J. Plant Biotechnology (2005) Vol. 7 (3), pp. 161~167

Journal of Plant Biotechnology

24-Epibrassinolide Modulate Cellular and Organogenic Response of Explants of *Brassica* Species, *in vitro* Culture

Andréa da S. R. Rocha[1], Camila M. Coutinho[1], Eugenia J. B. Braga[2], José A. Peters[2], Pedro Canisio Binsfeld*

[1]Centro de Biotecnologia e [2]Departamento de Botanica da Universidade Federal de Pelotas, UFPel, Campus Universitario, Caixa Postal, 354, CEP 96010-900 - Pelotas, RS, Brazil

Abstract

Brassinosteroids are steroidal plant hormones and are known to modulate physiological and cellular response in a wide range of plant species. Considerable insights has been achieved of the physiological role of brassinosteroid in Brassica species in the past few years, but their effect on direct organogenesis has not been extensively studied. In this sense, under optimal basal media and growth conditions we tested the cellular and organogenic response of 24-epibrassinolide (EBL) in a variable concentration (0.1 to 5.0 μM) and Zeatin (Z) (1.0 to 100 μM) and their synergic effect on hypocotyl explants of cauliflower and broccoli. The isolated EBL accelerated cell elongation and promotes direct organogenesis. One micromolar EBL + 10 μM of Z was the most efficient combination for cell elongation, cell differentiation as well as for organogenesis. A suppressing effect on root induction was confirmed for all the tested hormone levels. The general results indicate a synergic effect of EBL-Z and EBL potentates Zeatin activity, at least in certain tissues. Besides de genetic factors, we can speculate that the natural hormone concentration in the explants might affect the responses by application of exogenous growth regulators. Experiments with new plant growth regulators, like brassinolide, are important aiming to maximize or accelerate plant regeneration for *in vitro* multiplication or for genetic transformation.

Key words: Brassinolide, Cell elongation, Cell growth, Callus, Organogenesis, Shoot induction

* Corresponding author, E-mail: binsf@terra.com.br
Received Apr. 16, 2005; Accepted Sep. 26, 2005

Introduction

Steroid hormones are signaling molecules important for normal growth, development and differentiation of multicellular organisms. Brassinosteroids (BRs), which include brassinolide (BL), are a relatively new group of natural plant steroid hormones that are found in many plant species. It is widely accepted that BRs are important regulators of plant growth and development (Grove et al. 1979; Friedrichsen and Chory 2001; Sasse 2003; Kinoshita et al. 2005). However, in comparison to the other classical plant hormones, such as auxin and cytokinin, relatively little is known about BRs as modulators and its potential role in the regulation of organogenesis in the in vitro experiments.

Recent studies on BR-insensitive and BR-deficient mutants have convincingly confirmed evidence for an essential role of BRs in plant growth and development (Clouse and Sasse 1998; Sasse 2003). Physiological responses of BRs include effects on elongation and fission, cell division, vascular development, hormonal balance, enzyme activity like H+-pump activation, activation of protein and nucleic acid synthesis, stress modulation, cell cycle regulation and effect on hormonal balance (Clouse and Sasse 1998; Khripach et al. 2000; Sasse 2003; Bishop 2003; Symons and Reid 2004). BRs causes pronounced elongation of hypocotyls of dicots (Clouse 1996), but they can inhibit as well promote primary root extension and lateral root formation (Mussig et al. 2003). At molecular level, both animals and plants use steroids as signaling molecules principally recognized by members of the nuclear receptor superfamily of transcription factors. In plants, the kinase receptor BRI1, localized on the plasma membrane, is a crucial component of a receptor complex for BRs (Kinoshita et al. 2005). Besides signaling molecules, BRs act on transcriptional by binding directly to the promoter of hormone

responsive genes and post-transcriptional regulation by mRNA stabilization as well as regulation cyclin genes (Clouse and Sasse 1998; Sasse 2003; Bishop 2003; Kinoshita et al. 2005).

Brassinolide used *in vitro* culture promote cell proliferation in tobacco BY-2 cells (Miyazawa et al. 2003), in the presence of auxin and cytokinin, increased in 50% the cell number in cultured explants of *Helianthus tuberosus* (Clouse and Zurek 1991), increases the rate of cell division of Chinese cabbage (Nakajima et al. 1996) and *Petunia hybrida* protoplasts (Oh 2003). Promote cell elongation in cell suspension cultures of carrot (Bellincampi and Morpurgo 1988). Brassinolide promote callus growth and regeneration in *Spartina patens* (Lu et al. 2003), improves embryogenic tissue initiation in conifers (Pulmann et al. 2003) or somatic embryogenesis of *Cocos nucifera* (Azpeitia et al. 2003) and promotes adventitious shoot regeneration from cauliflower hypocotyl segments (Sasaki 2002). In the presence of BL, explants curvature, swelling and splitting of the internodes, which was attributed to increase cell division on the explants (Ho 2003). EBL promoted cell enlargement and induces adventitious root formation in hypocotyl segments of soybean (Sathiyamoorthy and Nakamura 1990).

Although many BRs, such as EBL are commercially available and employed in some tissue culture experiments, more accurate studies on tissue-specific and species-specific effects, concentrations and the dual (synergic or antagonistic) effect with growth regulators are desired. In this paper, we report that EBL in presence or absence of Zeatin can be effectively used to modulate cellular growth, shoot bud induction and plant regeneration of *Brassica* species *in vitro* culture.

Materials and Methods

Plant material and chemicals

The *Brassica* species used in this study includes cauliflower (*Brassica oleracea* var. *botrytis* L. cv. Piracicaba Precoce) and broccoli (*Brassica oleracea* var. *italica* Plenck cv. Green storm). The seeds were purchased from ISLA. The 24-epibrassinolide (EBL) was obtained from the Institute of Plant Molecular Physiology and Biotechnology (University of Bonn, Germany). The MS medium with vitamins was purchased from Duchefa Biochemical BV (Harlen, NL). The gelling agent Agar was purchased from Vetec Quimica Fina Ltd, Brazil. The cytokinin, Zeatin (Z) was a product of Riedel-deHaen (Sigma-Aldrich). A stock solution of 1 mg/mL was prepared from EBL dissolved in absolute methanol and Zeatin dissolved in 1N NaOH.

Seed sterilization and plant growth

Seeds of cauliflower and broccoli were surface-sterilized by immersion in alcohol 70% (v/v) for 30 seconds, followed by 6 min in sodium hypochlorite solution, 1% (v/v) final concentration (with a few drops of tween 20) and finally rinsed three times in sterile distilled water. For tissue culture assay, the aseptic seeds were germinated and grown for 8 days on flasks containing 1×MS medium (Murashige and Skoog 1962) + 2% (w/v) sucrose and 0.7% Agar, in a dark growth chamber at 24±1℃. For explant isolation, uniform etiolated plants were selected and the central segments of 4-5 cm from the etiolated hypocotyls (9-10 cm) were detached. These segments were sliced into experimental unit explants of 5-mm-long sections.

Tissue culture assay

For cellular and organogenic response evaluation, the sliced 5-mm-long sections of hypocotyl explants were placed randomly onto the basal growth media. Each Petri dish (90 ×20 mm, J.Prolab) represent a single treatment in which seven segments of each species were growh in 20 ml of MS medium with mineral salts and vitamins (Duchefa, M0222^{tm}), 2% (w/v) sucrose, and 0.7% Agar (Vetec^{tm}). All these basal media were adjusted to pH 5.8±0.1 before autoclaving for 15 min at 120℃. The media were supplemented with two different plant growth regulators, EBL and Z (Table 1). The first treatments consist of EBL in variable concentration (0, 0.1, 1.0 and 5 μM), the second treatments consist of a constant concentration of EBL (1.0 μM) and variable concentration of Zeatin (0, 1.0, 10 and 100 μM) and the third treatments consist of Zeatin in a variable concentration (0, 1.0, 10 and 100 μM). The experiment for cell elongation and proliferation was conducted for 8 days in a dark growth chamber at 24±1℃. The experiment for root and shoot induction and plant regeneration was conducted for 8 weeks in white fluorescent (*GE*, Universal) light with a photoperiod of 16 h light (40 μE $m^{-2}s^{-1}$) and 8 h dark at 25±1℃. Rege-

Table 1. Basal media used in the different treatments, with their respective concentrations of 24-epibrassinolide (EBL) and Zeatin (Z), shown in micromolar (μM).

Treatments	Basal Media	Hormones	Treatment levels (μM)
Control	MS	absence	0.0
First	MS	EBL	0.1; 1.0; 5.0
Second	MS	EBL + (Z)	1.0 + (1.0; 10.0; 100.0)
Third	MS	Z	1.0; 10.0; 100.0

nerated shoots after 8 weeks in culture were excised and cultivated in hormone free MS medium for root induction.

Experiment evaluation and analysis

Each data point was replicated seven times, and each complete experiment was repeated two times in a completely randomized design. The explants of the experiment were observed using a stereomicroscope (Zeiss Stemi SU11), during the experimental period. The experiment was evaluated for the following culture-response-variables: Cell elongation, cell-division and proliferation, root induction and development, shoot induction and plant regeneration. The relative growth was calculated according to the following equation (Fw=final weight; Iw=inoculum weight), RG= [(Fw-Iw)/Iw] x 100. The data were submitted to statistical analysis and the results presented as the average percentages with standard deviation.

Results and Discussion

In our study, the postulation was that EBL is an important modulator for cellular and organogenic response of cauliflower and broccoli explants. The experimental results allowed the corroboration of this postulation for cell elongation, cell division, vascular cell growth, root growth suppression as well as played a key role by bud induction and shoot regeneration. Since it is well known that EBL is involved in plant stress control by the accumulation of heat-shock proteins (Dhaubhadel et al. 2002; Sasse 2003), we also attribute a positive effect on the down regulation of tissue culture stress by using EBL which probably minimize the stress of the tissue culture process.

Effect of 24-epibrassinolide on cellular response

As predicted for BRs physiological activity, EBL promotes pronounced elongation of hypocotyl slices of cauliflower and broccoli (Figure 1A). The general cellular response, measured on cell elongation, cell division, cell proliferation, root growth suppression (Figures 1B, 1C and 3) showed remarkable differences between cauliflower and broccoli for all the evaluated variables. Since the growth conditions were strictly identical, we attribute these differences between the species to their genetic variability, to the physiological state of the explants and exogenous hormone concentration. Probably the natural hormone concentration in the explants was different, leading to different responses by application of exogenous EBL and Zeatin.

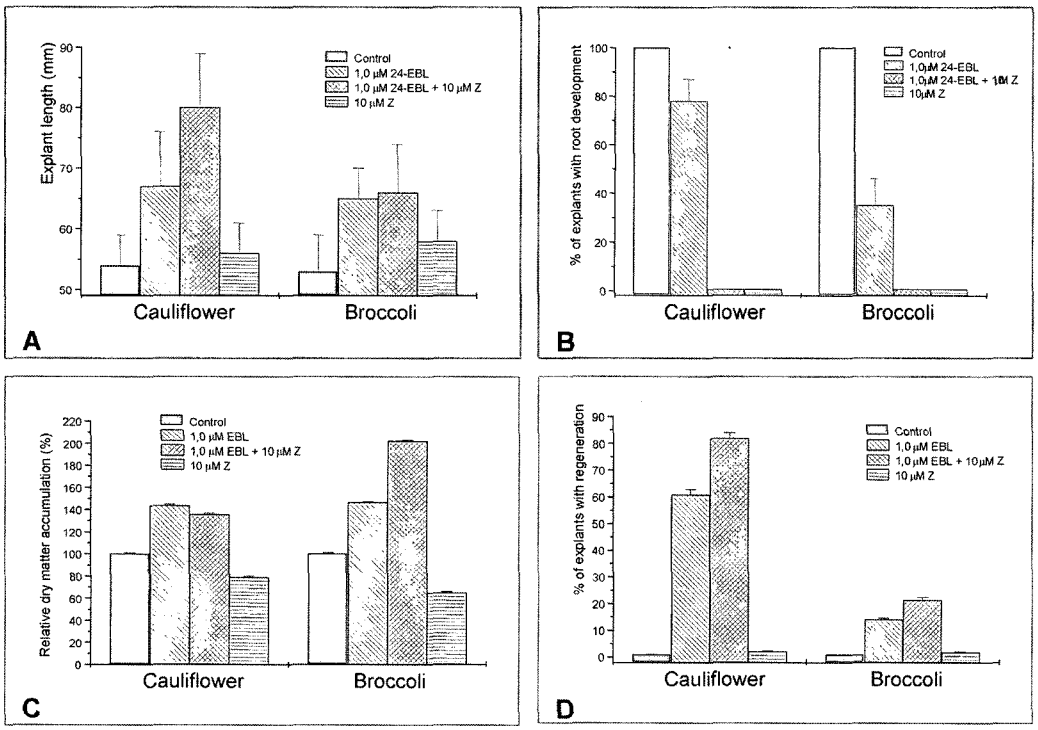

Figure 1. Effects of 24-epibrassinolide (EBL) and Zeatin (Z) on cauliflower and broccoli explants. (A) Cell elongation measured on explant length, (B) effect of EBL and Z on root growth and development, (C) relative cell growth measured on the dry matter accumulation, (D) efficiency of shoot bud induction and regeneration on different treatments. Average values (±standard deviation) from two independent experiments are shown.

In both species, EBL was more active than Zeatin for cell elongation, cell division and cell proliferation (Figure 1), showing to be an efficient substitute for cytokinin in plant cell growth. Early reports corroborate that EBL modulate cellular response when used as a substitute for cytokinin in protoplast culture of *Petunia hybrida* (Oh 2003) and in callus culture and suspension cells of *Arabidopsis* and epibrassinolide induced a up-regulation of the cyclin gene *cycD3*, resulting in cell proliferation independent of auxin presence (Hu et al. 2000; Miyazawa et al. 2003). A constitutive over expression of *cycD3* gene affected the leaf anatomy markedly, suggesting a central role of this gene in the transition from cell proliferation to differentiation (Dewitte et al. 2003).

In spite of the efficient action of EBL, the most significant results were achieved when EBL + Zeatin were combined (Figure 1), reflecting an additive or synergistic responses for cell elongation, proliferation and differentiation. A synergic effect between several plant hormones has been reported for brassinolide (Sasse 1989). Besides these synergic effects, we also observed individual cell elongation (Figure 2A), a strong vascular cell proliferation (Figure 2B), pronounced cell division and callus growth (Figure 2C), root curling and suppressing effect on root differentiation and development (Figure 2G), as well as explants swelling and splitting and high cell proliferation related to a low cell differentiation in the highest concentration of (5 μM) EBL + (100 μM) Zeatin (Figure 2D). Frequently, in the highest concentration of EBL, an explants curvature was observed (Figure 2E) this was attributed to a local increased cell elongation (Symons and Reid 2004). The wide range of responses allows us to deduce that EBL is involved in signaling path that act or modulates gene expression or biochemical processes that change the hydralic and osmotic properties of cell membrane and cell wall, changing cellular response according to the intensity of the signals (Sasse 2003; Kinoshita et al. 2005). Morillon et al. (2001) hypothesized about the role of BL in the modification of the aquaporin activities and water-transport properties of cell membranes. Whatever the molecular signals, cell elongation is contingent upon transport of uncharged osmotic ions, water across the cell and vacuolar membranes and a plastic cell wall. Modulation of gene expression involved in activation of the cell cycle by cyclin genes up-regulation as well as the modulation of transcription factors of genes for expansins, extensions and aquaporins are mechanisms by which plant hormones, such as BRs promote cell elongation. It is also known that many of such genes are regulated by BRs (Kinoshita et al. 2005; Oh 2003; Sasse 2003).

Besides its known functions in cell elongation, several reports have also proposed that BL plays a key role in proliferation (Figures 1C, and 2C). It has also been shown that BL treatments enhanced the activities of RNA and DNA polymerases and increase the protein levels in the cell suggesting a direct action in nucleic acid and protein synthesis

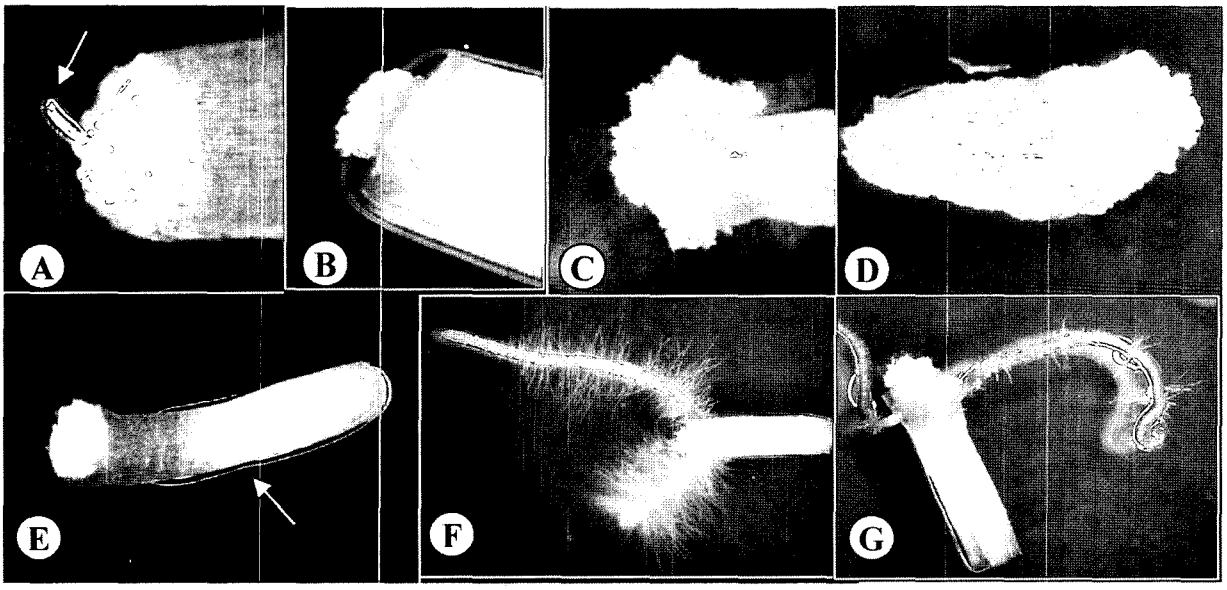

Figure 2. Photomicrographs of 24-epibrassinolide-treated explants showing cellular response: (A) individual evident cell elongation, (B) strong vascular cell proliferation, (C) pronounced cell division and callus growth, (D) cellular swelling and explant splitting in high 24-epibrassinolide concentration, (E) local cell elongation induce explant curvature in high 24-epibrassinolide concentration (F) absence of 24-epibrassinolide in the growth medium, induce a normal root and root hair development and (G) root curling and a suppressing or antagonistic effect on root differentiation and development was observed.

(Kalinich et al. 1985). Exogenous application of highly purified BL, caused not only cell elongation in the second internodes of bean, but also curvature, swelling and splitting of the internodes, as a result of increased cell division (Grove et al. 1979). Lu et al. (2003) reported that in *Spartina patens*, BL promoted cell division and callus growth at nanomolar concentrations. BL in the presence of auxin and cytokinin, caused at least a 50% increase in the total number of cells in cultured explants of *Helianthus tuberosus* after 24 h, showing a strong synergic effect of BL on cell proliferation (Clouse and Zurek 1991). Nakajima et al. (1996) and Oh (2003) also found a significant enhancement of cell-division rates upon the addition of EBL to the culture medium of Chinese cabbage mesophyll protoplasts and *Petunia hybrida* protoplasts.

Organogenic process requires a strict balance between cell proliferation and differentiation, which depends on a balanced interaction of plant hormones. In our experiment a synergic effect between EBL and Zeatin were beneficial for cell elongation, proliferation and cell differentiation

Effect of 24-epibrassinolide on organogenic response

For an efficient wide-ranging organogenic response, auxin and cytokinin are required. However, evidence continues to mouth that BRs may also play a significant role in cell differentiation and organogenic response. The present experimental results corroborate that EBL plays an important role on cell differentiation for shoot bud induction and regeneration but showed a suppressing effect for root growth. A significant difference between cauliflower and broccoli has been observed for bud differentiation, shoot regeneration and for root growth (Figure 1D). These differences probably have a genetic and physiological background leading to different responses by application of exogenous BRs. Gaudinova et al. (1995) and Oh (2003) have indicated that BR treatment altered the endogenous auxin and cytokinin levels in callus cultures, thus impact the modulation of cell differentiation (Mussing et al. 2003).

The EBL was effective for stimulation of bud induction and shoot regeneration from hypocotyl explants of cauliflower and broccoli (Figures 4A, 4B and 4C). The highest percentages of regeneration were obtained for 0.1 or 1.0 μM EBL in which 78 % of explants formed shoot buds. A maximal number of shoot buds per regenerating explant was achieved at 1.0 μM EBL, while a mean of 3.1 shoot buds per explant. No shoot buds were obtained from untreated explants. Zeatin was able to induce very low and isolated shoot buds in both species (Figures 1D and 4D).

A comparative analysis of EBL and Zeatin showed that the first was more effective for shoot bud induction and shoot regeneration (Figure 1D), suggesting that EBL can be applied as a substitute for cytokinin in regeneration of *Brassica* species which is similar to our findings for adventitious shoot regeneration in cauliflower (Sasaki 2002).

In spite of the efficient EBL action, in combination with Zeatin, the shoot bud induction and regeneration was strongly enhanced (Figure 1D), reflecting an additive or synergistic

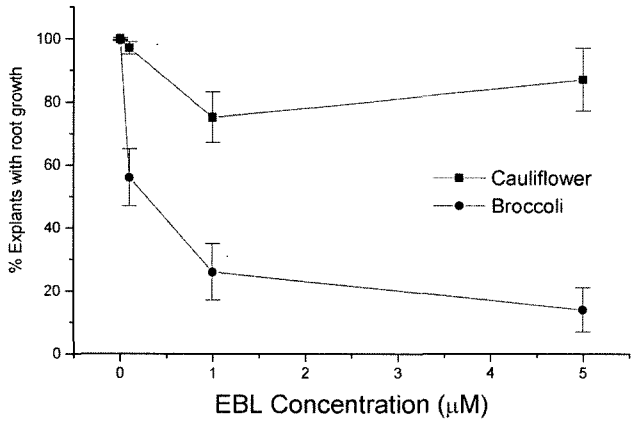

Figure 3. Effects of 24-epibrassinolide (EBL) on root growth suppression. Cauliflower and broccoli explants were cultured in the presence of various concentrations of EBL (0, 0.1, 1.0 and 5 μM) and compared with the control explants (hormone free medium). Average values (±standard deviation) from two independent experiments are shown.

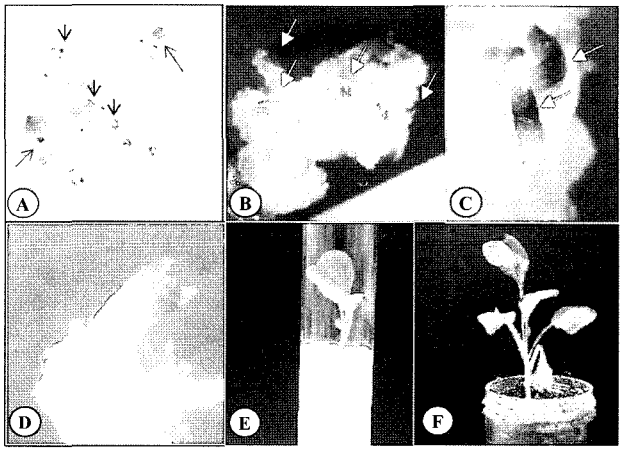

Figure 4. Photomicrographs of 24-epibrassinolide-treated explants showing organogenic response: (A) overview of explants of cauliflower (left) and broccoli (right) showing shoot bud induction, (B) cauliflower explant showing several shoot bud differentiation, (C) well differentiated shoot buds from explants growing in 1μM 24-epibrassinolide and 10 μM Zeatin, (D) individual shoot regeneration in absence of 24-epibrassinolide, (E) regenerated shoots were transferred to MS hormone free medium for root induction and (F) elongated and well rooted plantlets were transferred to the soil for acclimatization.

effect for shoot bud differentiation and regeneration. The best results, 7.3 shoot buds and 3.6 shoots per explant, were obtained cultivating the explants for 8 weeks in 1.0 μM EBL + 10 μM Zeatin. The Zeatin concentration at which the maximal effect achieved was lowered (10 μM Zeatin instead of 100 μM; note that 100 μM Zeatin was highly supraoptimal in the presence of EBL and no viable shoot had been regenerated with 1.0 μM EBL + 100 μM Zeatin).

Simultaneous administration of EBL and Zeatin results in additive effects. This synergic effect suggests that EBL makes more cells competent and more sensitive to Zeatin (i.e., for similar response they required less Zeatin) inducing more shoot buds per explant. It has been reported that BR change endogenous cytokinin levels in various plant species (Miyazawa et al. 2003), and EBL promotes accumulation of endogenous predominant cytokinins N-6-(-2-isopentenyl) adenine and *trans*-zeatin in tobacco callus tissue (Gaudinova et al. 1995). The demonstration of additive effects for shoot bud induction is a very promising result considering that brassinolide can be applied to overcome regeneration problems for recalcitrant species, as it was reported for *Malus prunifolia* (Willd.) Borkh *in vitro* clonal propagation (Schaefer et al. 2002).

Although BRs are known to regulate shoot growth, their role in the regulation of root growth is less clear. According to Mussing et al. (2003), exogenous BRs stimulate root growth at low (nanomolar level) concentrations. When higher concentrations are used (at micromolar level), inhibition of root growth can be expected, this may be the consequence of BR-induced ethylene synthesis. In absence of EBL, 100% of the hypocotyl explants of cauliflower and broccoli showed a vigorous root induction and growth, but in presence of EBL a pronounced suppression of root growth was observed (Figure 1B). As represented in Figure 3, the suppressing effect was highly specific and concentration dependent. Root suppression for cauliflower and broccoli was about 12% and 75% at 1.0 μM EBL respectively. This result makes the role of EBL in root organogenesis unclear, and much further investigation is required to solve this issue, including studies of the effect of EBL on genes controlling root induction. Shimada et al. (2003) observed that the levels of active BRs in *Arabidopsis* roots were significantly lower than the levels in shoots. This was notable in our experiment, since the high concentrations of EBL suppressed root induction and promote shoot bud differentiation. In the presence of Zeatin, root induction was completely inhibited (Figure 1B). These results are not surprising, since it is well documented that cytokinin slow down or fully suppress root induction in explants *in vitro* culture.

The regenerated shoots were transferred to MS hormone free medium for root induction (Figure 4E). About two weeks later, a mean of 4 roots of approximately 2.5 cm per shoot were observed in more than 80% of the transferred shoots, which were transferred to the soil for acclimatization (Figure 4F). Morphologically normal plantlets have been regenerated from hypocotyl explants of cauliflower (Sasaki, 2002). Plant organogenesis is essentially a post-embryonic process that requires a strict balance between cell proliferation and differentiation. This is subject to a complex regulatory network which, in some cases, depends on the action of a variety of plant hormones. Of these, auxins and cytokinins are those best documented as impinging directly on cell cycle control (Pullman et al. 2003). However, increasing evidence indicates that new group of hormones, like BRs also have an impact on cell cycle control by modulating cell cycle regulators.

In conclusion, results presented here confirm that EBL is effective for cellular and organogenic modulation in *Brassica* species and extend earlier reports of interactions between the important plant growth regulators BR and Zeatin. Although certain of these interactions appear to be antagonistic, the general results indicate that EBL potentiates Zeatin activity, at least in certain tissues. Elucidation of the molecular mechanisms will be required to understand how EBL and Zeatin modulate common targets involved in the control of cell elongation and cell division as well as organogenic response for plant regeneration. Experiments with new plant growth regulators, like brassinolides, are important in tissue culture aiming to maximize or accelerate plant regeneration for *in vitro* multiplication or genetic transformation.

Acknowledgements

Authors wishes to thank to Prof. H. Schnabl, Rheinische Friedrich-Wilhelms University of Bonn, Bonn, Germany, for supplying the EBL for this study.

References

Azpeitia A, Chan JL, Saenz L, Orpeza C (2003) Effect of 22(S),23(S)-homobrassinolide on somatic embryogenesis in plumule explants of Cocos nucifera (L.) cultured *in vitro*. The J Hortic Sci Biotech 78: 591-596

Bellincampi D, Morpurgo G (1988) Stimulation of growth in *Daucus carota* L. cell cultures by brassinosteroid. Plant Sci 54: 153-156

Bishop GJ (2003) Brassinosteroid mutants of crops. J Plant Growth Regul 22: 325-335

Clouse SD, Sasse JM (1998) Brassinosteroids: essential regulators of plant growth and development. Annu Rev Plant Physiol Plant Mol Biol 49: 427-451

Clouse SD (1996) Molecular genetic studies confirm the role of

brassinosteroides in plant growth and development. Plant J 10: 1-8

Clouse SD, Zurek DM (1991) Molecular analysis of brassinolide action in plant growth and development. In: Cutler HG, Yokota T, Adam G (eds), Brassinosteroids chemistry, bioactivity, and applications, American Chemical Society, Washington D.C, pp 122-140

Dewitte W, Riou-Khamlichi C, Scofield S, Healy JMS, Jacqmard A, Kilby NJ, Murray JAH (2003) Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. Plant Cell 15: 79-92

Dhaubhadel S, Browning KS, Gallie DR, Krishna P (2002) Brassinosteroid functions to protect the translational machinery and heat-shock protein synthesis following thermal stress. Plant J 29: 681-691

Friedrichsen D, Chory J (2001) Steroid signaling in plants: from the cell surface to the nucleus. Bioessays 23: 1028-36

Gaudinova A, Sussenbekova H, Vojtechova M, Kaminek M, Eder J, Kohout L (1995) Different effects of 2 brassinosteroids on growth, auxin and cytokinin content in tobacco callus-tissue. Plant Growth Regul 17:121-126

Grove MD, Spencer GF, Rohwedder WK, Mandava N, Worley JF, Warthen JD, Steffens GL, Flippen-Anderson JL, Cook JC (1979) Brassinolide, a plant growth promoting steroid isolated from *Brassica napus* pollen. Nature 281: 216-217

Hu Y, Bao F, Li J (2000) Promotive effects of brassinosteroids on cell division involves a distinct CycD30-induction pathway in *Arabidopsis*. Plant J 24: 693-701

Kalinich JF, Mandava NB, Todhunter TA (1985) Relationship of nucleic acid metabolism to brassinolide-induced responses in beans. J Plant Physiol 120: 207-214

Khripach V, Zhabinskii V, de Groot A (2000) Twenty years of brassinosteroids: steroidal plant hormones warrant better crops for the XXI Century. Ann Bot 86: 441-447

Kinoshita T, Cano-Delgado A, Seto H, Hiranuma S, Fujioka S, Yoshida S, Chory J (2005) Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. Nature 433: 167-71

Lu Z, Huang M, Ge DP, Yang YH, Cao XN, Qin P, She JM (2003) Effect of brassinolide on callus growth and regeneration in *Spartina patens* (Poaceae), Plant Cell Tiss Org Cult 73: 87-89

Miyazawa Y, Nakajima N, Abe1 T, Sakai A, Fujioka S, Kawano S, Kuroiwa T, Yoshida S (2003) Activation of cell proliferation by brassinolide application in tobacco BY-2 cells: effects of brassinolide on cell multiplication, cell-cycle-related gene expression, and organellar DNA contents. J Exp Bot 54: 2669-2678

Morillon R, Catterou M, Sangwan RS, Sangwan BS, Lassalles JP (2001) Brassinolide may control aquaporin activities in *Arabidopsis thaliana*. Planta 212: 199-204

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473-497

Müssig C, Shin GH, Altmann T (2003) Brassinosteroids Promote Root Growth in *Arabidopsis*. Plant Physiol 133: 1261-1271

Nakajima N, Shida A, Toyama S (1996) Effects of brassinosteroid on cell division and colony formation of Chinese cabbage mesophyll protoplasts. Jpn J Crop Sci 65: 114-118

Oh MH (2003) Brassinosteroids Accelerate the Rate of Cell Division in Isolated Petal Protoplasts of *Petunia hybrida*. J Plant Biotech 5: 63-67

Pullman GS, Zhang Y, Phan BH (2003) Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Rep 22: 96-104

Sasse JM (1989) Using PEST to study the interactions of brassinolide and other natural plant growth regulators. Proc Plant Growth Regul Soc Am 16: 82-87

Sasse JM (2003) Physiological actions of Brassinosteroids: An Update. J Plant Growth Reg 22: 276-288

Sasaki H (2002) Brassinolide promotes adventitious shoot regeneration from cauliflower hypocotyl segments. Plant Cell Tiss Cult Org Cult 7: 111-116

Sathiyamoorthy P, Nakamura S (1990) *In vitro* root induction by 2,4-epibrassinolide on hypocotyl segments of soybean (*Glycine* max (L.) Merr.). Plant Growth Reg 9: 73-76

Schaefer S, Medeiro SA, Ramirez JA, Galagovsky LR, Pereira-Netto AB (2002) Brassinosteroid-driven enhancement of the in vitro multiplication rate for the marubakaido apple rootstock [*Malus prunifolia* (Willd.) Borkh]. Plant Cell Rep 20: 1093-1097

Shimada Y, Goda H, Nakamura A, Takatsuto S, Fujioka S, Yoshida S (2003) Organ-specific expression of brassinosteroid-biosynthetic genes and distribution of endogenous brassinosteroids in *Arabidopsis*. Plant Physiol 131: 287-297

Symons GM, Reid JB (2004) Brassinosteroids do not undergo long-distance transport in pea. Implications for the regulation of endogenous brassinosteroid levels, Plant Physiology 135: 2196-2206