BIOLOGIA PLANTARUM 47 (3): 333-339, 2003/4

Effect of chilling on DNA endoreplication in root cortex cells and root hairs of soybean seedlings

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Abstract

Relative nuclear DNA contents in cortex parenchyma cells in root segments of 3- and 7-d-old soybean seedlings grown at 25 °C and in plants grown for 3 d at 25 °C, and then for 4 d at 10 °C, were determined with cytophotometry. Measurements revealed that in each variant the cortex cell nuclei with DNA content between 2C and 8C were in all the examined segments and nuclei with 8C - 16C DNA appeared in higher parts of roots. However, in chilled plant cells the number of 8C - 16C DNA nuclei was very low. Therefore, chilling inhibited endoreplication in comparison with plants grown at 25 °C for 7 d, and even reduced endopolyploidy level as compared to the initial seedlings, *i.e.* 3-d-old plants. DNA contents in root hairs grown at 25 °C (control) and in root hairs emerged at 10 °C were also determined. In controls 4C - 8C DNA nuclei predominated while in chilled plants an additional population of 2C - 4C DNA appeared. Thus a reduction of DNA synthesis was brought about by low temperature. The occurrence of an intermediate DNA contents besides those with full endoreplication cycles suggests the possibility of differential DNA replication. This suggestion seems to be supported by the lack of ^{3}H-thymidine incorporation into root hair nuclei at the examined developmental stage both in control and chilled root hairs. The same number, but larger, chromocentric lumps in polyploid cortex cell nuclei of higher root zones, in comparison to meristematic nuclei, suggests that endoreduplication process occurred.

Additional key words: DNA content, *Glycine max.*

Introduction

Endoreplication is a form of nuclear DNA polyploidization that results in multiple copies of a genome (D'Amato 1984). This process is widespread in plants, particularly in angiosperms (Joubès and Chevalier 2000). The constant tissue-specific pattern of endopolyploidy in different organs suggests that endoreplication cycles in plants constitute an essential part of the developmental programmes that are necessary for differentiation, morphogenesis, and for specialized function of given cells and tissues (Cebolla *et al.* 1999). Endoreplication in plants has often been correlated with cell growth, and it is widely accepted that there is a clear positive correlation between endoreplication level and cell elongation, and finally organ size (Lemontey *et al.* 2000). It is thought that endoreplication provides a mechanism to increase the level of genome expression. It seems that endoreplication is an important factor determining potential transcriptional and translational cell ability, and can also determine the cell participation in organ formation and function regulation (Kwiatkowska *et al.* 1990). Larkins *et al.* (2001) suggested that endoreplication is modulated by the content of growth regulators. As this process could be regarded as an 'alternative' cell cycle, hence, endoreplication requires down-regulation of M-phase cyclin-dependent kinases (M-phase promoting factor, MPF) and up-regulation of S-phase CDKs activity (Grafi 1998).

Another way of genome information increment is DNA amplification or differential replication (Nagl 1976). These processes take places at particular times and in special cell types during development and serve the purpose of providing the cells with supernumerary

Received 15 July 2002, *accepted* 7 May 2003.
Abbreviations: DABCO - 1,4-diazabicyclo-[2,2,2]octane; DAPI - 4',6-diamidino-2-phenylindole; PBS - phosphate buffered saline.
Acknowledgments: I am grateful to Prof. Maria Kwiatkowska for her valuable advice and comments during the experimental part and preparation of the manuscript.
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copies of selected genes to cope with a temporary and exceptionally high demand for transcripts of these genes (Wintersberger 1994).

Although endoreplication has been already described in many plant species, its physiological significance remains poorly understood. One of the biological roles of endoreplication is a probable involvement of endopolyploid cells in symbiosis and root nodule development in leguminous plants. Endopolyploid cells can be invaded and host the nitrogen-fixing bacteroids while also present diploid cells remain uninfected (Truchet *et al.* 1991). An increased DNA content in nuclei of root hairs containing infection threads in comparison with uninfected root hairs was also observed (Łotocka and Golinowski 1998). Moreover, Olszewska and Legocki (1989) discovered that within a root hair zone the cortex cells polyploid prior to the infection reacted to *Rhizobium* with an increase in DNA content through endoreplication.

Since both parenchyma cortex and root hair cells actively participate in symbiosis and root nodule formation it seemed justified to examine DNA content in these cells in soybean plants.

Furthermore, soybean is a chill-sensitive plant and low temperature reduces its growth and development. It was observed that chilling of soybean root zone considerably reduced plant infection with symbiotic bacteria (Zhang *et al.* 1995), nodule development and number (Stoyanova 1997), and atmospheric nitrogen fixation (Guy *et al.* 1997).

It seems probable that influence of low temperature on soybean root nodule development could be one of the reasons of limited soybean growth and yield in temperate climate. A hypothesis has been advanced that a suppressive effect of chilling on endoreplication process could be the reason of this phenomenon. That is why the studies on DNA content in root of chill-treated (10 °C) soybean seedlings have been undertaken.

Materials and methods

Plants: Seeds of *Glycine max* (L.) Merr. cv. Aldana (obtained from IHAR in Radzików) were germinated for 3 d at 25 °C (control) in darkness in Petri dishes on filter paper wetted with distilled water. The sections were marked every 2nd mm (1st cm of root) and every 3rd mm (remaining part of root) on 3-d-old seedling roots (Fig. 1). Some of these seedlings continued to grow at 25 °C and some were transferred to lower temperature (10 °C) for 4 d. DNA content in root hairs was determined in 3-d-old seedlings grown at 25 °C and in root hairs emerged at 10 °C during 14 d.

DNA staining and analysis: Roots were fixed in absolute ethanol and glacial acetic acid mixture (3:1) for 1 h at room temperature, then washed with ethanol and kept in 70 % ethanol until further procedure. For cytophotometry the material was hydrated gradually and hydrolysed in 4 M HCl for 90 min (this hydrolysis time was optimal for this material) and then stained in Schiff's reagent prepared from pararosaniline and processed according to the standard method. Tissue fragments containing parenchyma cells were excised from a median part of each segment and placed on microscope slides in 45 % acetic acid drop and squashed. After freezing and removing coverslips the slides were dried and embedded in Canada balsam.

To obtain root hair preparations the epidermis with root hairs was peeled off gently from the root surfaces. Slides were prepared as above.

Absorbance of Feulgen stained nuclei was measured at 565 nm using *Jenamed-2* microscope (*Carl Zeiss*, Jena, Germany) with the computer-aided *IMAL-512* system for image analysis according to Rosiak *et al.* (2002). DNA content was determined in arbitrary units (a.u.).

Values of 2C and 4C (C-DNA content in haploid nuclei) were calculated from telophase and prophase nuclei of root tip meristematic cells submitted to Feulgen reaction in the same bath together with the other experimental material. Cytophotometric measurements were carried out for 180 nuclei for every root cortex segment analysed. In the case of root hairs there were 160 nuclear measurements carried out for every variant.

For 4',6-diamidino-2-phenylindole (DAPI) fluorescence roots were fixed as above and macerated in pectinase solution for 30 min at 40 °C. After rinsing in distillate water the squashed preparations were made of root meristem and parenchyma tissue from higher root zones. Dried coverslips were immersed in DAPI at a concentration of 1 μg cm^{-3} for 10 min. The slides were then washed in distilled water and embedded in phosphate buffered saline (PBS)/glycerol mixture (9:1) with 2.3 % 1,4-diazabicyclo-[2,2,2]octane (DABCO). Stained DNA was visualized by fluorescence microscopy (*Nikon Optiphot-2*, Japan) equipped with *UV-2A* filter (λ = 360 - 460 nm).

Incubation in ^{3}H-thymidine: Seedling roots with root hairs grown both at optimal temperature and at chilling conditions were incubated in water containing ^{3}H-thymidine (1.85 Bq cm^{-3}; 662.3 GBq mmol^{-1}) for 60 min at 25 °C and 10 °C respectively. Material was fixed, stained and preparations were prepared as above. The slides were covered with photosensitive emulsion and exposed in darkness at 8 °C. Following 24 d the autoradiograms were developed.

Results

DNA content in nuclei of root cortex parenchyma cells: DNA content measurements in root cortex of 3-d-old seedlings grown at 25 °C, 7-d-old seedlings grown at 25 °C, and of seedlings grown at 25 °C for 3 d and then chilled (10 °C) for 4 d were performed for 7 corresponding segments (I, II, III, IV, V, IX and XII) which were localised in different developmental zones, starting from meristematic up to root hair zone. Individual segments expanded and translocated along with the seedling growth and development (Fig. 1, Table 1). Measurements of marked segments' lengths showed the highest growth rate in segment I, lower in segments II and III, and no growth in higher zones in 7-d-old seedlings grown at 25 °C; in chilled seedlings' root growth rate was drastically reduced especially in segment I.

DNA content in root cortex cells was analysed in tissue sections excised from the middle of each examined segment. Cytophotometric measurements revealed that in all examined root segments in 3-d-old seedlings grown at 25 °C there were cells containing 2C nuclei and nuclei with increased DNA content in comparison with telophase and prophase nuclei of meristematic cells. A progressive increase in DNA content was observed in higher and higher root zones. However, endopolyploidy level became stabilized in segment IX, *i.e.* at 20th mm of root length (measured from root tip). Nuclei with DNA content between 4C and 8C predominated. Cells with DNA content between 8C and 16C were the least numerous. The highest DNA content, nearly 16C, was observed in nuclei in segments IX and XII (Fig. 2).

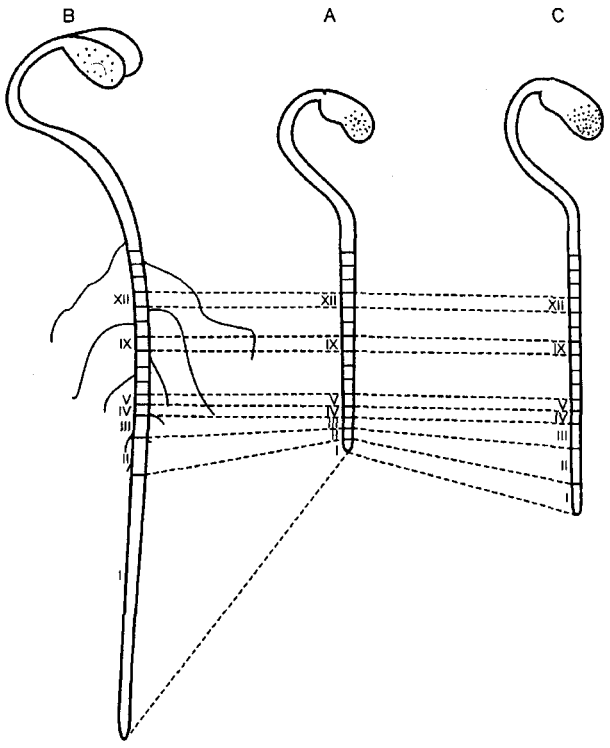

Fig. 1. Soybean seedlings with segments marked on roots where DNA content was determined. *A* - 3-d-old seedling grown at 25 °C, *B* - 7-d-old seedling grown at 25 °C, *C* - seedling grown at 25 °C for 3 d and then transferred to 10 °C for 4 d.

Table 1. Lengths [mm] of selected segments (I - XII) on roots of soybean seedlings grown 3 d at 25 °C (initial length), 7 d at 25 °C and 3 d at 25 °C + 4 d at 10 °C in which DNA content was determined. Means ± SE, $n = 6$.

Segment	3 d at 25 °C	7 d at 25 °C	3 d at 25 °C + 4 d at 10 °C
I	2	58 ± 5.2	4 ± 1.0
II	2	6 ± 1.5	4 ± 1.3
III	2	3 ± 0.7	3 ± 0.7
IV	2	2 ± 0.0	2 ± 0.0
V	2	2 ± 0.0	2 ± 0.0
IX	3	3 ± 0.0	3 ± 0.0
XII	3	3 ± 0.0	3 ± 0.0

DNA content in root cortex of 7-d-old seedlings grown at 25 °C was similar to that in 3-d-old plants, *i.e.* from 2C up to nearly 16C, and the maximal polyploidy level was found already in segment IV *i.e.* at 67th mm from the root tip. The nucleus population with DNA content between 4C and 8C appeared in all analysed root segments of 3- and 7-d-old seedlings (Fig. 2).

In root cortex cells of plants subjected to 4-day chilling DNA content ranged from 2C to 8C in all segments analysed, and only in few nuclei it was a little over 8C (Fig. 2). In these plants, as in control, the cell population with nuclear DNA content between 4C and 8C appeared. These results show that chilling caused a reduction of endopolyploidy level in comparison with 7-d-old seedlings grown at 25 °C, and even decrease in polyploidy level in relation to initial plants, *i.e.* 3-d-old seedlings.

DNA content in nuclei of root hairs: Nuclear DNA contents were measured in root hairs emerged at control and chilling conditions. The analysis of histograms (Fig. 3) has shown that in control DNA content in nuclei ranged from slightly over 2C to nearly 8C with 4C - 8C nuclei being the most numerous, in chill treated root hairs a population with DNA level between 2C - 4C appeared while the number of 4C - 8C nuclei, characteristic of control, diminished. Chill-induced increase in the number of 2C cells and an appearance of 2C - 4C population, which was absent from control, suggest that low temperature caused the reduction of DNA synthesis dynamics in root hairs. The presence of cell populations

with intermediate DNA contents between completed replication cycles both in control cells and those subjected to chilling suggests either a continuation of DNA synthesis or a possibility of a differential DNA replication in the analysed root hairs. That is why the studies on ^{3}H-thymidine incorporation to root hairs emerged in optimal temperature and in chilling conditions were carried out. These studies were performed on root hairs which were at the same developmental stage as those in which DNA content was determined. It has been

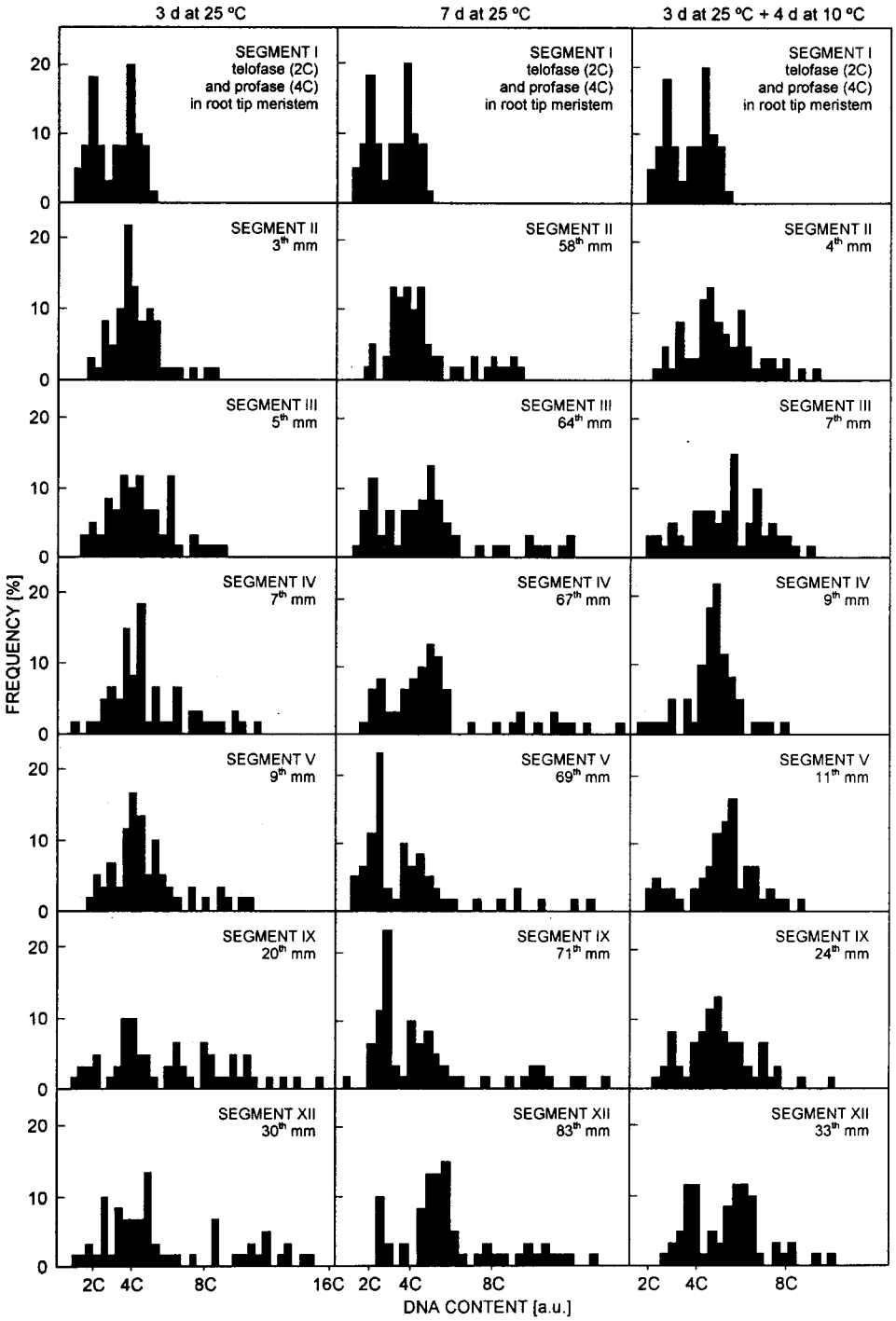

Fig. 2. Frequency distribution [%] for DNA content [a.u.] in nuclei of root cortex cells in individual segments at defined distance from root tip in 3-d-old seedlings grown at 25 °C, 7-d-old seedlings grown at 25 °C and seedlings grown at 25 °C for 3 d and then transferred 10 °C for 4 d.

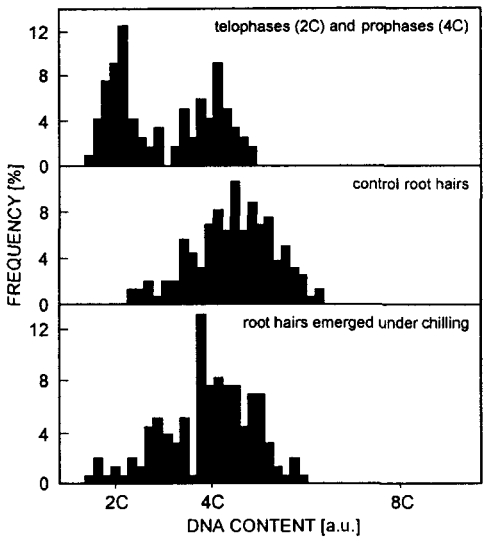

Fig. 3. Frequency distribution [%] for DNA content [a.u.] in nuclei of root hairs emerged at 25 °C and 10 °C.

found out that root hair nuclei both in control and chilled plants did not incorporate ^{3}H-thymidine at this developmental stage, which supports a hypothesis about a differential DNA replication.

Number and size of chromocentres: Chromocentric lumps in root meristematic cells and in parenchyma cells of higher zones of root after DAPI-stained DNA were analysed. Observations revealed that number of chromocentres both in meristematic and parenchyma nuclei is the same. However chromocentric lumps from parenchyma nuclei are larger than those from meristematic ones (Fig. 4).

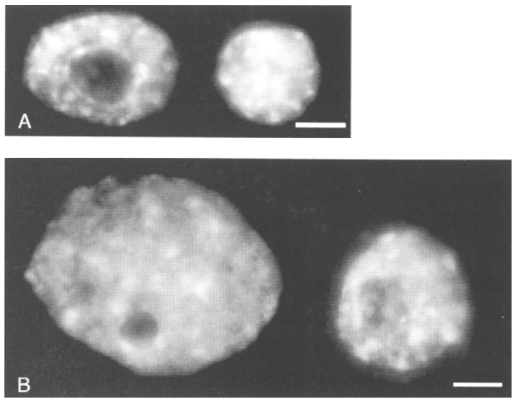

Fig. 4. Fluorescence of DAPI-stained nuclei of root meristematic cells (*A*) and polyploid nuclei of root parenchyma cells (*B*). *Bars* = 10 μm.

Discussion

Cytophotometric measurements revealed that soybean plants contained 2C cells and cells with increased DNA content in root cortex parenchyma, starting from segment II from the tip to the last analysed segment (30th mm from the tip) in seedlings grown at optimal temperature. DNA content was gradually increasing in higher and higher root zones and nearly reached 16C. It is highly probable that in soybean, similarly as in most of angiosperms, nuclei become polyploid through the endoreduplication way (D'Amato 1989). Occurrence of the same number, but bigger, chromocentric lumps in polyploid nuclei of higher segments of root, in relation to meristematic nuclei, suggests that not endomitotic but endoreduplication process took place.

In control root hairs 2C nuclei and those with higher DNA content were observed. It is supposed that the presence of diploid cells together with polyploid ones, in differentiated plant tissues gives them a chance of potential further growth, wound healing and regeneration (Melaragno *et al.* 1993).

It seems interesting that a nuclear population with the highest endopolyploidy level, *i.e.* 8C - 16C DNA, disappeared from root cortex of chill-treated seedlings. It seems that this phenomenon cannot result only from chill-induced decrease in endoreplication dynamics or inhibition of consecutive endoreplication rounds since endopolyploidy level in plants in which endoreplication was inhibited should be at least equal to that in 3-d-old plants (*i.e.* 2C - 16C) which were not chill-treated. This intriguing phenomenon and a way of endopolyploidy level decrease is difficult to explain and needs further studies. It may be assumed that one of the reasons for polyploidy level decrease was the induction of endopolyploid root cortex cells to mitotic divisions in response to chilling.

There exists an opinion that once a cell begins cycling endomitotically, it loses the potential to return to the mitotic cycle. However, endopolyploid nuclei could be induced to divide occasionally, *e.g.* under stress conditions as tissue wounding (Barlow 1978). Galbraith *et al.* (1981) suggest that the processes of DNA synthesis, mitosis and cell division are not mandatory linked, but that several rounds of gene replication can occur prior to the onset of several mitoses and cell divisions. In their experiments with a culture of tobacco leaf protoplasts they observed that polyploid nuclei were eliminated by subdivision of large polyploid cells into clusters of small diploid cells. The same phenomenon in the similar system, but the culture was under induction of plant growth regulators, was identified by Valente *et al.* (1998).

Besides those mentioned above, other reasons for reduction of DNA content in endopolyploid cells were also proposed. Kowles and Phillips (1985) suggested that the decrease in DNA level observed at later stages of maize kernel endosperm development could be due to DNA degradation. There are also assumptions that a

decrease in DNA contents may be caused by an increased DNase activity or progressive chromatin condensation producing its change of colourability (Damsz and Łuchniak 1988).

In the case of root hairs, an increase in the number of 2C nuclei (in comparison with control) and decrease in 4C - 8C DNA nuclei following 10 °C treatment prove that chilling caused, similarly as in root cortex cells, a decrease in endoreplication dynamics. Moreover, the presence of nuclei with intermediate DNA content between 4C and 8C nuclei in control root hairs, and between 2C and 4C, and between 4C and 8C DNA nuclei in chilled seedlings seems to prove that either the differential DNA replication or continuation of S phase in full endomitotic cycle took place in these cells. It is known that ^{3}H-thymidine incorporation into a tissue without mitotic activity indicates DNA replication. However, autoradiographic studies showed this isotope incorporation neither into control nuclei nor into chill-treated ones at the developmental stage during which the experiment was carried out. Therefore, it seems that in this case a differential DNA replication occurred took which happened earlier. However, a reliable confirmation of this assumption could be obtained with the use of molecular methods.

On the basis of these results a conclusion can be drawn that low temperature generally decreases DNA endoreplication dynamics in root cortex parenchyma cells and root hairs of soybean plants. This fact may contribute to poor soybean development in chilling conditions since cells with lower content of genetic information can weaken signal emission towards symbiotic bacteria, and it is known that root hairs and root cortex cells actively participate in symbiotic processes and root nodule formation (Fehér *et al.* 1998). Besides, the fact that bacteria choose particular cells of root hairs and cortex is probably not random as root nodules of leguminous plants consist of endopolyploid cells as well 2C DNA ones and these former cells can be invaded and host the nitrogen-fixing bacteroids while the latter cells remain uninfected (Truchet *et al.* 1991).

Even though bacterial Nod factors affect 2C or 4C cortex cells and induce their mitotic divisions resulting in nodule primordium formation (Foucher and Kondorosi 2000), polyploid cells present in this tissue could influence these former ones, according to Barlow's hypothesis (Barlow 1978) that polyploid cells affect the activity of adjacent 2C cells and coordinate their further development.

An assumption, that in soybean at optimal conditions genes responsible for nodulation process increase their expression owing to multiplication of their number through endoreplication and/or amplification of DNA while these processes decrease under chilling which results in a handicapped symbiotic ability in temperate climate, seems to be well-founded.

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