interphase nuclei from each sample were scored. The percentage of nuclei in different cell cycle phases were estimated according to the C values of the cells, i.e., 0-2.2C (G_1), 2.2-3.6C (S-phase), 3.6-4.8C (G_2) and >4.8C was calculated for ploidy histogram (Evans and Van't Hof, 1974). The second program, i.e., VIDAS (Germany) has been designed for cell/nuclear area measurement (Taha and Francis, 1990). The measurement of cell/nuclei area was fulfilled from 150 cells or nuclei. Eventually, all graphs were transferred to Excel program for multiple comparisons.

RESULTS AND DISCUSSION

According to the Table 1, MI in the cells of primary root of seedling as well as the *in vitro* cloned root was higher than that of in the E and NE cells. In contrast, Cdt in the E and NE cells was higher than the cells of roots. Comparing the E and NE cells, the E cells with higher MI need a shorter time to complete cell cycle. It also seems that, with the exception of the PRC cells, there is no significant difference between mitotic time of various tissues.

Figure 3 shows the distribution of DNA (C-value) in the interphase cells of different tissues, which its information has been shown in Table 2.

Table 1: Cell parameters in various tissues of sugar beet: (PRS) Primary Root of Seedling; (PRC) primary root of *in vitro* cloned plant; (E) and (NE) Embryogenic and Non-Embryogenic calluses, respectively

	Cdt(h)			Mt(h)	
Tissue	L = 0	L = 0.25	MI (%)	L = 0	L = 0.25
PRS	24.5	36.7	4.10±1.83	1.45	2.17
PRC	121.0	130.0	3.52±1.91	6.14	6.60
E	69.0	75.0	1.10±1.01	1.09	1.20
NE	184.0	204.0	0.93±0.73	2.47	2.74

Cdt: Cell doubling time, MI: Mitotic Index, Mt: Mitosis time, L: The fraction of the cells, which do not divide

Table 2: The percentage of nuclei in interphase and polyploid in different tissues of sugar beet: (PRS) Primary Root of Seedling; (PRC) primary root of *in vitro* cloned plant; (E) and (NE) Embryogenic and Non-Embryogenic calli, respectively

	Cell cycle phase (%)			
Cell line	G_1	S	G_2	Polyploidy (%)
PRS	52.70	28.00	7.4	11.00
PRC	51.90	26.70	12.4	12.00
E	35.00	25.00	22.6	17.40
NE	37.14	5.19	15.2	42.50

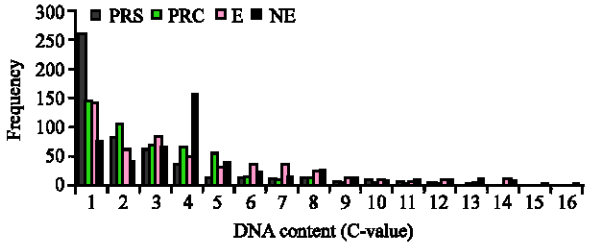

Fig. 3: Distribution of DNA (C-value) of interphase cells in various tissues of sugar beet: PRS: Primary Root of Seedling, PRC: Primary Root of *in vitro* Clone, E and NE: Embryogenic and Non-Embryogenic cells, respectively

Based on Fig. 5 and Table 2, different tissues consist of different cell populations during interphase. The PRS and NE callus were recognized as tissues with the highest (28%) and lowest (5.19%) population of S phase, respectively. In the E cells, this amount was 4.82 fold more than the NE ones. In all tissues, two categories of cell cycling populations were distinguished: the first one had 2C value of DNA content and the second contained more than 2C. The most remarkable difference of the tissues was the high population of polyploidy in the callus cells. In the NE cells, it consists of 42.5% which was higher than that of in the E-cells (17.4%).

Comparison of frequency distribution of nuclear and cell areas of different tissues have been shown in Fig. 4 and 5, respectively. Nuclear and cell areas of the NE cells were 72.40±39 and 2434±34 μm^2, respectively, which were higher than those of the E cells (48.31±29 and 857.9±30 μm^2, respectively) (Fig. 4, 5 and Table 3).

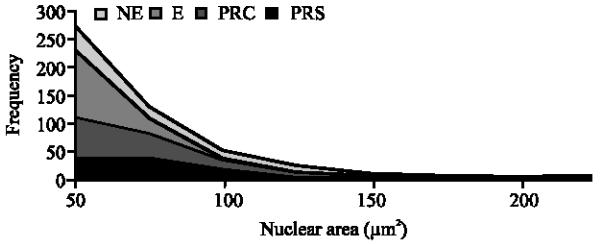

Fig. 4: Frequency of nuclear areas in various tissues of sugar beet: PRS: Primary Root of Seedling, PRC: Primary Root of *in vitro* Clone. E and NE: Embryogenic and Non-Embryogenic cells, respectively

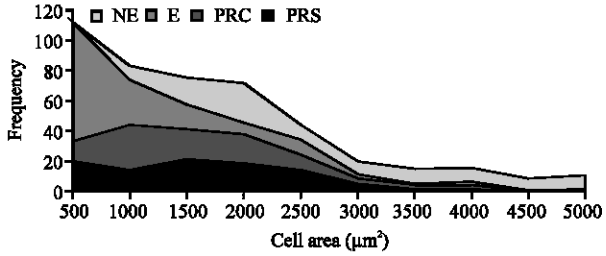

Fig. 5: Frequency of cell areas in various tissues of sugar beet: PRS: Primary Root of Seedling, PRC: Primary Root of *in vitro* Clone. E and NE: Embryogenic and Non-Embryogenic cells, respectively

Table 3: The mean cell, Nuclear (N) and Cytoplasm (C) areas and the proportion of cytoplasm to nuclear (C/N) of different tissues in sugar beet through image analysis: (PRS) Primary Root of Seedling; (PRC) primary root of *in vitro* cloned plant; (E) and (NE) Embryogenic and Non-Embryogenic callus, respectively

Tissue	Cell (µm²)	N (µm²)	C (µm²)	C/N
PRS	68.07±15	41.11±12	26.96	0.65
PRC	360.02±24	71.33±24	288.69	4.05
E	857.90±30	48.31±29	809.59	16.76
NE	2434.00±34	72.40±39	2361.60	32.62

The method that was used in this study for cell doubling time estimation may be considered as direct method, that the average of doubling time within the meristem or part of the meristem is measured using scoring of mitosis phases by observation of single cell nuclear (Verma, 2001). In indirect method, which is widely used for animal tumors, estimation of mitotic activity and cell doubling time are based on cell growth i.e., increasing of weight or cellular mass volume in a timed period (Hasegawa *et al*., 2000). The point we wish to stress, is that cell growth and division are in principle quite separate processes and also in typical meristematic cell cycles where they alternate and maintain a steady state, there must be specific mechanisms to coordinate them (Cavalier-Smith, 1985). As a result of positive correlation between cell volume or genome size and cell cycle length (Cavalier-Smith, 1978), which was clearly confirmed here (Table 1 and 2), estimation of cell doubling time or the rate of mitotic activity on the basis of cell growth may caused a great mistake.

Comparing Table 2 with Table 1 shows that in the NE cells with a large cell surface, the mitotic activity of the cells is the slowest one, which is conferred by the lowest population of S phase cells. If MI and Cdt are estimated by the indirect method, the results would probably be different and the E callus containing cells with smaller size (than the NE callus) may be categorized as a tissue with the longer cell doubling time.

The general topic of cell cycle in plant cell cultures and its relation with chemical inhibitors have been studied by several workers (Gould, 1984; Planchais *et al*., 1997). Omission of auxin leads either to a rapid cessation of growth and cell division or in suitable cultures to organogenesis or embryogenesis. In the embryogenic culture, removal of 2,4-D produced a marked reduction in cell cycle time, enhancement of mitosis, concomitant with the onset of morphogenesis (Bayliss, 1985).

Previous studies on sugar beet were indicated that the omission of auxin using TIBA as an anti auxin leads to somatic embryogenesis (Kulshreshtha and Coutts, 1997; Ehsani-Moghaddam *et al*., 2000). The present study revealed that the use of TIBA caused a significant reduction in the duration of Cdt i.e. about 115 h (if all of cells divide) or 129 h (if only 75% of the cells actively divide) in the E cells (Table 1, compare the E and NE cells). As in no steps of the NE callus production, auxin has been used, it seems that the omission of auxin in the cell culture of sugar beet is not enough for changing of cell behavior toward differentiation and use of an inhibitor for blocking of endogenous auxins, which is highly produced by sugar beet normal callus (Kevers *et al*., 1999b) is recommendable.

The MI as an index for cell activity and proliferation (Taha and Francis, 1990) was increased from 0.93 ± 0.73 in the NE cells to 1.1±1.01 in the E cells, suggesting higher cell activity of the E cells than the NE ones, which also agrees with reduction of the Cdt in the E cells. Based on the results shown in Table 1, with the exception of the PRC cells, there is no significant difference among the mitotic duration of various cells, which means interphase was the main source of variation within the cell doubling time data.

If we accept that the cell doubling time, necessarily dependent on cell activity, then cells with lower activity should have a longer duration of cell cycle (Clowes, 1961). In this respect, the NE callus may be considered as a tissue with the lower cell activity than the E cells (Table 1). However, this result does not confirm

ultrastructural studies of the cells, which before showed the amount of those organelles that are usually dealing with cell activity (Avers, 1978) in the NE cells are higher than the E ones (Ehsani-Moghaddam and Taha, 2005). One explanation for this contradiction may be obtained from the comparison of the polyploidy population of the cells. Table 2 shows that the percentage of polyploidy or in the other word endoreduplication (Nagl, 1978) in the NE cells is the highest among the tissues. It was found that nearly all non-meristematic cells have undergone endo-cycle, e.g., 70-80% in *Beta vulgaris* (Butterfass, 1966). The genome of endo-cycling nuclear is very often not completely replicated during a cycle. In such a case, polyploidization leads to a DNA amount that is not only quantitatively, but also qualitatively, different from the basic diploid genome (Nagl *et al*., 1985). Therefore, if the endo-cycling of the nuclei and also the needed energy and substances used for elongation of the huge NE cells to be taken into consideration, evidently the NE cells should be considered as cells with highest activity.

It is believed that cells with high proliferation require a certain minimum DNA content independent of the 2C value of a given species (Nagl *et al*., 1985). Thus, it seems that after five times of subculture, the polyploidy population of the NE callus and the DNA content of their cells were increased, so that the NE cells were completely lost the ability of a continuous proliferation. In fact, cell division is controlled by a serine threonine protein kinase, termed Cyclin-Dependent Kinase (CDK) that, in association with other proteins, mediates cell division and DNA replication (Huntley and Murray, 1999). In yeast, for instance, cells decide to exit or continue the mitotic cycle in the G_1 phase, at a checkpoint named start (Shen and Gigot, 1997). In a typical plant tissue i.e., seedling root meristem, which the majority of cells are proliferating (Webster and McLeod, 1980) with different pattern of cell division (Evans *et al*., 2001), if cells miss n cycles and remain in G_0 (Kelly and Trewavas, 1985) they would be 2n times longer than proliferative ones (Green, 1976). Therefore, in a non-proliferative cells with longer length, cell length is related to the cell cycle duration as well as the missed cycles, so that the longer the cell, the longer the cell cycle duration.

On the other hand, elongation of non-proliferative cells at the end of the first cycle could affect on nucleus organization, which may be followed by the loss of more cell cycle. Based on Table 2, in the typical meristematic cells i.e., the PRS, with highest cell activity and proliferation, the proportion of C/N is only 0.65 whereas in tissues with the highest quiescent cells i.e., the NE callus it is 32.62. In contrary to the meristematic cells, in which there is little space around the nucleus and positioning of the nucleus within the central portion of the division plane is usually not an issue (Wick, 1991) in the NE cells it appears to be critical. As cell elongation continues and the rate of cytoplasm/nucleus will increase, orientation of nucleus and consequently cell wall formation faces with more disruption.

In conclusion, changing of gene expression pattern brought about by the cultural environment (Gaspar *et al*., 2000), makes a karyotipic variations such as endoreduplication and increasing of nucleus volume. Apart from biochemical behavior changes, the main effect of such variations is probably cytoskeleton alterations including misoriented cell plates (Verma, 2001) incomplete cell division and cell wall formation, loosening of cell-to-cell junction, consequently production of a non-proliferative cell with a huge nucleus and cytoplasm size, which has repeatedly left cell cycle and lost the ability of cooperation with other cells for differentiation.

CONCLUSION

Changing of gene expression pattern brought about by the cultural environment, made karyotipic variations such as increasing cell-doubling time, decreasing mitotic index, endoreduplication and increasing of nucleus volume in non-embryogenic cells. Apart from biochemical behavior changes, the main effect of such variations was incomplete cell division, loosening of cell-to-cell junction, consequently production of a non-proliferative cell with a huge nucleus and cytoplasm size, which had repeatedly left cell cycle and lost the ability of cooperation with other cells for differentiation.

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