Phytochemistry 69 (2008) 2172–2179

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The natural compound benzoxazolin-2(3*H*)-one selectively retards cell cycle in lettuce root meristems

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ARTICLE INFO

Article history:
Received 31 January 2008
Received in revised form 21 May 2008
Available online 1 July 2008

Keywords:
BOA phytotoxicity
Cyclic hydroxamic acid
G_2/M checkpoint
Flow cytometry
Mitotic activity
Natural plant product

ABSTRACT

Benzoxazolin-2(3*H*)-one (BOA) is a natural plant product that is phytotoxic to target plant species, inhibiting germination and growth and causing oxidative damage. We investigated its effects on the root meristems of seedlings of lettuce (*Lactuca sativa*) by means of light and transmission electron microscopy, flow cytometry, and conventional determination of mitotic index. Flow cytometry analyses and mitotic index showed a retard of cell cycle in BOA-treated meristems with selective activity at G_2/M checkpoint.

1. Introduction

4-Hydroxy-1,4-benzoxazin-3-ones are cyclic hydroxamic acids that are produced by certain Gramineae and have allelopathic and other chemoecological activities (Sánchez-Moreiras et al., 2004). Present in healthy plants as 1,4-benzoxazine glucosides, they can be cleaved from the sugar moiety after injury and protect the plant from disease, insects and weeds (Hofman and Hofmanova, 1971; Friebe, 2001). The main cyclic hydroxamic acid of rye (*Secale cereale*), 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), undergoes thermal degradation to a more stable cyclic hydroxamic acid, 2(3*H*)-benzoxazolinone (BOA), which is also active. BOA was first isolated as a natural antifungal from rye seedlings. Since then its protective activity and detoxification by fungi have been studied by a number of researchers as have its allelopathic effects on the seedlings of various species; and it is on this latter activity, and the response of target plants, that research on BOA has focused in recent years (Burgos et al., 2004; Baerson et al., 2005; Sánchez-Moreiras and Reigosa, 2005; Batish et al., 2006).

In this way, seedling growth inhibition in BOA-treated seedlings has been reported by many authors. Wolf et al. (1985) reported dose-dependent inhibitions on root and coleoptile growth in velvetleaf seedlings in the presence of BOA, Pérez and Ormeño-Núñez (1993) in *Avena fatua* seedlings, Chiapusio et al. (1997) in *Lactuca sativa* seedlings, and Singh et al. (2005) in mung bean seedlings. Barnes and Putnam, 1987 detected an additional necrosis of the apical root meristem closely resembled evident symptoms in lettuce when germinated in the soil/residue Petri dish bioassays; BOA treatment turned black the lettuce root meristems. Burgos et al. (2004) reported also effects at microscopic level in cell organization of cucumber root tips after treatment with 3 mM BOA.

The growth-limiting effects of various kinds of biotic and abiotic stress to which seedlings may be subjected have been related to their effects on the regulation of elongation and cell division (see, for example, Sacks et al., 1997; Burssens et al., 2000; West et al., 2004; Peres et al., 2007). Hitherto, however, there has been no research on whether the inhibition of seedling growth by BOA may be due to alteration of these processes. The interest on the physiological significance of BOA phytotoxicity at seedling stage was the base for the present work. In this way, the main goal of this study was establishing a link between an easily observable phytotoxic effect (radicle growth inhibition in the presence of a physiologically traceable BOA concentration) and a cellular-based explanation (altered cell cycle).

In the work described here we used flow cytometry with cell synchronization (Blanco Fernández et al., 2001), together with conventional determinations of mitotic index, to study the effects of physiologically realistic BOA concentrations on the cell cycle of root tip meristem cells of seedlings of lettuce (*L. sativa* cv. Great Lakes). As far as we know, this is the first time its root meristem cells have been studied by flow cytometry preceded by cell cycle synchronization.

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doi:10.1016/j.phytochem.2008.05.014

2. Results

2.1. Seedling growth

The use of pre-germinated seedlings in growth bioassays was a great advance to detect the effects on growth process avoiding previous resistance. When seeds, previously treated with the phytotoxic compound, are used for growth bioassays, the retard on germination can overlap effects on growth, confusing data interpretation. This experimental procedure, the use of pre-germinated seedlings to evaluate effects on growth and development, allowed the precise establishment of BOA phytotoxic activity.

Seedling root growth was unaffected by BOA concentrations $\leqslant$0.3 mM, but declined progressively as concentration increased thereupwards (Fig. 1). IC_{50} was 0.9 mM and IC_{80} 4 mM.

2.2. Morphology

Six hours exposure to 1 mM BOA inhibited root growth and root hair formation (Fig. 2a). After exposure, BOA-treated roots had thicker sub-apical regions than control roots, no root hairs, and a characteristic brown coloration. TEM images (Fig. 2b) showed meristem cells of BOA-treated roots to have significant fewer and larger vacuoles than those of control roots.

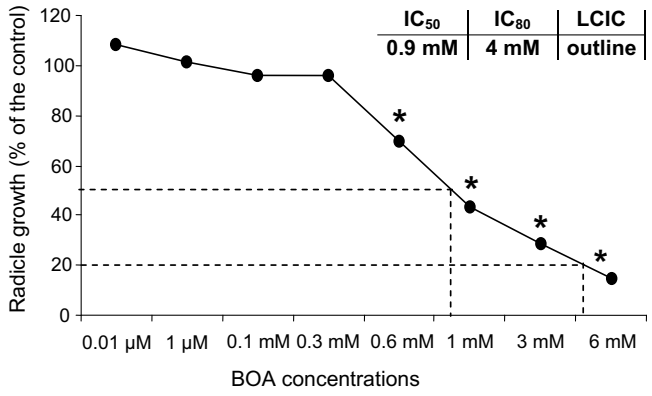

Fig. 1. Radicle growth of lettuce seedlings (mean % growth, expressed as a % of the control value) treated with various concentrations of BOA (from 0.01 µM to 6 mM). The table shows the corresponding IC_{50} and IC_{80} values. Asterisks represent significant differences with respect to the control ($p \leqslant 0.05$).

2.3. Cell cycle analysis

Fig. 3 shows cell cycle progression data in 4 h treated- (1 mM BOA) and control- (distilled water) cells from unsynchronized lettuce meristems. Overall cycle duration was about 6 h for both treatments when meristems treated with water or BOA, without previous HU synchronization, were analyzed. These first analyses showed evidences about the accumulation in G_2 of 1 mM BOA-treated cells after 4 h treatment, suggesting a potential effect of this phytotoxic compound on the G_2/M checkpoint. However, the amount of cells having division in unsynchronized meristems is so low that becomes impossible to demonstrate statistically this effect. Due to this low division activity, our group used the inhibitor hydroxyurea (HU) to increase the number of mitotic cells and to prove a clear effect of this natural compound on cell cycle of lettuce plants.

Fig. 4, which shows DNA content histograms of non-BOA and BOA-treated root tips 2 h after the removal of hydroxyurea, confirms the synchronization of the actively dividing cell population. The G_1/G_0:S:G_2 ratios at these times (57:15:28 and 55:19:26, respectively), together with population profiles recorded at later times (Fig. 4; 4, 6, 8 and 10 h), the previously determined composition of unsynchronized root tip cell populations (about 10–12% S, 10% G_2), and the mitotic index results (see below), are in keeping with the interpretation that a cohort comprising about 25% of the initial population of non-mitotic cells progressed through the cell cycle from G_1/S to G_2/M in synchronized fashion, but that synchrony was not maintained into a second cycle.

At time 2 h after removal of hydroxyurea, the DNA content histogram of BOA-treated root tips was similar to that of control tips. At time 4 h, however, the proportion of non-mitotic control cells in phase G_2 was 27.2%, and that of non-mitotic BOA-treated cells 33.4% (Fig. 4). Over the next 6 h, the G_2 population of control cells fell steadily to a roughly normal level of 12.4% as the synchronized cohort progressed through mitosis and the G_1 phase, but the fall in the BOA-treated G_2 phase population was much less pronounced and occurred almost exclusively between 4 and 6 h after the initiation of BOA treatment. Cell cycle progression was so slow in BOA-treated meristems that the number of cells in G_2 was the double of control meristems after 10 h treatment. Flow cytometry showed increasingly significant arrest on cell cycle progression delaying the entry of cells in mitosis by blocking the cell cycle at G_2/M transition. This effect on cell cycle progression of treated root meristems appeared clearly 10 h after BOA exposition (Fig. 4). Further

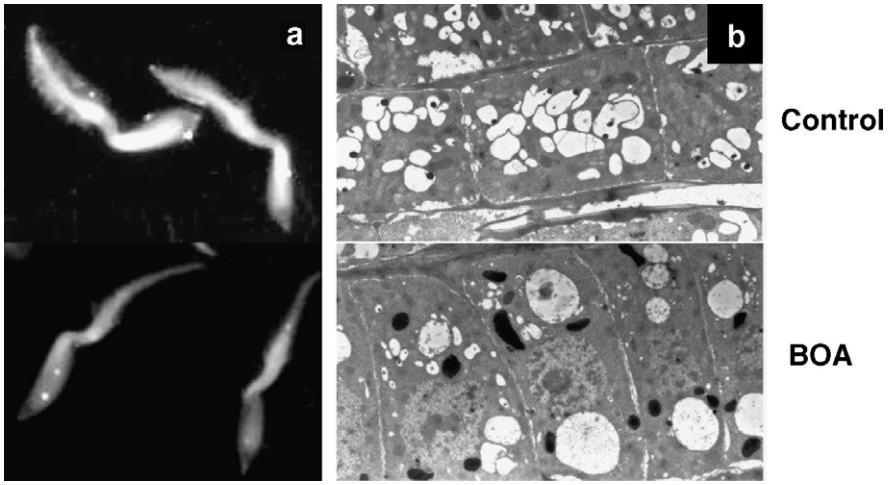

Fig. 2. (a) Pictures of control and 1 mM BOA-treated lettuce seedlings and (b) transmission electron microscopic images of lettuce root tip cells grown for 6 h in distilled water (controls) or 1 mM BOA. Pictures from Aliotta, G. and Cafiero, G.

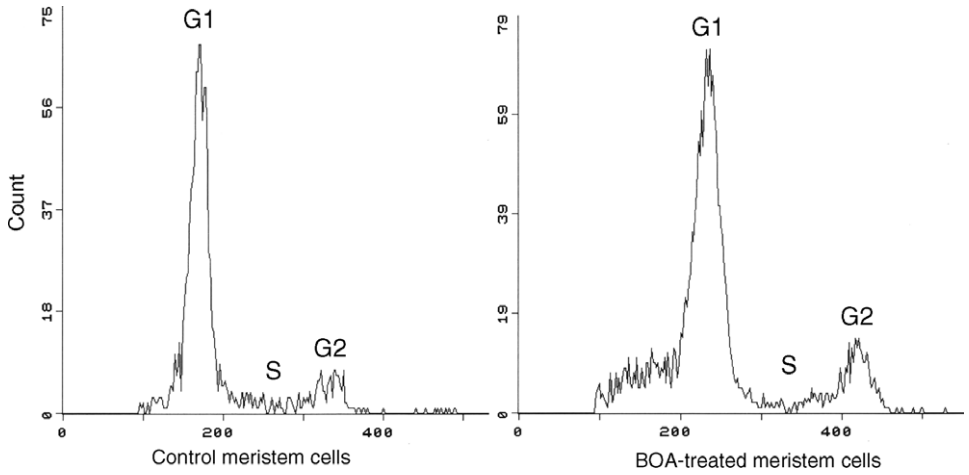

Fig. 3. Comparative cell cycle analysis (DNA content) of 4 h control and 1 mM BOA-treated lettuce meristems. This experiment was done in unsynchronized cells from 3 mm long seedlings. Raw data are shown here because the low amount of dividing cells made impossible their statistical treatment.

light is thrown on this behavior by the mitotic index results presented below.

2.4. Mitotic activity

The staining procedure described under experimental allowed easy identification of cells in the various phases of mitosis (Fig. 5). Mitotic index confirmed flow cytometry results, but gave additional information showing a retard (and not only a blockage) in cell cycle progression for BOA-treated meristems compared to control meristems.

The mitotic index of control root tips was zero 2 h after removal of hydroxyurea, peaked sharply at 36% at time 4 h, and by time 8 h had fallen to less than 10%. By contrast, the mitotic index peak of BOA-treated root tips was lower (18%), later (at time 6 h) and broader (2–12 h), showing that BOA caused a variable delay in their progression as they passed through the G_2/M transition, apparently with the result that the rate of exit from G_2 became equal to the rate of entry (Fig. 4). With flow cytometry results, we only detected an impasse in treated meristems before mitosis; however, at time 14 h, both BOA-treated and control root tips had regained normal mitotic index values.

Control of cell cycle in response to BOA resulted in a modulation of cell division activity followed by an adaptive growth response.

3. Discussion

We can conclude that the selective effect of BOA on cell cycle at G_2/M checkpoint in lettuce root meristems is an important mechanism of action of this compound, which explains the previously detected seedling growth inhibition.

3.1. Seedling growth inhibition and morphology

For investigation of the effects of an exogenous agent on root growth, treatment of pre-germinated seedlings is preferable to treatment of seeds because in this latter case effects on root growth may be confounded with effects on germination. In this study, BOA dose-dependently slowed or limited the growth of pre-germinated lettuce seedling roots, and suppressed the growth of root hairs.

Microscopy suggested that the slowed growth is probably not due to an effect on cell elongation, but to an effect on cell division in the apical meristem, which displayed cells that were larger, and had fewer and larger vacuoles, than the meristem of control roots. Burgos *et al.* found that cucumber roots treated with 0.3 mg/mL BOA showed decreased production of root hairs and lateral growth, enlargement of cortical cells above the root tip, and multiple columns of cells growing in width but not in length (Burgos et al., 2004). The present results show that lettuce seedling roots suffer similar effects, even at physiological concentrations of BOA (0.1 mg/mL). The smaller number and larger size of vacuoles in the meristem cells of BOA-treated roots may reflect a detoxification process whereby BOA is neutralized and sequestered in vacuoles, as in *Arabidopsis* (Baerson et al., 2005); large vacuoles in white mustard roots exposed to the secondary metabolites gramine and hordenine have also been implicated in lytic detoxification processes (Liu and Lovett, 1993).

3.2. Unaffected mitotic activity

In this study, treatment with 1 mM BOA lowered the numbers of cells in all stages of mitosis except prophase, which was observed in more BOA-treated than control cells (results not shown). However, BOA did not cause chromosomal alterations detectable by simple staining and microscopy. In particular, metaphase and anaphase configurations, which can be affected by certain plant secondary metabolites (Dayan et al., 1999), were quite normal in appearance, and no condensation abnormality was detected in prophase or metaphase. Thus the effects of BOA at the G_2/M transition, which recall those of other natural compounds (Chui-Wah et al., 2005; Kuras et al., 2006), appear not to be associated with direct effects on the machinery of mitosis.

3.3. Cell cycle retard

The control of cell division in plants involves numerous factors, including plant hormones and growth regulators, the machinery of cell cycle regulation (cyclins, cyclin-dependent kinases and their inhibitors, etc.), and the evolution of plant-specific processes such as cell wall metabolism (Menges et al., 2002).

In recent years, light has begun to be thrown on the way in which progression through the cell cycle may be modulated as a coordinated part of the response to various kinds of biotic and abiotic stress. For example, Reichheld et al. (1999) found evidence of a mechanism in tobacco cells that responds quantitatively to redox status signals by modulating the expression of both core cell cycle genes and antioxidant genes. Reichheld et al. demonstrated also a variety of plant responses in front of oxidative stress via regulation

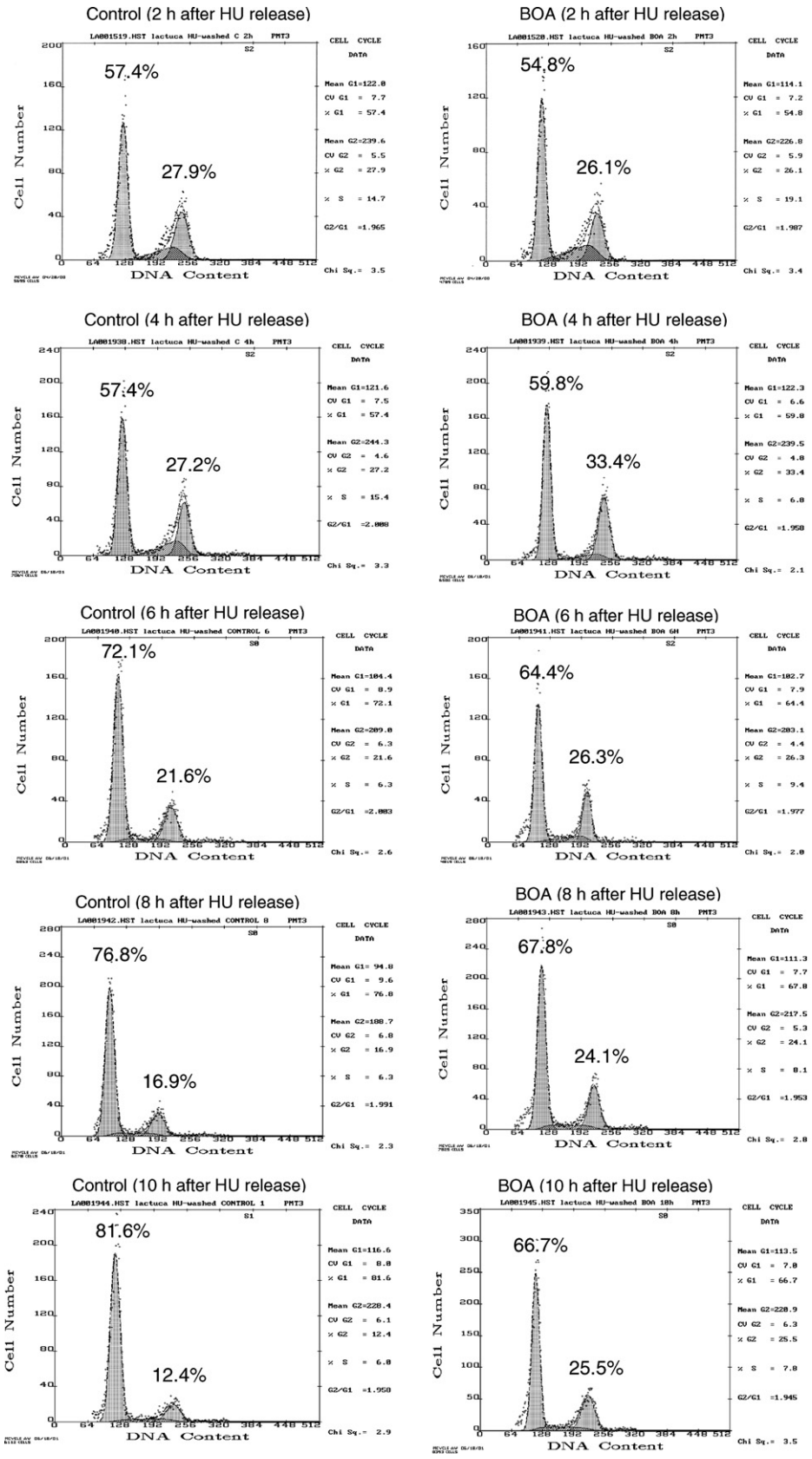

Fig. 4. Flow-cytometric DNA content histograms of hydroxyurea-synchronized lettuce root tips 2, 4, 6, 8 and 10 h after removal of the synchronization agent and transfer to distilled water (controls) or 1 mM BOA solution.

of different checkpoints in cell cycle depending on the oxidative stress degree. Cell cycle arrest as consequence of the retard of DNA replication and the delay of the start of mitosis has been associated with an inhibition of the activity of cyclin-dependent

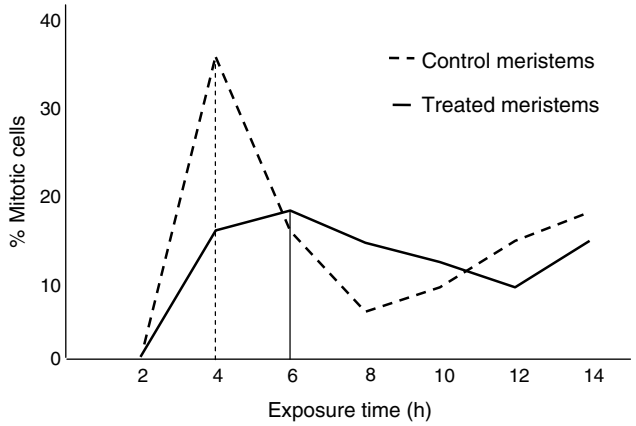

Fig. 5. Mitotic index of hydroxyurea-synchronized lettuce root tips at 2-h intervals for 14 h after transfer to distilled water (controls) or 1 mM BOA solution.

kinases, cell cycle gene expression, and a concomitant activation of stress genes (Reichheld et al., 1999; De Veylder et al., 2007; Peres et al., 2007). When DNA stress checkpoints are activated, the induction of DNA-repair genes and the inactivation of genes required for mitosis go on. This signaling cascade after biotic or abiotic stress affects plant metabolism and "stimulates cell cycle checkpoints, results into impaired G_1 to S transition, slows down DNA replication, and delays entry into mitosis" (Peres et al., 2007), which will result in cell cycle arrest.

In previous work (Sánchez-Moreiras and Reigosa, 2005) we showed that mature lettuce plants respond to BOA as to an oxidative stressor, and we hypothesized that this response involves the generation of reactive oxygen species (ROS) and early senescence. It has also been shown that BOA-treated *Arabidopsis* seedlings activate protein families known to participate in cell rescue and defense; most of them through detoxification mechanisms (Baerson et al., 2005). In this studies, the upregulation of defensive genes was accompanied by the downregulation of genes involved in development and cell biogenesis (Sánchez-Moreiras, 2006), including cyclins, the regulatory proteins that interact with cyclin-dependent kinases (CDKs) to control the course through the various stages of the cell cycle and promote both G_0/G_1/S and S/G_2/M progression (Qin et al., 1996; Torres Acosta et al., 2004) Some studies detected also higher cyclin mRNA contents in G_2 to M phases (Mironov et al., 1999) along the cell cycle. The retardation of passage through the G_2/M checkpoint that was observed in the present study is in keeping with these earlier findings.

The findings of this study of lettuce seedling roots suggest that it is cell division rather than cell elongation that is affected by BOA. This is in keeping with earlier reports that BOA inflicts oxidative damage (Baerson et al., 2005; Sánchez-Moreiras and Reigosa, 2005; Batish et al., 2006), and with the hypothesis that by altering redox status, and thereby causing raised levels of ROS, BOA triggers the activation of stress-inducible genes, the possibly dose-dependent closure of cell cycle checkpoints, and the accumulation of antimitogenic hormones such as abscisic acid and jasmonic acid (Liu and Lovett, 1993; Świątek et al., 2002; Kadota et al., 2004).

Specifically, this work supports the hypothesis that in lettuce seedling root meristems BOA selectively retards cell cycle at G_2/M checkpoint.

4. Experimental

Aqueous solutions of 2-benzoxazolinone (BOA; from Aldrich Chemical Co., Ref. 157058) of pH 6.0 were made up at concentrations of 1×10^{-5}, 1×10^{-3}, 0.1, 0.3, 0.6, 1, 3 and 6 mM using distilled water. *L. sativa* cv. Great Lakes (California) was used as target species in the present work (Macías et al., 2000).

4.1. Seedling growth

Growth bioassays were designed to be fast and reproducible with a target species that shows easy germination conditions, and fast and homogeneous growth. After the bioassays, growth dose-response curves were established. To characterize the root growth response of the lettuce seedlings used in this work when exposed to BOA, and thereby establish the range of BOA concentrations to be used in the subsequent experiments, dose-response curves were constructed as follows (Rice, 1984; Inderjit and Dakshini, 1995).

For each treatment level, 25 pre-germinated seeds of *L. sativa* (radicle length: 1–3 mm) were placed in a Petri dish (9 cm ∅) on Whatman 3 MM paper soaked in 4 ml chemical solution or distilled water (control). The Petri dish was then placed in a thermostatically controlled chamber (6/18 h photoperiod at 150 μmol m^{-2} s^{-1} and 20/25 °C of temperature) for radicle growth. Radicle length was measured 48 h after BOA exposition. Statistically significant differences between BOA treatments and controls with respect to growth response were identified by ANOVA followed by LSD tests. Mean growth response, expressed as a percentage of the mean growth response of controls, was plotted against BOA concentration to determine IC_{50} and IC_{80} (the concentrations required for 50% and 80% inhibition, respectively) and the LCIC (lowest complete inhibition concentration). These measurements established the range of BOA toxicity and determined the concentration for further cell cycle analyses.

4.2. Morphological examination

Root tips excised from seedlings grown as described above for 6 h on Whatman paper soaked in distilled water or BOA solution of concentration 1 mM (just slightly greater than the IC_{50} of BOA; see Section 2) were fixed for 2 h at room temperature in a 3% solution of glutaraldehyde in 0.065 M phosphate buffer of pH 7.4, and were then passed through a 2% solution of OsO_4 in 0.1 M phosphate buffer of pH 6.8 at 4 °C, dehydrated with ethanol and propylene oxide, and embedded in Spurr's resin. Thin sections, cut with a diamond knife on a Supernova microtome, were stained for 5 min in 2% aqueous uranyl acetate followed by 10 min in lead citrate (at room temperature), and were examined by transmission electron microscopy (TEM) using a Philips CM12 apparatus at 80 kV.

This study was done in collaboration with Dr. Giovanni Aliotta and Dr. Gennaro Cafiero from the Life Science Department in the 'Seconda Università degli Studi di Napoli' (Italy) and the Interdepartmental Center of Ultrastructural Biology (CIRUB) in the University of Naples "Federico II" (Italy), respectively.

4.3. Cell cycle analysis

In the present work, and for the first time, *L. sativa* root meristems were synchronized using the inhibitor hydroxyurea, after checking the overall duration of cell cycle in unsynchronized cells. HU reversibly halts the cell cycle in the G_1 and early S phases by inhibiting ribonucleotide reductase, and hence the production of deoxyribonucleotides (Doležel et al., 1999).

Seedlings with roots 1–3 mm long were obtained by germinating seeds in the dark for 20 h on moistened filter paper at 27 °C. These seedlings were transferred, 20 per dish, to Petri dishes 9 cm in diameter containing filter paper moistened with 5 ml of 2.5 mM HU (pH 6.0). After 6 h incubation in the dark at 27 °C, the seedlings were cleansed of hydroxyurea by three washes in distilled water of pH 6.0 (Coba de la Peña and Sánchez-Moreiras, 2001).

The exposition to HU induced blockage and accumulation of nuclei in G_1 phase. After inhibitor removal, a synchronous cell population, around 25% of total recorded nuclei, was detected in progression through S and G_2 phases (%S is about 10–12% in asynchronous lettuce meristems). In synchronized plant cell cultures, S nuclei can represent more than 50% of total population. Fig. 6 shows some steps of synchronous cell cycle progression at different times after HU removal, showing both raw flow cytometry histograms (left) and the corresponding Multicycle-treated data (right). HU synchronization is favoring the accumulation of cells during cell cycle making its analysis much easier. Immediately after HU removal, $G_0 + G_1$ phase represents the 79% of detected nuclei, G_2 phase is around 8%, and S phase represents around the 12% (Fig. 6a). Start and advance of synchronized nuclei in S phase (29.6% of detected nuclei) was already detected 30 min after HU washing (Fig. 6b). One hour later, synchronized nuclei population began to incorporate into G_2 phase (Fig. 6c). Finally, all synchronized nuclei were incorporated into G_2 phase 2 h after HU release (Fig. 6d). G_2 population, usually near 10% in non-synchronized meristems, reached amounts near 25%. After this time, mitosis started with several metaphasic chromosomes.

In the experimental procedure establish to measure BOA effects, synchronized seedlings were transferred to other Petri dish to start the treatment immediately after HU washing. The seedlings were washed dedicatedly three times with distilled water at pH 6.0 to remove inhibitor. Seedlings were then transferred to Petri dishes containing filter papers moistened with 4 ml of either distilled water of pH 6.0 (controls) or a pH 6.0 solution of BOA at a concen-

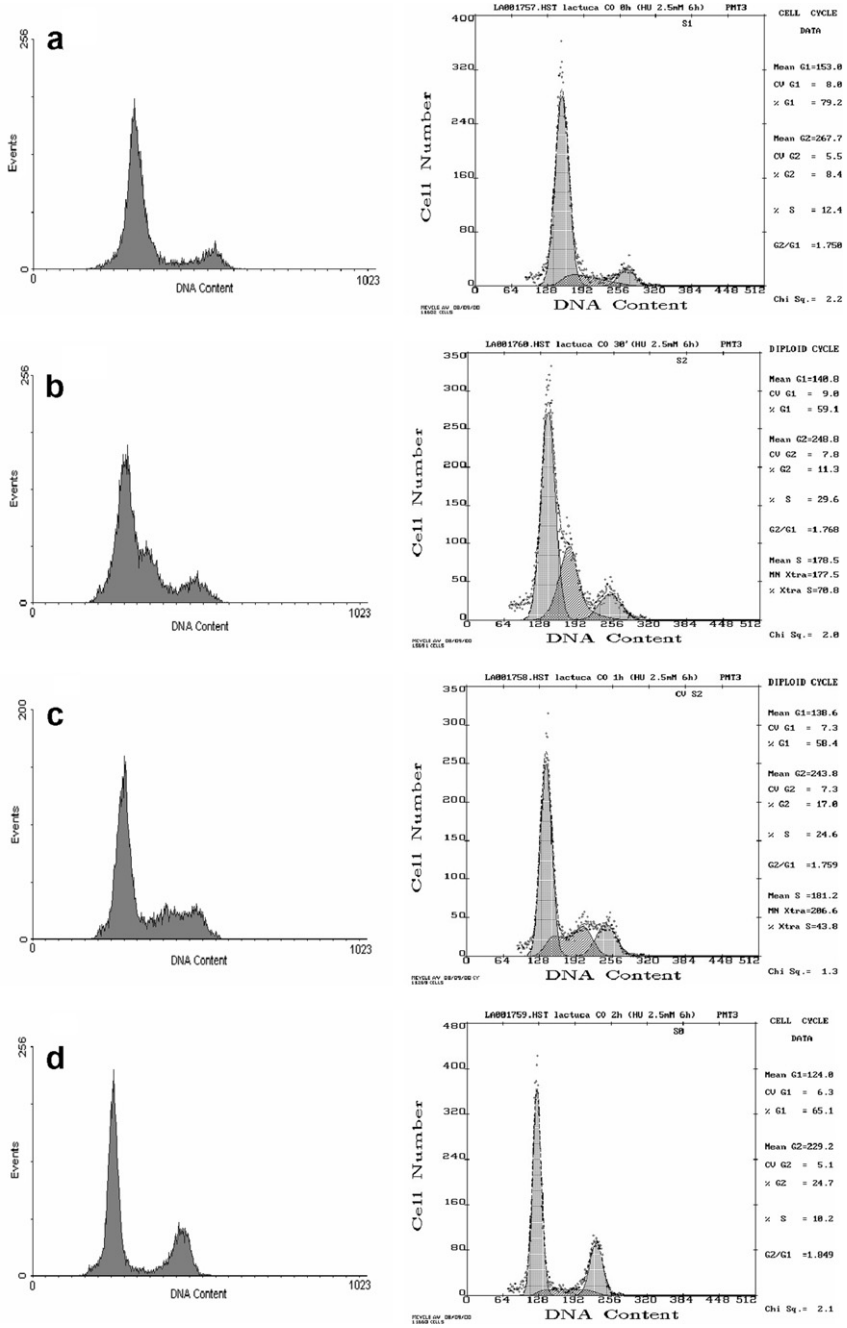

Fig. 6. Flow-cytometric DNA content histograms of hydroxyurea-synchronized lettuce root tips immediately after removal of the synchronization agent (a), and after 30 min (b), 1 h (c) and 2 h (d). Right graphics show raw data and left graphics show multicycle processed data.

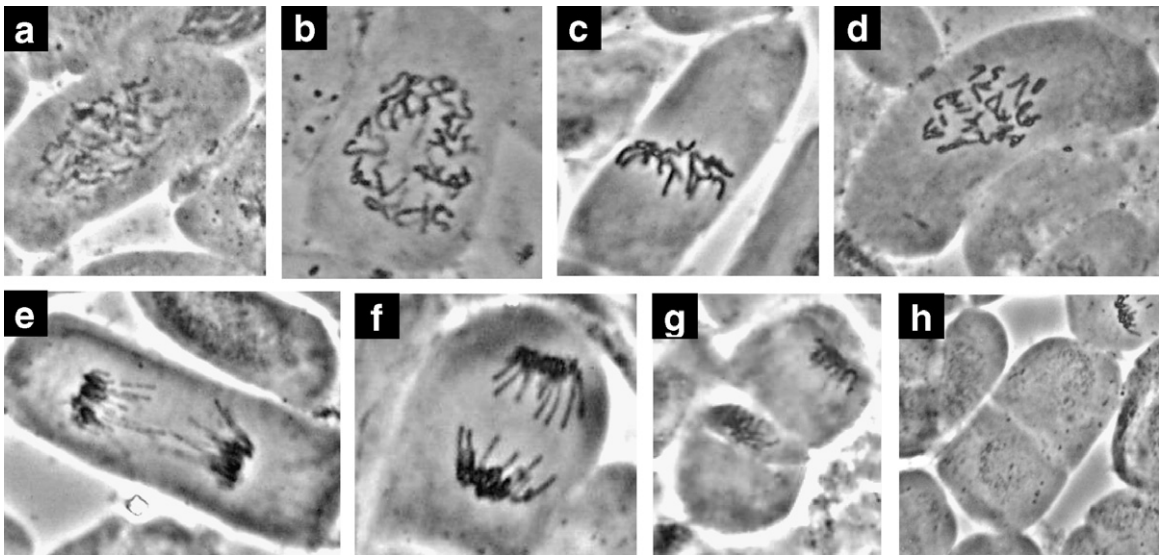

Fig. 7. Optical photomicrographs of lettuce seedling root tip meristem cells during mitosis and cytokinesis after 1 mM BOA treatment: (a,b) prophase, the nuclear envelope begins to disintegrate and chromosomes appear as two identical chromatids joined at the centromere; (c,d) metaphase, the chromosomes move along the mitotic spindle to occupy the equatorial plane; (e,f) anaphase, sister chromatids separate and migrate to opposite poles of the mitotic spindle; and (g,h) Telophase and cytokinesis, a new nuclear envelope forms around each set of chromosomes and new cell wall sections are constructed to separate the daughter cells.

tration of 1 mM (just slightly greater than its IC_{50}; see Section 2); and incubated in the dark at 27 °C. For the next 14 h, 40-seedling samples of BOA-treated and control seedlings were taken every 2 h and were simultaneously processed as described below. This experimental procedure was replicated three times after independent HU synchronizations and BOA treatments.

The apical 1 mm of the root of each seedling was removed and chopped in 700 μl of a 1:1:1 (v/v/v) mixture of Tween 20, β-mercaptoethanol, and Galbraith's nuclear buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS, 0.1% (w/v) Triton X-100; pH 7.0). The resulting mixture was filtered twice through 30 μm mesh nylon filters, and the filtrate (500 μl) was collected in a microcentrifuge tube, treated with 5 μl of 1% RNase solution followed immediately by 30 μl of a 10 mg/ml solution of the nucleic-acid-specific dye ethidium bromide, and incubated for 30 min at room temperature before cell DNA contents were evaluated by flow cytometry using a Coulter Elite apparatus with a 488 nm excitation laser. For each sample, at least 10,000 nuclei were analyzed. Histogram profiles were calculated from DNA content histograms using Multicycle software (from Phoenix Flow Systems, San Diego, CA, USA). This program allows a suitable estimation of peak shape, CV of each nuclei population, the percentages of nuclei in the G_0/G_1, S and G_2 phases, background subtraction, and chi-square (χ^2) estimation of fitting between raw data and estimated data. With these analyses $G_0 + G_1$, S and G_2 populations were estimated in control and BOA-treated seedlings.

4.4. Mitotic activity

Since the flow-cytometric method described above distinguishes only intact nuclei, it is insensitive to mitotic cells. For a correct evaluation of G_2, M, and G_1 lengths, mitotic indices (percent of mitosis) were evaluated complementarily to flow cytometry. In this way, with the study of flow cytometry and mitotic index, the knowledge of the accurate stage of every cell cycle phase was possible. Thanks to this technique we could easily detect the complete mitotic event (Fig. 7), which allowed differing prophase (a,b), from metaphase (c,d), anaphase (e,f) and telophase (g,h).

Mitotic indices were determined by conventional staining methods as follows. The roots of seedling samples obtained and synchronized as above were fixed over 24 h in a 6:3:1 mixture of acetic acid, chloroform and ethanol containing iron traces, and were then stored at −20 °C for at least 3 days in fresh fixative with no iron traces (Sánchez-Moreiras et al., 2001). The roots were then macerated for 25 min with 1 N HCl at 60 °C and stained for 10 min with Schiff's reagent, after which the root tips were immersed in a little acetic acid, their meristems were excised and counterstained with a drop of acetocarmine on a microscope slide, the slide was heated, the meristems were squashed under a coverslip to separate their cells, and 1000 cells were examined under a microscope to determine the mitotic index. Three replicate slides were examined per treatment.

Acknowledgements

Authors thank Nieves Redondo and Marina Horjales for allowing and helping us to carry out this work in their laboratory; Ana Martínez and Alfonso Blanco for helping with cell staining in the mitotic activity study; Claudia Cárcamo and Oliver Weiss for helping with flow cytometry; and Giovanni Aliotta (Life Sciences Department, Second University of Naples) and Gennaro Caffiero (CIRUB, Federico II University, Naples) for their collaboration with microscopy and beautiful photomicrographs.

This work has been supported by the Project BFI2000-0987 from the Spanish Ministry of Science, and the Project PGIDT00PX30114PR from the Galician Government. Dr. Sánchez-Moreiras worked with a Grant (FPI) from the Spanish Ministry of Education.

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