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Cell cycle activity and β-tubulin accumulation during dormancy breaking of *Acer platanoides* L. seeds

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Abstract

Cell cycle events in embryo axes of Norway maple (*Acer platanoides* L.) seeds were studied during dormancy breaking by flow cytometric analyses of the nuclear DNA content and by immunodetection of β-tubulin. Most embryonic nuclei of dry, fully matured seeds were arrested in the G_2 phase of the cell cycle. In addition, the lowest content of β-tubulin was detected in dry, mature seeds. Imbibition in water and cold stratification resulted in a decrease in the number of nuclei in G_2, and a simultaneous increase in β-tubulin content. In germinated seeds the content of β-tubulin was the highest and the number of cells in G_2 was the lowest. Both cell cycle events preceded cell expansion and division and subsequent growth of the radicle through the seed coat. The anatomical investigation has proved that the main reason for decrease in the number of nuclei in G_2 is mitosis, started with seeds germination (radicle protrusion). The activation of the cell cycle and the β-tubulin accumulation were associated with embryo dormancy breaking.

Additional key words: development, DNA, flow cytometry, germination, microtubule, Norway maple.

Introduction

Both DNA synthesis and microtubule organization, have been extensively studied in seeds, taking into consideration cellular events during embryogenesis, as well as endosperm formation, organogenesis and postgerminative growth (Clayton 1985, Barlow 1993, Raghavan 1997). The progression through the cell cycle is associated with changes in the specific organization of the microtubular cytoskeleton (Hussey *et al.* 1990). The expression of β-tubulin is dependent on the developmental stage of the analyzed tissue (Hussey *et al.* 1988). In maize (*Zea mays* L.) roots, the progression of the cell cycle through the G_1 phase was dependent on the turnover of the microtubular cytoskeleton (Baluška and Barlow 1993).

After reaching physiological maturity, seeds of many trees, including *Acer platanoides* L., enter a state of deep dormancy, and for germination to occur they require moist, cold treatment (stratification). Despite many years of research on tree seeds, the mechanisms of dormancy breaking at low temperature are virtually unknown. The process of seed dormancy breaking involves many parallel and concomitant pathways (Ross 1996). The theories include mechanisms mediated by endogenous growth regulators (Ross and Bradbeer 1971, Tomaszewska 1976, Szczotka and Tomaszewska 1979, Wareing 1982) and consequent quantitative and qualitative effects on gene expression (Slater and Bryant 1987, Taylor *et al.* 1993, Mullen *et al.* 1996, Jarvis *et al.* 1997).

It has also been proposed that seed dormancy may be controlled by blocking or unblocking of the synthesis of particular proteins involved in specific processes (Satoh and Esashi 1979). In our earlier publications (Pawłowski *et al.* 1997, Pawłowski and Szczotka 1997, 2001) we presented qualitative and quantitative changes in protein synthesis in embryo axes and cotyledons of *Acer platanoides* seeds. We found by 2-dimensional electrophoresis two proteins associated with dormancy breaking and numerous unassociated proteins. The highest increase in the number of proteins was observed

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after hydration. The proteins associated with dormancy breaking in the last weeks of cold stratification appeared just before seed germination. In cotyledons we observed one of these two proteins. This protein is probably associated with dormancy breaking in both embryo axes and cotyledons.

Two interrelated processes mediate the transition between the quiescent and active status of seeds: cell division and cell growth. In embryos of fully matured, quiescent non-dormant dry seeds of tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum* L.), most of the meristematic nuclei are arrested in the G_0/G_1 phase of nuclear cycle (2C amount of DNA). Upon imbibition in water, an induction of the 4C signal (phase G_2 of the cell cycle) was observed before visible germination, indicating that nuclear replication precedes radicle protrusion (Baiza *et al*. 1986, Bino *et al*. 1993, Lanteri *at al*. 1993, Liu *et al*. 1997). Górnik *et al*. (1997) demonstrated that DNA replication is not a prerequisite for radicle protrusion. However, further seedling development, including root growth and root hair development, is dependent on DNA replication.

Protein synthesis is an essential prerequisite for seed germination (Pawłowski *et al*. 1997, Hodurková and Vyskot 2003). Cell cycle activity is associated with spatial and temporal dynamics of microtubules and microtubule-associated proteins (Hepler and Hush 1996). Structurally, microtubules consist of α- and β-tubulin heterodimers. Rearrangements of microtubules involved in establishing cell division planes are known to start immediately after DNA synthesis, during G_2 (Gunning and Sammut 1990). Nuclear DNA contents and dynamics of β-tubulin accumulation have been studied during development and germination of tomato seeds. About halfway during seed development, seed moisture content drops and cell cycle activity halts, which is reflected in a decrease in the frequency of G_2 cells and a reduction in β-tubulin content (Liu *et al*. 1997). In dry mature tomato seeds, β-tubulin content is very low, but the protein rapidly accumulates in the embryonic root tip cells upon imbibition, preceding DNA replication and protrusion of the root through the testa (Bino *et al*. 1992, 1993, De Castro *et al*. 1995). It has been concluded that the induction of cell cycle activity is an early parameter of initiation of seed germination (Bino *et al*. 1993, De Castro *et al*. 1995). The increase in the number of cells in G_2 is due to the fact that mitosis and cytokinesis do not take place before radicle protrusion (Argerich and Bradford 1989).

Imbibition and low temperature activate many cellular and molecular mechanisms that break the dormancy. The main events that are initiated, include synthesis of proteins, DNA and RNA (Bewley and Black 1994). Research on dormant seeds of two tree species – *Acer platanoides* (orthodox - desiccation-tolerant) and *Acer pseudoplatanus* (recalcitrant - desiccation-sensitive) – revealed a high level of 4C in dormant seeds. In the orthodox seeds of *Prunus avium* L. (cherry), most of nuclei have 2C DNA. (Finch-Savage *et al*. 1998). In the late stages of cold treatment during stratification, nuclear DNA levels change in the orthodox *Acer platanoides* and *Prunus avium*, whereas in the recalcitrant *Acer pseudoplatanus*, no change has been recorded.

In this paper, we report on the role of cell cycle activation during dormancy breaking of *Acer platanoides* seeds. The cell cycle activity and β-tubulin accumulation was studied in dormant dry seeds, and in imbibed seeds subjected to cold stratification (which breaks dormancy). Warm treatment (which does not break dormancy) was a control.

Materials and methods

Full mature Norway maple (*Acer platanoides* L.) seeds were collected in the autumn of 1997 in the Kórnik Arboretum. The seeds were dried at 21 °C until they reached 10 % moisture content, and later stored at -3 °C in sealed plastic bags. To break their dormancy, the seeds were stratified at 2 °C for 13 weeks (maximum percent of germination) in closed plastic trays on filter paper moistened with water. The control was set up in the same way but at 15 °C. The germination test (four replicates of 50 seeds each) was carried out according to the conditions recommended by the International Seed Testing Association (1993).

For analysis of nuclear DNA in dry seeds, seeds stratified at 2 or 15 °C and in germinated seeds (just germinated), 1 mm of the distal part of the embryo root tip was dissected from each seed. Each sample was composed of 15 tips, with 4 replications. Fresh material was prepared by chopping with a sharp razor blade in a nuclear isolation buffer containing 15 μg dm^3 RNase as described by Bino *et al* (1993). The buffer containing nuclei from the chopped tissue was filtered through an 88 μm nylon filter. To detect DNA, fluorescent dye propidium iodide was added and the level of DNA was measured immediately. Flow cytometric performance and data processing were conducted as previously described (Bino *et al*. 1993). The flow cytometer *Coulter Epics XL-MCL* (*Coulter Corp*., Miami, USA) was used. DNA content is proportional to the fluorescent signal and is expressed as arbitrary C values in which the 1C value comprised the DNA content of the unreplicated haploid chromosome complement. Using the signals obtained from fresh tissues, the gain settings were adjusted so that

signals of all intact nuclei were registered within the channel range. MultiCycle for Windows Cell Cycle Analysis software v. 3.0 (*Phoenix*, *Flow Systems Inc.*, San Diego, USA) was used for data analysis and correction of the background noise. One-way analysis of variance (*ANOVA*) and Tukey-Kramer HSD test was used to analyze differences between the samples (significant at $P < 0.05$).

For analysis of β-tubulin content (Western blotting method), 15 root tips were isolated every 2 weeks from seeds stratified at 2 °C in water. Protein extraction, electrophoresis, electroblotting and immuno chemiluminescence detection of β-tubulin were performed according to de Castro *et al*. (1995), with some modifications. For electrophoresis we used precast 8 - 18 % gradient polyacrylamide ExcelGel (*Amersham Pharmacia Biotech*, Uppsala, Sweden). The proteins were electroblotted for 2 h instead of overnight. The incubation periods of the immunoblot in blocking solution and in the mouse monoclonal anti-β-tubulin antibody solution (*Boehringher Mannheim*, Germany, clone KMX-1) were increased to 2 h. The experiment was repeated three times.

For analysis of mitotic activity longitudinal sections of embryo axes of dry, cold-stratified and warm-treated seeds were prepared. Material was fixed in a solution of ethanol and acetic acid (3:1); dehydrated in an ascending series of ethanol and then embedded in *Technovit* (*Heraeus Kulzer GmbH*, Germany). Sections, 9 μm thick made by a rotary microtome, were stained with toloudine blue for observation of mitoses and photographed under a light microscope.

Results

After reaching maturity, the dormant *Acer platanoides* seeds require for germination a cold moist treatment (stratification). The cold-treated seeds started germination after week 8 of stratification and reached maximum of germination (83 %) in week 13. Rest of seeds had remained dormant and did not germinate (non-germinated seeds). The warm-treated seeds remained in a state of deep dormancy and failed to germinate until the end of the experiment (Fig. 1).

The flow cytometry of nuclear DNA contents of radicle tip cells from dormant dry seeds revealed two peaks (Fig. 2). The first peak corresponded with the 2C DNA level at the prereplication stage of nuclear division (G_0 or G_1 stage), while the second peak matched the 4C DNA level (G_2 stage), whereas the small third peak matched 8C DNA (effect of endoreduplication -

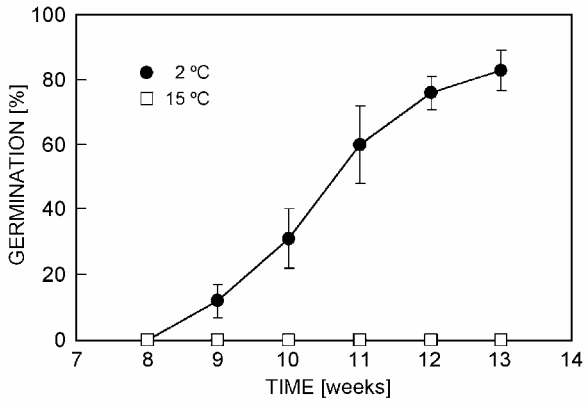

Fig. 1. Effect of cold stratification and warm treatment on germination of *Acer platanoides* seeds. *Error bars* represent SE, $n = 4$.

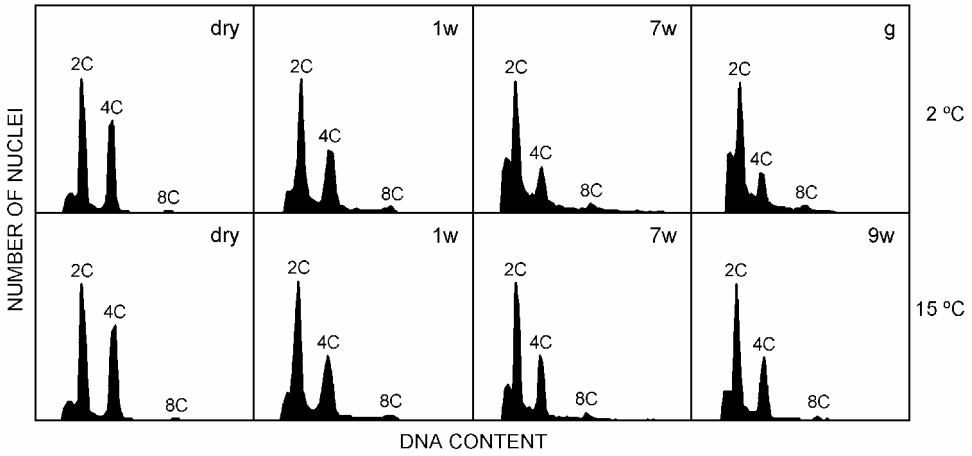

Fig. 2. Flow cytometry histograms of DNA contents of embryo axes of *Acer platanoides* seeds during cold stratification (2 °C) and under control conditions of warm treatment (15 °C). A minimum of 10 000 nuclei were considered for analysis of all samples (g - germinated seeds, w - week).

Table 1. Percentage of nuclei with 2C, 4C, 8C DNA and in the S phase in *Acer platanoides* seed embryo axes (g - germinated seeds, n - non-germinated seeds). Seeds stratified at 2 or 15 °C for different duration. Different letters show means that are significantly different at $P < 0.05$.

DNA	2 °C							15 °C		
	0	1	3	5	7	9	g	n	1	7	9
2C	43.3c	45.8bc	44.4c	45.7bc	49.8abc	53.5a	51.5ab	46.8abc	45.8c	45.9bc	47.6abc
4C	49.5a	44.4abc	42.6bc	43.8bc	45.1ab	39.5a	31.0d	42.5bc	44.4abc	42.3bc	44.0bc
8C	1.5c	2.4c	2.3c	2.7c	2.4c	3.7bc	7.0a	2.4c	2.4c	5.6ab	2.5c
S	5.7ab	7.4ab	10.7a	7.8ab	2.7b	3.3b	10.5a	8.3ab	7.4ab	6.2ab	5.9ab

Table 2. Ratios of 4C/2C and 8C/2C in embryo axes of *Acer platanoides* seeds during cold stratification and warm treatment (g - germinated seeds, n - non-germinated seeds). Different letters show means that are significantly different at $P < 0.05$.

DNA	2 °C							15 °C		
	0	1	3	5	7	9	g	n	1	7	9
4C/2C	1.15ab	0.97abc	0.96abc	0.96abc	0.91bc	0.74cd	0.60d	0.91bc	0.97abc	0.92bc	0.93bc
8C/2CS	0.04c	0.06c	0.05c	0.06c	0.05c	0.07bc	0.13a	0.05c	0.07c	0.12ab	0.05c

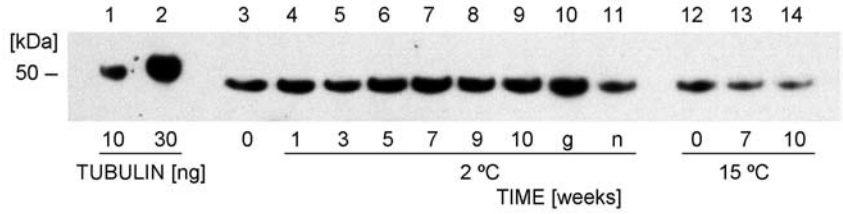

Fig. 3. Immunoblot showing the β-tubulin accumulation pattern in dry and stratified *Acer platanoides* seeds at 2 °C and 15 °C (g - germinated seeds, n - non-germinated seeds).

endopolyploid). During cold stratification at 2 °C, the 4C peak decreased gradually. First of all, the 4C DNA peak decreased in week 1, next in week 7 and was the lowest in germinated seeds (week 13). At the temperature of 15 °C, which did not break the dormancy and seeds did not germinate, the 4C peak decreased also in week 1, but in the following weeks it remained on the same level.

Nuclei with 2C and 4C DNA were found in 43.3 and 49.5 % of cells of dry seeds, respectively (Table 1). During cold stratification the percentage of nuclei with 4C DNA decreased to 42.6 % in week 3, then slightly increased (to 45.1 %) in week 7. In week 9 the percentage of nuclei with 4C decreased again (to 39.5 %), and was the lowest in germinated seeds (31 %). In control (15 °C), the decline in percentage of nuclei with 4C DNA was not observed, as their level was nearly constant. In week 9, the percentage of nuclei with 4C DNA amounted to 44 %. Changes in percentage of nuclei with 8C DNA were observed in week 9 of cold stratification (beginning of germination) and more significant in germinated seeds. During warm temperature treatment, in week 7 also an increased percent of nuclei with 8C DNA was observed. The percentage of nuclei in the S phase of the cell cycle was significantly higher in germinated seeds in comparison with week 9 of cold stratification.

Calculation of the ratios 4C/2C DNA and 8C/2C DNA showed changes taking place in the cell cycle during dormancy breaking of *Acer platanoides* seeds (Table 2). The high 4C/2C ratio in dry seeds decreased in week 1 of stratification at 2°C as well as during treatment at 15 °C. During warm treatment it later remained similar till the end of the experiment. During cold stratification, the ratio 4C/2C DNA decreased in week 7, and the lowest value was observed in germinated seeds. The ratio 8C/2C DNA remained on a similar level until week 9 of cold stratification, in germinated seeds was observed an increased in this ratio. In week 7 of warm treatment ratio 8C/2C reached a significantly higher level.

β-Tubulin was detected in radicle tips of dry dormant seeds and during all stages of stratification (Fig. 3.). In dry seeds the content of β-tubulin was low. Futher the content of β-tubulin increased (1 - 7 week of cold stratifi-

cation). In the beginning of germination, weeks 9 and 10, the amount of β-tubulin slightly decreased and a maximum accumulation was observed in germinated seeds.

In non-germinated seeds (also stratified at 2 °C, but had remained dormant and did not germinate), the concentration of β-tubulin was similar to that in dry seeds. Under control conditions, where seeds were subjected to the temperature of 15 °C (which does not break dormancy), a gradual decrease in the content of β-tubulin was observed, and the lowest content was noted in week 10 of warm treatment.

Longitudinal sections of embryo axes of dry, cold-stratified and warm-treated seeds (each two weeks) were observed with respect to mitotic activity of cells. Only in the meristematic part, in the protoderm of embryo axes of germinated seeds we observed various phases of mitotic division: metaphase, anaphase and telophase (Fig. 4). In previous weeks of stratification, preceding radicle tip emergence, mitotic divisions were not observed.

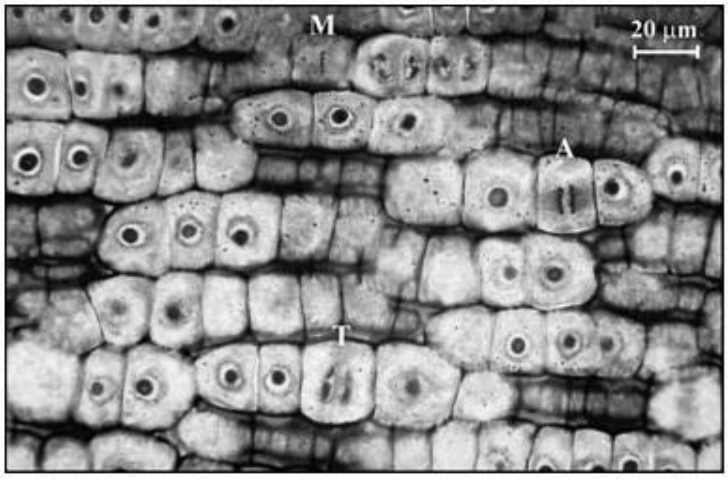

Fig. 4. Longitudinal section of embryo axes of germinated *Acer platanoides* seeds with marked phases of mitosis (M - metaphase, A - anaphase, T - telophase).

Discussion

The results of cell cycle activity analysis showed that more cells of embryo axes of dry, dormant Norway maple seeds were in the G_2 phase (with 4C DNA) than in the G_1 phase (with 2C DNA) of the cell cycle. Cells of dry seeds with a high level of 4C indicate that during maturation the cell cycle is activated. DNA synthesis leading to increase in a level of 4C occurred during seed development as result of replication towards mitosis, which is needed for tissue differentiation and organogenesis (Kudo and Kimura 2001). Cells arrested in G_2 were characteristic for differentiated tissue or deeply quiescent embryos (Sgorbati *et al*. 1989).

A similar high level of 4C was observed by Finch-Savage *et al.* (1998) in Norway maple seeds collected in year 1995. However, seeds collected in 1994 had most of cells arrested in the G_1 phase (60 %). In dry seeds analyzed in our study the 4C/2C ratio was 1.1, but in seeds used by Finch-Savage *et al*. (1998) it was only about 0.65. The reason of such differences maybe associated with harvesting of seeds. In our experiment seeds were harvested directly from the tree when the seeds were not yet dry but were maturated (Pawłowski and Kalinowski 2003). In the work of Finch-Savage *et al*. (1998) seeds were collected after shedding. On the other hand the number of nuclei in G_2 phase in both investigations was similar relatively and higher then in non-dormant maize (Baiza *et al*. 1986), pepper and tomato seeds (Bino *et al*. 1993, Lanteri *at al*. 1993, Liu *et al*. 1997). In slow dried, desiccation-tolerant carrot (*Daucus carota* L.) somatic embryos, Tetteroo *et al*. (1995) noticed a reduced proportion of 4C DNA nuclei, as compared with non-dried embryos.

The influence of temperature on cell cycle activity of Norway maple seeds became visible only in week 7 of cold stratification, when we observed an decrease in the number of nuclei in G_2 in relation to G_1. This decrease was observed before radicle protrusion. It can be concluded that dormancy breaking of seeds was associated with changes on the nuclear DNA level. In germinated seeds the number of cells in G_2 in relation to G_1 significantly decreased. Besides mitotic divisions (anatomical investigation indicates that the reason for this decrease in germinated seeds was mitosis), a reduction in the number of 4C nuclei during stratification appears to

be more properly related to the increase in 8C nuclei, including the respective increase in the percentage of S-phase nuclei. In other words, the decrease of 4C/2C ratios may represent a shift of 4C nuclei towards 8C nuclei, which increased the 8C/4C ratio in the completion of endoreduplicative DNA synthesis events. In Norway maple seeds, Finch-Savage *et al*. (1998) observed at the end of stratification an increase in the 4C/2C DNA ratio. This increase in 4C/2C ratio was caused probably by DNA replication, and the ratio reached a level similar to that observed in our investigations. This maybe was a need (increase in number of cells in G_2) for completion of the developmental mode during stratification, before dormancy could be broken and seeds allowed to enter germination and cells in mitotic events. The decrease in 4C/2C ratio observed in germinated seeds in the present study was similar to that reported by these authors. They suggest that germination was preceded by DNA replication. Similar changes were observed by other researchers in seeds of various plant species, including pepper (Lanteri *et al*. 1993), tomato (Bino *et al*. 1992), and maize (Deltour 1985, Dandoy *et al*. 1987). In present study we did not observe an increase in 4C/2C ratio, but only increase in 8C/2C ratio suggesting that DNA replication could have appeared before germination. Our previous investigation (Pawłowski *et al*. 1997) in which we observed increase in the total DNA content in nuclei of Norway maple seeds at the end of stratification supports this conclusion.

The presence of nuclei with 8C DNA, suggests that not all replication may be associated with cell division, but may result from endoreduplication, the amplification of the genome in the absence of mitosis. This endoreduplication might reflect a need for increase in transcription in the subsequent stages of seed germination (Kudo and Nakamura 2001). Endoreduplication of DNA is associated with differentiation of cells during plant development (Bassi 1990).

Warm temperature treatment, which does not break dormancy of *Acer platanoides* seeds, did not change the 4C/2C ratio. We have observed only in week 7 an increase in the 8C/2C ratio caused probably by endo-reduplication. Warm temperature initiated amplification of the genome but did not break dormancy and did not initiate mitotic divisions. Seeds remained dormant and did not germinate in unfavorable conditions of increased temperature.

Changes in cell cycle activity are associated with changes in microtubule organization. Research on dormancy breaking in *Acer platanoides* seeds showed that this process is associated with an increase in accumulation of β-tubulin, one of two (with α-tubulin) components of microtubules. During cold stratification, which breaks dormancy, the amount of β-tubulin increased and was the highest in germinated seeds. In dry seeds, β-tubulin accompanied high percentage of cells in G_2 phase. Gunning and Sammut (1990) observed a similar relationship in root tips of wheat (*Triticum vulgare* Vill.). In seeds which are not characterized by physiological deep dormancy, most of the nuclei are arrested in the G_1 phase of the cell cycle and the level of β-tubulin is low (Bino *et al*. 1992, 1993, de Castro *et al*. 1995, Liu *et al*. 1997).

The increase in β-tubulin at the beginning of stratification (in week 1) of *Acer platanoides* seeds was caused by imbibition of embryo cells. Simultaneously we observed a small decrease in the number of cells in G_2 in relation to G_1. The increase in β-tubulin accumulation before germination and the change in cell cycle activity, *i.e*. a decrease in the number of cells in G_2 in relation to G_1 in week 7, was probably caused by the influence of cold stratification. In germinated seeds the level of β-tubulin was the highest and was correlated with the highest decrease in the number of cells in G_2 and with the beginning of mitotic divisions.

Bergervoet *et al*. (1999) observed variable contents of β-tubulin also in axillary buds of Norway maple during dormancy breaking. Warm temperature broke the dormancy of buds and β-tubulin content increased. The similarity of changes in buds and seeds is rather difficult to explain because of differences in dormancy type between them. It can only be concluded that increased β-tubulin contents precede visible growth in both kinds of tissues of Norway maple.

In conclusion, cell cycle activity and β-tubulin accumulation preceded cell expansion and division and subsequent growth of the radicle through the seed coat. The activation of the cell cycle and the β-tubulin accumulation were associated with embryo dormancy breaking and could be the indicators of the process of dormancy release in seeds.

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