Chapter 7

PHYSIOLOGICAL EFFECTS RELATED TO BRASSINOSTEROID APPLICATION IN PLANTS

M.M.A. GOMES

Instituto Superior de Tecnologia em, Ciências Agrárias/Fundação de Apoio à Escola, Técnica do Estado do Rio de Janeiro (FAETEC), Av. Wilson Batista, s/n, Parque Aldeia, Campos dos Goytacazes-RJ, CEP28070-620, Brazil*; e-mail: maramag@ig.com.br*

Abstract: Brassinosteroids are plant hormones whose functions have been discovered in the past years. In order to confirm scientifically the biological effects caused exclusively by these compounds, different tools can be used**,** such as BR-deficient or BR-perceptive mutants, molecular studies, biological assays, application of brassinosteroid biosynthesis inhibitors, endogenous quantification and exogenous application. This work aims at relating the physiological effects in plants when exposed to different dosages and analogues of brassinosteroids during different phases of development (germination, flowering, fructification) and when submitted to biotic and abiotic stress (pathogens, water stress, saline stress, hypoxia, temperature, heavy metals and pesticides) as well as the particularities related to tropisms, circadian rhythms and interactions with other plant hormones. The use of brassinosteroids with the objective of increasing crop yield in the field and to improve the quality of the seedlings has also received attention in recent papers. The main objective of this chapter is to discuss the physiological effects that occur in cells, tissue or whole plants when submitted to brassinosteroid applications, taking into account the possible mechanism of action of these compounds and their practical use in agriculture**,** describing the analogues and the dosages used in field and laboratory experiments during the last 10 years.

Key words: brassinosteroid concentration, brassinosteroid analogue, germination, flowering, plant tissue culture, plant stress

Abbreviations: BR-brassinosteroid, BL-brassinolide, EBL-epibrassinolide, HBL-homo-brassinolide, POD-peroxidase, CAT-catalase, SOD-superoxide dismutase

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1. INTRODUCTION

This chapter aims at providing information for researchers that work with exogenous brassinosteroid application. The use of this compound has to be evaluated in order to establish how plants function when BRs are present or to discover the metabolic route involved in a plant's response to hormones or even to prevent toxic effects in plants caused by a variety of stressing situations. Sometimes, the BR concentration used in a plant does not apply to other plants or the developing stage varies the responsiveness dose. When this compound is applied exogenously, it should have a practical use in agriculture as well as in plant propagation for instance, as a constituent of plant culture medium or to establish seedlings in the field or seed priming. The use of exogenous BRs to alleviate plant stress can not be forgotten as it can be used as an antistress agent in a wide range of biotical and abiotic stress conditions (heavy metals, herbicides, saline stress, and drought stress). There is also some information on the increase of plant yield by brassinosteroids application but this is not yet commercially viable.

The use of exogenous BRs to study plant metabolism is desirable but it has to be taken into account that the concentrations used are not always compatible with the endogenous concentrations that can actually be found in plants, so the effects may be confusing. The use of BR plant mutants or endogenous quantification are more appropriate to verify gene expression or changes in plant metabolism caused by BRs because the concentrations applied to explain biological effects may suppress, repress or super express some genes.

There are also problems relating to hormone volatilization if it is spray-applied, the use of surfactants also should be considered to promote best adherence to plant surface. The time of the day and the quality of light should be taken into account as BRs sometimes act in darkness but not under light conditions or, on the contrary, it works in light but not in dark conditions depending on the expected response, so it is very important to decide which time of the day it will be applied. The moment of application is also fundamental because studies have proved that there are significant differences if BR is applied before, after or at the same time of the stressing agent. If it is applied before the stressing agent, it can protect the plant from this agent but, on the other hand, when applied after this external factor, the effects can be deleterious or vice-versa. Besides, BRs endogenous concentrations vary during the day and also according to the plant ontogeny, which causes significant differences in endogenous concentrations among plant organs and plant species. Few plants in Plant Kingdom were tested for BRs, the most common results being for plants of the *Brassica* species, *Arabidopsis thaliana* and some crops with agricultural interests.

We have selected here a series of studies that took place in the last decade in which BRs application was used as a tool to explain plant's behavior to this hormone. Although the BRs concentrations used are commonly very low, there were detected slight differences among the responsiveness even when a varied series of low concentrations get the same response. So, it is important to take into account the concentrations and the mode of application that other researchers have used to improve the desirable results. This work is dedicated to researchers that believe that this plant hormone is responsible for a lot of plant responses and that it can be used in crops in several situations but, at the same time, they know that there are still gaps to be filled in.

2. BRASSINOSTEROID APPLICATION TRIGGERS SEVERAL RESPONSES RELATED TO PLANT GROWTH AND DEVELOPMENT

Brassinosteroids have biological effects at low concentrations and they are found in gymnosperms, alga, monocotyledons (Liliopsida) and dicotyledons (Magnoliopsida) plants in different organs, such as leaves, floral buds, seeds, fruits, stems and roots. It can affect a great variety of developmental process during plant growth and development (Table 1).

Table 1. Effects and concentrations of brassinosteroid and brassinosteroid analogues on plant growth and development in different plant species

Species	Concentrations	BR	Organ	Mode of application	Physiological effects analyzed	Author
Arabidopsis thaliana	10^{-10}, 10^{-8} and 10^{-6} M	BL	Seed	Medium constituent	Growth	Tanaka *et al.* (2003)
Arabidopsis thaliana	0.1 and 0.5 nM 10 nM	24-EBL 24-ECAS	Seed	Medium constituent	Root growth	Müssig *et al.* (2003)
Arabidopsis thaliana	1–100 nM	BL	Seedling	Medium constituent	Lateral root formation	Bao *et al.* (2004)
Arabidopsis thaliana	100 nM	EBL	Seed	Medium constituent	Hypocotyl growth, apical hook	De Grauwe *et al.* (2005)
Arabidopsis thaliana	0.05 and 0.1 µM	24-EBL	Seed	Medium constituent	Root growth	Golovatskaya (2008)
Arabidopsis thaliana	2 µM	BL	Inflorescence stalk	Medium constituent	Ethylene production	Arteca and Arteca (2008)
Vigna radiata	10^{-8}, 10^{-6}, 10^{-4} M	28 HBL	Seed	Seed soaking	Photosynthesis, Enzyme activities	Fariduddin *et al.* (2003)
Vigna radiata	0.0001, 0.01, 1 µM	28-HBL	Leaves (25 day-age)	Spray	Enzymes activities, Photosynthesis, protein and chlorophyll content	Fariduddin *et al.* (2004)
Vigna radiata	1 µM 0.01 µM	28HBL	Seed Leaves	Seed soaking Spray	Photosynthesis, enzymes activities, chlorophyll content, yield	Fariduddin *et al.* (2008)
Pisum sativum	0.1 µM	EBL	Detached pea shoot	Medium constituent (incubation for 20 minutes)	Protein	Fedina *et al.* (2008)

(*continued*)

(*continued Table 1.*)

Species	Concentrations	BR	Organ	Mode of application	Physiological effects analyzed	Author
Cucumis sativus	0.1 mg/L	24-EBL	Leaves	Spray application	Photosynthesis	Yu *et al.* (2004)
Brassica oleraceae	0.001 µM 10 µM	EBL	Cotyledon	Cotyledon incubation for 3 days	Growth, chlorophyll content, anthocyanin acontent	Çag *et al.* (2007)
Lupinus albus *Lupinus angustifolius* *Lupinus luteus*	10^{-9} M 10^{-9} M	EBL HBL	Seed	Seed soaking	Protein and amino acid content	Kandenlinskaya *et al.* (2007)
Oryza sativa	10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M	BL	Coleoptile	Medium constituent	Lamina joint inclination and coleoptile elongation	Jeong *et al.* (2007)
Wolffia arriza	10^{-9} M	24-EBL	Culture cells	Addition to cells	Growth, photosynthetic pigments, protein and sugar content	Bajguz and Asami (2005)
Chlorella vulgaris	10 nM	BL	Culture cells	Addition to cells	Growth	Bajguz and Asami (2004)
Glycine max	0.21, 2, 1, 21 µM 0.22, 2.2, 22 µM 0.22, 2.2, 22 µM	24-EBL MH-5 BB-6	Seedling	Medium constituent	Growth	Mazorra *et al.* (2004)
Ananas comosus	0.1, 0.3, 0.5 1 mg/L	BB-16	Micropropagated seedling	Spray	Growth	Catunda *et al.* (2008)
Citrus reshni	0.1, 0.5, 1.0 mg/L	BB-16	Seedling	Spray	Stem diameter	Altoé *et al.* (2008)
Passiflora edulis	0.1 mg/L (successive applications)	BB-16	Plant (6 months)	Spray	Yield (number of fruits)	Gomes *et al.* (2006)
Opuntia ficus-indica	0.1 and 10 mg/L (BB-6) 0.001 and 10 mg/L (BB-16)	BB-6 BB-16	Cladodes	Spray	Yield (number of vegetative buds) and growth	Cortes *et al.* (2003)
Lycopersicon esculentum	10^{-8} M	28-HBL	Roots	Roots dipped	Enzymes, chlorophyll content, yield (number of fruits)	Ali *et al.* (2006)
Allium cepa	0.005 ppm, 0.05 and 0.5 ppm	24-EBL	Bulb	Medium constituent	Root length, number of mitoses	Howell *et al.* (2007)
Hordeum vulgare	0.1, 0.5 and 1.0 µM	28-HBL	Seeds	HBL-supplemented distilled water	Primary root growth, mitoses activity, protein content, antioxidant enzymes activities	Kartal *et al.* (2009)

There are many studies relating the effects of brassinosteroid application on *Arabidopsis thaliana* plant development, so they will be related together in the next paragraphs.

Exogenous BL (10^{-10}, 10^{-8} and 10^{-6} M) remarkably promoted the growth of hypocotyls and cotyledonous leaf-blades in a dose-dependent manner (Tanaka *et al.*, 2003). The BL-induced hypocotyls elongation was shown only in conditions of light. Hypocotyls seedlings that were treated with 10^{-6} M of BL became 2.4 times longer than untreated seedlings. In darkness, BL did not promote but rather inhibited hypocotyls elongation at concentrations higher than 10^{-8} M. On the other hand, BL-induced elongation of cotyledonous leaf blade was found in both dark and light, although BL was less effective in darkness. Taproot elongation was severely inhibited by exogenously applied BL in a dose dependent fashion in both dark and light conditions. The inhibition of taproot growth in the light was observed when seedlings were treated with BL concentrations greater than 10^{-10} M. Conversely, in darkness, taproot growth was inhibited when treated with 100-fold higher concentrations of BL (10^{-8} M) at a minimum. The authors suggest that the results indicate that responsiveness of organs to BL differs among organs. Besides, cytological observation disclosed that BL-induced hypocotyls elongation was achieved through cell enlargement rather than cell division. Furthermore, a serial experiment with hormone inhibitors showed that BL induced hypocotyl elongation not through gibberellins and auxin actions. However a synergistic relationship of BL with gibberellins A_3 and indole-3-acetic acid (IAA) was observed on elongation growth in light-grown hypocotyls, even though gibberellins have been reported to be additive to BR action in other plants. These authors showed that BRs act on light-grown hypocotyl elongation independent of, but cooperatively with, gibberellins and auxin (Tanaka *et al.*, 2003). Müssig *et al.* (2003) showed that low concentrations of 24-epicastasterone (10 nM) and 24-EBL (0.1 and 0.5 nM) promoted root elongation in *A. thaliana* wild-type plants up to 50% and in BR-deficient mutants up to 150%. The growth stimulating effect of exogenous BRs is not reduced by the auxin transport inhibitor 2,3,5,-triiodobenzoic acid. BR-deficient mutants show normal gravitropism and 2,3,5-triiodobenzoic acid or higher concentrations of 2,4-dichlorophenoxyacetic acid (2,4D) and naphthalene acetic acid (NAA) inhibit root growth in the mutants in the same extent as in wild-type plants. They verified that simultaneous administration of EBR and 2,4D results in largely additive effects but exogenous gibberellins do not promote root elongation in the BR-deficient mutants and the sensitivity to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid is not altered. Thus, the root growth stimulating effects of BRs appears to be largely independent of auxin and gibberellins action. Bao *et al.*(2004) verified that BRs are required for lateral root development in *Arabidopsis* and that BRs acts synergistically

with auxin to promote lateral root formation. The authors showed that the number of lateral roots increased in response to 1–100 nM BL with 10 nM being optimal, inducing nearly an eightfold increase in lateral root formation but the elongation of primary roots was inhibited by 1–100 nM BL. Auxin is necessary for lateral root development and lateral root emergence can be blocked by the auxin transport inhibitor N-(1-naphthyl) phthalamic acid (NPA). The authors found that 2 μM NPA not only inhibited lateral root formation in wild-type seedlings but also reduced BL promotion of lateral root formation, so these results indicate that BRs promote lateral root development by increasing acropetal auxin transport.

Dark-grown *Arabidopsis* seedlings develop an apical hook by differential cell elongation and division, a process driven by cross-talk between multiple hormones like auxins, ethylene and gibberellins as they interact in the formation of the apical hook. In the light, a similar complexity of hormonal regulation has been revealed at the level of hypocotyls elongation (De Grauwe *et al*., 2005). These authors analyzed the involvement of brassinosteroids in auxin-and ethylene controlled process in the hypocotyls of both light-and-dark-grown seedlings. They showed that BR biosynthesis is necessary for the formation of an exaggerated apical hook and that either application of BRs or disruption of BR synthesis alters auxin response, presumably by affecting auxin transport, eventually resulting in the disappearance of the apical hook. When EBL (100 nM) was applied to light-grown hookless mutants or light-grown PIN mutants, EBL increased hypocotyls length even when ACC was supplied together with EBL. When treated with EBR, the relative hypocotyl elongation in the mutants was comparable with that of the wild type. However, when ACC and EBR were applied simultaneously, a small synergistic effect was observed in all hookless mutants, whereas this was not the case for the wild type. Thus, these components may act in a signal transduction route either upstream or independently of BRs.

The effects of GA_3, 24-EBL and their combination on morphogenesis of *Arabidopsis thaliana* (L.) Heynh (7-day-old seedlings) were studied by Golovatskaya (2008). The cotyledons shape and size were dependent on 24-EBL and the root length was both GA_3 and EBL regulated, indicating organ specificities in the responses to these hormones. Simultaneous treatment of dark-grown plants with GA_3 (0.01–1.0 μM) and EBL (0.05–0.1 μM) exerted an additive stimulatory effect on the root growth of *det-2* (BR-deficient mutant), reduced the inhibitory effect of EBL on hypocotyl elongation of *ga4-1* (GA deficient mutant) and enhanced the effect of EBL on hypocotyls and cotyledon elongation of *det-2*. The authors suggest that localization and quality of morphogenetic responses of *Arabidopsis* plants to exogenous GA_3 and EBL are linked to the dynamics and the ratio of endogenous hormone levels in the plant, inactivation and destruction of exogenous hormones

during their transport in the plant, as well as on the degree of overlapping of the hormone responses.

The effects of varying concentrations of IAA alone or in combination with BL or 6-benzylaminopurine, a cytokinin, on ethylene production were evaluated in order to determine the relationship between IAA (0–100 μM) and BL (2 μM) or BAP (10 μM) in *A. thaliana* inflorescences (Arteca and Arteca, 2008). Inflorescences treated with BL alone had no effect on ethylene production. However, when BL was used in combination with IAA there was a dramatic increase in ethylene production above the induction promoted by IAA alone so, there was a synergistic effect due to the co-application of these hormones in ethylene production.

Mung bean (*Vigna radiata* L. Wilczek cv. T-44) seeds soaked in 28-HBL (10^{-8}, 10^{-6}, 10^{-4} M) for 4, 8 or 12 hours enhanced net photosynthetic rate, leaf chlorophyll content, carbonic anhydrase activity, carboxylation efficiency, stomatal conductance and seed yield at harvest being the best combination the concentration of 10^{-6} M for 8 hours, the others concentrations were either very low or supra optimal for most of the parameters (Fariduddin *et al.*, 2003). When 28-HBL (0.0001, 0.01, 1 μM) and kinetin (0.01, 1, 100 μM) were applied to the leaves of 25-day old plants of mung bean (*Vigna radiata* L. Wilczek), the activities of nitrate reductase and carbonic anhydrase, chlorophyll and total protein contents and net photosynthetic rate in the leaves and pod number and seed yield increased at harvest (Fariduddin *et al.*, 2004). Other study with this plant tested the effects of 28-HBL supplied to the seeds (1.0 μM) and/or to the foliage (0.01 μM) of mung bean (*Vigna radiata* L.Wilczek) plants (Fariduddin *et al.*, 2008). It was observed that the activities of carbonic anhydrase and nitrate reductase, leaf chlorophyll content, net photosynthetic rate, stomatal conductance, carboxylation efficiency, dry mass, pod number and seed yield at harvest increased significantly over the control, irrespective of the mode of application. However the plants raised by seed soaking and also received foliar application of HBL had most successful results. Detached pea (*Pisum sativum* L.) shoot was incubated in a medium supplemented with 0.1 μM EBL for 20 minutes and then submitted to a protein quantitative assay (Fedina *et al.*, 2008). This analysis revealed that the phosphorylation of PY20 phosphotyrosine polypeptides was changed under the action of EBL. The results indicate that eight of these proteins belong to the Calvin Cycle enzymes so the observed changes in phosphorylation of these proteins may partly explain the effects of BRs on photosynthesis. The effects of 24-EBL spray application on gas exchange, chlorophyll fluorescence characteristics, Rubisco activity and carbohydrate metabolism were investigated in cucumber (*Cucumis sativus* L. cv. Jinchun No. 3) plants (Yu *et al.*, 2004). EBR significantly increased the light-saturated net CO_2 assimilation rate from 3 hours to 7 days after spraying, with 0.1

mg.L^{-1} EBR proving most effective. EBR-treated leaves also had a higher quantum yield of PSII electron transport than the controls, which was mainly due to a significant increase in the photochemical quenching (q_p), with no change in the efficiency of energy capture by open PSII reaction centers (F'_v/F'_m). It was concluded that EBR increases the capacity of CO_2 assimilation in the Calvin Cycle, which was mainly attributed to an increase in the initial activity of RUBISCO. Different concentrations of EBL (0.001, 0.1 and 10 μM) were tested in excised red cabbage (*Brassica oleraceae* L.) by incubating the cotyledons on those solutions for 3 days (Çag *et al.*, 2007). There was a significant increase in chlorophyll content of cotyledons incubated in 0.001 μM compared to the control and the concentration of 10 μM showed the lowest peroxidase activity and the optimal concentration for anthocyanin concentration was 10 μM. The concentration of 0.001 μM EBL promotes growth on cotyledons, whereas 10 μM inhibits it and 0.01 μM causes no significant effect on cotyledon growth. Presowing treatment of seeds of lupine (*Lupinus angustiflolius*, *Lupinus luteus* L., *Lupinus albus* L.) of various species and cultivars with EBL (10^{-9} M) and HBL (10^{-9} M) caused an increase in protein content and a change in the proportion of some amino acids (Kandenlinskaya *et al.*, 2007). These changes in protein metabolism correlated with an increase in the concentration of indole acetic acid and a decrease in the content of abscisic acid.

Jeong *et al.* (2007) verified the responses of lamina joint inclination and coleoptiles elongation of rice (*Oryza sativa* L.) to exogenous BL under light or dark conditions. Both responses were more pronounced under darkness, implying that BR signalling is inhibited by light. The authors suggest that phytochrome B acts as a negative regulator of BL-regulated growth and development processes in rice.

The use of brassinazole, an inhibitor of BR biosynthesis, to prove the involvement of BRs on plant development is well known and exogenous BR is used to restore the effects caused by brassinazole. The application of 24-EBL (10^{-13}–10^{-6} M) to *Wolffia arrhiza* cultures, an aquatic monocotyledon, stimulated the growth and increased the content of photosynthetic pigments, sugar and protein and the concentration of 10^{-9} M showed the greatest effect (Bajguz and Asami, 2005). Addition of Brz2001 (10^{-6}–10^{-4} M) to *Wolffia arrhiza* cultures, a kind of brassinazole, inhibits growth after 7 days of cultivation and this inhibition could be reversed by the addition of EBL so BR is important for *Wolffia arrhiza* growth and development. Cultured *Chlorella vulgaris* Beijerinck cells with 0.1–10 μM Brz2001 inhibits their growth during the first 48 hours of cultivation in the light and this inhibition is prevented by the co-application of BL (10 nM) (Bajguz and Asami, 2004). This result suggests that the presence of endogenous BRs during the initial steps of the *C. vulgaris* cell cycle is indispensable for their normal growth in

light. In darkness, a treatment with 10 nM BL promotes growth through the first 24 hours of culture but during the following 24 hours the cells undergo complete stagnation. Brz2001, was also used to block the growth of roots, hypocotyls and epicotyls of soybean (*Glycine max* cv. Cubasoy 27) seedlings producing a dwarf phenotype (Mazorra *et al.*, 2004). The application of 24-EBL (0.21, 2.1 and 21 μM) completely reversed the inhibitory effects caused by brassinazole and two growth-promoting brassinosteroid analogues tested (MH-5 and BB-6, 0.22, 2.2 and 22μM of each) partially overcame the Brz2001-induced growth defects but MH-5 proved to be more effective and the largest reversible growth reduction was obtained with 0.22 μM MH-5 and 2.2 μM BB-6. BB-6 and MH-5 have biological activity despite presenting the spirostane side chain instead of the 22α,23α-dihydroxicholestane characteristic of the natural brassinosteroids.

BB-16 (0.1, 0.3, 0.5 and 1.0 mg.l), a spirostanic analogue of brassinosteroid, was applied to micropropagated seedlings of Imperial pineapple (*Ananas comosus* L. Merrill) to evaluate the development under two substrates (Plantmax® and a mix of composting sugar cane bagasse and filter cake, CC) (Catunda *et al*., 2008). The plants that were cultivated on CC substrate and sprayed with $0.1 mgL^{-1}$ BB-16 produced 2.8 times more dry matter than the control cultivated in Plantmax substrate. Besides, this treatment showed higher growth of shoots with greater number of leaves, rosette diameter, leaf width, fresh and dry matter production at 150 days after planting, the authors suggest that BR would influence carbohydrate translocation from root to shoot because CC is richer in organic compound when compared to Plantmax. This analogue (BB-16), at 0.1, 0.5 and 1.0 $mg.L^{-1}$, was also used to verify the development of "Cleopatra" orange rootstock (*Citrus reshni* Hort ex Tanaka) mycorrhized and non-mycorrhized, BB-16 promoted an enhancement in the diameter of the stem which is an important parameter for plant grafting (Altoé *et al.*, 2008). Successive applications of BB-16 ($0.1 mg.L^{-1}$) to yellow passion fruit plants (*Passiflora edulis* f. *flavicarpa*) after the appearance of the first flowers enhanced yield when applied during three successive weeks (81.5 fruits/plant) when compared to the control (53.5 fruits/plant) (Gomes *et al.*, 2006). BB-6 and BB-16 applied to the cladodes of *Opuntia ficus-indica* L. Mill. var. lutea stimulated larger number of vegetative buds under both greenhouse and field conditions and promoted precocity, accelerating growth during the first stages of vegetative bud development, but did not alter the morphology of the harvested cladodes (Cortes *et al.*, 2003).

The quantities of nitrate reductase, carbonic anhydrase and content of chlorophyll in leaves were significantly higher than control in 30 and 60 days old plants of tomato (*Lycopersicon esculentum* Mill.) whose roots (20 days old seedling) were dipped in 28-HBL (10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M), there were more fruits in BR-treated plants than in control (Ali *et al.*, 2006).

Besides, the fruits at ripening had higher levels of lycopene and β-carotene, being the treatment 15 min feeding of 10^{-8} M the best of all them (Ali *et al.*, 2006).

Low doses of 24-EBL (0.005ppm) nearly doubled the mean root length and the number of mitoses over that of controls of onion (*Allium cepa*) root tips (Howell *et al.*, 2007). Intermediate doses of EBL (0.5 ppm) also produced mean root lengths and number of mitoses that were significantly greater than those of the controls. Seeds of barley (*Hordeum vulgare* L.) germinated between filter papers in 0.1, 0.5 and 1.0 μM HBL-supplemented distilled water, the HBR-seedlings showed significant increases in the primary root growth (twofold increase in 1.0 μM HBR), the roots treated with HBR showed more mitotic activity, mitotic abnormalities and significant enlargements at the root tips when compared with control material (Kartal *et al.*, 2009). HBR application to barley seeds decreased total soluble protein content, superoxide dismutase, catalase and peroxidase activities significantly at 1.0 μM HBR concentration.

2.1 Brassinosteroid interactions with other plant growth substances

Plant metabolism involves responses that are regulated mainly by hormones and environment. The involvement of a unique substance to explain such intricate processes does not exist, so it is a consequence of a complex mechanism that involves hormones, phytochromes, DNA synthesis, gene expression. The next paragraphs will relate the influence of applied BR and other applied plant growth regulators in plant physiology (Table 2).

When auxin (10^{-5} M) and BL (10^{-7} M) were applied exogenously to *Zea mays* L. cv. Golden Cross Bantam plants, both increased ethylene production (Yun *et al*., 2009). When these hormones were tested simultaneously, the increase in the level of ethylene was greater than the sum of effects by each one. Such a positive interaction was also recorded for changes in the activity of ACC synthase and the expression of its gene. For ACC oxidase, however, the two hormones had no apparent influence. When applied separately neither affected root elongation nor proton extrusion. However, when given in combination, both phenomena occurred. These results suggest that BL interacts with IAA to promote ethylene biosynthesis and elongation in roots. Therefore it is possible that BL acts by inducing auxin, which then stimulates both ethylene production (at the early stage) and root development.

A high concentration of BL induced abnormal shoot shape in rice (*Oryza sativa* L. cv. Tai Nguyen) seedlings, that is, the newly developed leaf sheath

Table 2. Effects and concentrations of brassinosteroids and other hormones on hormone interactions

Species	Hormones and concentrations applied	Organ which received BR	Mode of application	Physiological effects analyzed	Author
Zea mays	Auxin (10^{-5} M) BL (10^{-7} M)	Root tissue	Growth medium constituent	Ethylene production	Yun *et al.* (2009)
Oryza sativa *Echinochloa crus-galli*	BL (0.1, 1.10 μM) Ethephon (1.2, 5.5 mM)	Root	Root soaking	Shoot shape Leaf sheath length Number of leaves	Chon *et al.* (2008)
Vicia faba	BL (10 mM) ABA (50 mM)	Detached leaf	Incubation solution constituent	movement	Haubrick *et al.* (2006)
Tabebuia alba	BL (0.052, 0.104, 0.208 mM) GA3 (0.072, 0.1443 and 0.2887 mM) IAA (0.1427, 0.2854, 0.5708 mM)	Seedling (104 d-age)	Spray	Development and leaf anatomy	Ono *et al.* (2000)

was shorter than the old sheath and increased the number of leaves under light (Chon *et al*., 2008). In this same study, the number of leaves of barnyard grass (*Echinochloa crus-galli* L.) increased at 0.1 μM BL while the number of leaves of rice increased at 1.0 μM. BL strongly stimulated ethylene production and the amount of ethylene and the third leaf sheath length in rice seedlings were negatively correlated. The effect of ethephon on leaf sheath was very similar to that of BL. The results indicate that BL-induced abnormal shoot growth of rice seedlings was probably mediated by ethylene production.

When analyzed the regulation of stomatal movement of *Vicia faba* L. cv. Longpod, BL did not oppose the effect of ABA (Haubrick *et al*., 2006). On the contrary, BL (10 mM) modulated stomatal aperture by promoting stomatal closure and inhibiting stomatal opening. BL inhibited inwardly rectifying K^+ currents of *Vicia faba* guard cell protoplasts in a manner similar to ABA. The effects of BL and ABA (50 mM stock in 95% ethanol) applied together were not additive, suggesting that these two hormones may function in interacting pathways to regulate stomatal apertures and guard cell physiology.

The effects of gibberellin, GA_3 (0.072 mM, 0.1443 mM and 0.2887 mM), auxin (0.1427 mM, 0.2854 mM, 0.5708 mM) and BL (0.052 mM, 0.104 mM, 0.208 mM) in different combinations were evaluated on the development and leaf anatomy of *Tabebuia alba* (Cham.) Sandow seedlings (Ono *et al*., 2000). The results showed that GA_3 plus BL produced the highest stem and petiole growth rates and also produced a significant development of lateral

buds but BL application alone stimulated petiole growth but not stem growth, thus indicating the influence of both hormones to promote stem growth and lateral bud development in *Tabebuia alba*.

2.2 Does exogenous BR change the endogenous concentration of other plant hormones?

It is expected that brassinosteroid application may change other endogenous plant hormones because it is well known that the level of a hormone in a tissue will alter the plant response by regulating endogenous levels of other hormones. Some researchers have studied how BR can affect other endogenous hormone levels in organs or cells (Table 3).

Table 3. Effects of brassinosteroids on the endogenous concentrations of other plant hormones

Species	Hormones and some concentrations applied	Organ which received BR	Mode of application	Endogenous hormone quantified	Author
Triticum aestivum	24-EBL (0.4 nM and 0.4 μM)	Root seedling	Root incubation medium	Cytokinin Auxin Abscisic acid	Aval'baev *et al.* (2003)
Pisum sativum	BL (200 ng/2μL)	The oldest unexpanded internode of 21 day-old plants	2μL applied to the internode	GA_{20} BRs IAA	Jager *et al.* (2005)

Aval'baev *et al*. (2003) studied the effects of 24-EBL (0.4 nM and 0.4 μM) on the dynamics of the concentration of auxins, cytokinins and abscisic acid in the seedlings of wheat (*Triticum aestivum* L. cv. Moskovskaya 35). The results showed that neither of the EBL-concentrations induced changes in the concentration of IAA or ABA in seedlings roots. On the other hand, even 1 hour after application of EBL, there was an almost twofold increase in the rate of accumulation of cytokinins and such an enhanced level was verified through the period of experiment and they suggested that the growth stimulation activity of EBL in wheat seedlings was primarily due to the effects of this agent on the cytokinin metabolism in plants.

The application of BL (200 ng/2 μl 100% ethanol) to *lkb* (BR-deficient mutants) pea plants (*Pisum sativum* L.) reduced GA_{20} levels and metabolism studies revealed a reduced conversion of GA_{19} to GA_{20} in EBL-treated (1 μM) lkb plants (Jager *et al*., 2005). These results indicate that BRs actually negatively regulate GA_{20} levels in pea. Although GA_{20} levels are affected by BR levels, this does not result in consistent changes in the level of the bioactive GA, GA_1. It appears that the BR growth response is not mediated by changes in

bioactive GA levels, thus providing further evidence that BRs are important regulators of stem elongation.

2.3 Seed germination is enhanced by exogenous brassinosteroids

It is supported that brassinosteroids have the ability to induce seed germination as they are naturally occurring substances in seeds and are probably as important as gibberellins and abscisic acid in the control of this process (Table 4).

Seed germination of *Nicotiana tabacum* L. cv. Havana 425 is determined by the balance of forces between the growth potential of the embryo and the mechanical restraint of the micropylar endosperm (Leubner-Metzger, 2001). In contrast to the gibberellin (GA_4), the BL did not release photodormancy of dark-imbibed photodormant seeds. BL and GA_4 promoted endosperm rupture of dark-imbibed non-photodormant seeds, but did not appreciably affect the induction of class I β-1,3-glucanase (βGLU I) in the micropylar endosperm. Promotion of endosperm rupture by BL was dose-dependent and 0.01 μM was most effective. It is proposed that BRs promote seed germination by directly enhancing the growth potential of the emerging embryo in a GA and βGLU I-independent manner.

Wheat seeds (*Triticum aestivum* L. cv. HD2204) were soaked in aqueous solutions of 28-HBL (10^{-10}, 10^{-8} and 10^{-6} M) for 8 hours and the α-amilase levels were increased in HBR-treated seeds, the most effectives dosages were 10^{-10} and 10^{-8} M, the levels of catalase and peroxidases also increased in seedlings whose seeds were treated with HBR (Hayat and Ahmad, 2003a). These authors used the same concentrations of 28-HBL in seeds of *Lens culinaris* cv. Pusa-6 and they verified that HBR-treated plants decreased root length and nodule number per plant but increased nitrate reductase activity and the most effective concentration was 10^{-8} M (Hayat and Ahmad, 2003b).

Broomrapes (*Orobanche spp*.) are serious root parasitic weeds that cause great damage to crop production. Different plant growth regulators were used to verify the potential of germination of this species (Song *et al*., 2005, 2006). Exogenous gibberellins, BL (1 $mg.L^{-1}$) and fluridone, inhibitor of carotenoid biosynthesis, significantly increased the broomrape seed response to a germination stimulant (Gr24, 10^{-6} M) even when seeds were first conditioned at a suboptimal temperature and under water stress. Exogenous GA_3 and BL could restore the germination of *Orobanche spp*. seeds. This may be due to breaking of the secondary dormancy, which is induced by the suboptimal temperature and by water stress.

Table 4. Effects of brassinosteroids in different developmental stage of plants

Species	Developmental stage	BR and concentrations applied*	Organ	Mode of application	Physiological effects analyzed	Author
Nicotiana tabacum	Seed germination	BL (0.01 µM)	Seed	Filter paper wetted with BL and other compounds	Endosperm rupture of dark-imbibed non-photodormant seeds	Leubner-Metzger (2001)
Triticum aestivum	Seed germination	28-HBL (10^{-10}, 10^{-8}, 10^{-6} M)	Seed	Seed soaking	Increase in α-amylase levels	Hayat and Ahmad (2003a)
Lens culinaris	Seed germination	28-HBL (10^{-10}, 10^{-8}, 10^{-6} M)	Seed	Seed soaking	Decrease in root length, nodule number and increase in nitrate reductase activity	Hayat and Ahmad (2003b)
Orobanche spp.	Seed germination	BL (0.5–1.0 mg/L)	Seed	Petri dishes wetted with BL and other compounds	Increased seed germination	Song *et al.* (2005) Song *et al.* (2006)
Vitis vinifera	Fructification	BL (200 ng/5 µL)	Berries	Application of 5µL to each berry	Accelerated ripening, increase on total soluble solids	Symons *et al.* (2006)
Cucurbitaceae family (zucchini, melon, cucumber)	Flowering and fructification	EBL (0.1, 1, 10 µM) EBL (0.1, 10 µM)	Plants Seedling	Pipetting 250µL onto the apical meristem and developing leaf 20µL applied	Differences in the appearance of female and male flowers, ethylene production	Papadopoulou and Grumet (2005)
Cucumis sativus	Flowering and fructification	24-EBL (0.02, 0.2, 2 µM)	Unpollinated ovaries	Spray on to unpollinated ovaries at anthesis	Parthenocarpic growth, expression of cell-cycle related genes	Fu *et al.* (2004)
Pharbitis nil	Flowering	BL (0.01 and 1.0 µM) CAS (0.01 and 1.0 µM) BL (0.1–10 µM)	Cotyledon Shoot apices (*in vitro*)	Soft paintbrush Medium constituent	Flowering inhibition	Kesy *et al.* (2003)
Oryza sativa	Flowering and fructification	BL (2.1×10^{-9} M and 2.1×10^{-8} M)	Whole rice plants	Spray application	Panicle ripening, endogenous hormone quantification, growth	Saka *et al.* (2003)
Litchi sinensis	Flowering and Fructification	BL (0.5, 0.75 and 1.0 mg/L)	Trees (6-y-old)	Spray before anthesis and at early fruit stage	Enzymes activities, pectin content, Ca level	Peng *et al.* (2004)
Lycopersicon esculentum	Flowering and fructification	28-HBL (0.1, 1.0 and 3.0 µM) 24-EBL (0.1, 1.0 and 3.0 µM)	Pericarp discs	Petriplates supplied with BRs	Carbohydrates, lycopene, chlorophyll and ascorbic acid content	Vardhini and Rao (2002)

Triticum aestivum	Senescence	EBL (0.001, 0.1, 10.0 μM)	Leaves segments	Petri dishes containing EBL	Enzymes activities, chlorophyll content	Çag et al. (2007)
Zea mays	Tropism	BL (10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M)	Primary roots	Immersion of root caps	Gravitropic curvature	Chang *et al.* (2004)
Arabidopsis thaliana	Tropism	BL (10^{-10},10^{-9}, 10^{-8} and 10^{-7} M)	Seed	Medium constituent	Gravitropic curvature, root length	Kim *et al.* (2007)
Zea mays	Tropism	BL (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M)	Root	Immersion of root caps	Gravitropic curvature	Kim *et al.* (2000)
Brassica napus	Tropism	BL (0.1 μM)	Root	Medium constituent	Polar auxin transport, endogenous IAA quantification	Li *et al.* (2005)
Pisum sativum	Tropism	BL (10^{-13} to 10^{-8} M)	Root	Root solution	Gravitropic curvature	Amzallag and Vaisman (2006)
Arabidopsis thaliana	Tropism	BL (1.0 nM)	Seedling	Growth medium constituent	Hypocotyl curvature Phototropism	Whippo *et al.* (2005)
Arabidopsis thaliana	Circadian rhythms	HBL (20 μM)	Seedling	Medium constituent (imaging microtiter plates)	Modulation of circadian periodicity	Hanano *et al.* (2006)

2.4 How exogenous BR application may regulate flowering and fructification?

Brassinosteroid spraying at flowering generally leads to a significant increase in the production of various crops and it is reported in several article papers (Table 4).

Exogenous EBL application (200 ng dissolved in 100% ethanol) in grape (*Vitis vinifera* L. cv. Cabernet Sauvignon) significantly promoted ripening, while brassinazole, significantly delayed fruit ripening (Symons *et al*., 2006). Using the first appearance of coloring (anthocyanin production) in the berry skin as an indicator for the onset of ripening, the authors showed that EBR significantly promoted *véraison* while brassinazole delayed *véraison*. Total soluble solids measured in berries 28 days after the first treatment (13.4ºBrix) was greater than in control plants (12.7ºBrix) and brassinazole treated plants (11.7ºBrix) indicating that BRs also stimulate sugar accumulation. These results provide evidence that changes in endogenous BRs levels influence this key developmental process.

BR application in species of the Cucurbitaceae family was tested in order to identify different phenotypes in relation to sexual expression. In this family a phase of male flowers precedes either female or bisexual flower production. In this study EBL application (0.1, 1, 10 μM) caused a significant decrease in time of appearance of the first female flowers in monoecious cucumber plants and increased the total number of female flowers on the main stem. Increasing concentrations had a stronger effect (Papadopoulou and Grumet, 2005). EBL application in cucumber, melon and zucchini caused an increase in ethylene production suggesting that BR effect can be mediated by ethylene. The concentration of 10 μM epi-BL leads to an ethylene production comparable to that induced by 5 ppm ethefon. The treatment with 5 ppm ethefon was sufficient to increase femaleness of cucumber plants but not zucchini plants suggesting that the difference in response to EBL treatment may reflect differences in the sensitivity to ethylene (Papadopoulou and Grumet, 2005).

In a work conducted by Fu *et al*. (2008) 24-EBL application (0.02, 0.2 and 2.0 μM) in cucumber (*Cucumis sativus* L.) unpollinated ovaries induced parthenocarpic growth accompanied by active cell division in Jinchun No. 4, a cultivar without parthenocarpic capacity, whereas brassinazole treatment inhibited fruit set and, subsequently, fruit growth in Jinchun No.2, a cultivar with natural parthenocarpic capacity, and this inhibitory effect could be rescued by the application of EBR. RT-PCR analysis showed both pollination and EBR induced expression of cell-cycle related genes (CycA, CycB, CycD3.1, CycD3.2 and CDKB) after anthesis. BRs triggered active cell division associated

with increased transcripts of cell cycle-related genes, especially that of cyclin D3 genes. These results indicate that BRs play a regulatory role in early fruit development of cucumber plants. It is noteworthy that fruit development is a complex process and BRs could cross talk with other hormones such as auxins and gibberellins.

In order to verify the effects of exogenous BRs on the flowering induction of *Pharbitis nil Chois* cv. Violet, a short-day plant, BL and castasterone in the concentrations of 0.01 and 1.0 μM were applied to the cotyledons (Kesy *et al*., 2003). Both BRs used inhibit flowering, forming less number of flowers in relation to control plants and flowering inhibition was depended on the concentration and the method of BR application as well as the length of the inductive dark period. In plants regenerated from sub-induced apices treated with BL (1 and 10 μM), the flower formation was inhibited completely. These authors suggest that BR can be acting similar as auxin because this hormone is proved to inhibit flowering of this short-day plant when applied exogenously.

BL applied to rice (*Oryza sativa* L. cv. Nippon bare) promoted panicle ripening (Saka *et al*., 2003). These authors analyzed if exogenous BR application at the meiosis and flowering stages affected the endogenous levels of abscisic acid, auxin or ethylene. When brassinolide (2.1×10^{-9} and 2.1×10^{-8} M) were applied to the whole rice plants by spraying twice, 10 days before heading and on the day of heading, the free-IAA content slightly increased and greatly increased the bound-IAA content at the milk ripe stage in field conditions (22–33ºC) and in low temperature (17–22ºC). BL slightly decreased the ABA content of the spikelet at the milk-ripe stage in field conditions and slightly increased it in the low temperature condition. The rate of ethylene production was markedly high at the milk-ripe stage and low at the dough-ripe stage (21 days after heading) in field conditions. BL treatment clearly increased the rate of ethylene production from the panicles under both light and dark conditions at the milk-ripe stage. BL treatment also increased panicle weight and grain weight. BL promotes the assimilate translocation and accumulation of carbohydrates in the panicle and it may promote ripening by regulating the amounts of endogenous hormones such as auxins, abscisic acid and ethylene, not only in field conditions but also at low temperature conditions. Under low temperature condition, BL may maintain or rescue the sites of action of the other endogenous hormones mentioned as auxin and abscisic acid to promote grain filling after anthesis in rice plants.

Litchi trees (*Litchi chinensis* cv. nuomoci) were sprayed with 0.5, 0.75 and 1.0 $mg.L^{-1}$ BL at full blossom and at early fruit stage (Peng *et al*., 2004). The enzyme activities of pectin methylesterase (PME) and polygalacturonase (PG) increased and showed the same trend under BR treatments. The content of water soluble pectin remained at a higher concentration during early stages

of development of treated fruit compared to control fruit (water-prayed). Calcium concentration of fruit pericarp was higher in treated fruit than control fruit and showed significant dosage response. The cellulase activity was inhibited by BL treatment and BL reduced fruit cracking compared to control. The rise of PME and PG activities in fruit from trees treated with BL might reflect the rise of pectin metabolism, which may be related to cell division and cell elongation resulting in fruit growth. Meanwhile, the increase in calcium during early stages of fruit development could provide a good basis of fruit pericarp development and the final increase in protopectin content in the pericarp might thus guarantee the good quality of the fruit pericarp. The results showed that BL sprayed before anthesis may play an important role in increasing the commercial value of litchi fruits (Peng *et al*., 2004).

The application of 28-HBL (0.1, 1.0 and 3.0 μM) and 24-EBL (0.1, 1.0 and 3.0 μM) to tomato (*Lycopersicon esculentum* Mill.) pericarp discs resulted in increased levels of lycopene and lowered chlorophyll levels (Vardhini and Rao, 2002). Brassinosteroid-treated pericarp discs exhibited decreased ascorbic acid and increased carbohydrate contents. Fruit ripening as induced by brassinosteroids was associated with an increase in ethylene production. This study revealed the ability of BRs in accelerating fruit-senescence.

2.5 Senescence is also a process regulated by brassinosteroid

Senescence is controlled by phytohormones and the involvement of auxins, ethylene and cytokinins is well documented but it is likely that BR influence this process. Saglam-Çag (2007) showed that EBL accelerated senescence in wheat (*Triticum aestivum* L.) leaves segments especially at high concentration. An increase in peroxidase activity (at 0.1 μM) and a decrease in protease activity (at 10 μM) were detected. Application of 0.1 and 10 μM was effective in accelerating chlorophyll breakdown while 0.001 μM EBL treatment showed the highest chlorophyll content of leaves inhibiting the chlorophyll loss.

2.6 New insights related to tropism and circadian rhythms when exogenous brassinosteroid is applied in plants

When BL (10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}M) was applied exogenously to maize (*Zea* (*Zea mays* L.cv. Golden Cross Bantam) primary roots, it reduced the presentation time and lag period for the gravitropic response, whereas ethylene increased them (Chang *et al*., 2004). Ethylene increases the rate of gravitropic curvature

in a dose-dependent manner. If AVG, a specific action inhibitor of ACC synthase, is applied to the primary roots, it reduces the gravitropic curvature in the presence and absence of BL. The authors suggested that BL is involved in the gravitropic response in maize primary roots via ethylene production, but it acts in a way that differs somewhat from that of ethylene. It is possible that BL affects protein kinase activity, since the protein kinase inhibitors, staurosporine and H89, reduce BL-increased gravitropic response. The effect of brassinosteroids on plant gravitropism was verified by Kim *et al*. (2007) in the primary roots of *Arabidopsis thaliana* ecotype Columbia and Wassilewskija and BR-related mutants. Exogenously applied BL (10^{-10} M) increased both gravitropic curvature and length of primary roots, whereas at higher concentrations (10^{-9}, 10^{-8}, 10^{-7} M), BL further increased gravitropic curvature while it inhibited primary root growth. IAA is the primary hormone involved in the gravitropic response of plants. BL may activate the gravitropic response through a combination of its effect on polar IAA transport and IAA biosynthesis, resulting in the modulation of endogenous IAA levels in both upper and lower side of roots. Kim *et al*. (2000) showed that the stimulatory effect of BL is more pronounced in the presence of IAA, suggesting that BL increases the sensitivity of maize (*Zea mays* L.) roots to IAA but in *A. thaliana* the dose-dependent increase in the root gravitropic curvature was not as clearly evident in the presence of BL than the control upon IAA treatment. Kim *et al*. (2007) suggested that BL interacts negatively with IAA in the regulation of plant gravitropic response and root growth, and its regulation is achieved partly by modulating biosynthetic pathways of the counterpart hormone. Kim *et al*. (2000) also demonstrated the occurrence of endogenous BR (castasterone) in the primary roots which provides the first evidence of BR in plant roots. Analysis of the polar auxin transport capacities were analyzed in response to BL (0.1 μM) treatment to explore the potential interactions between them in *Brassica napus* ecotype Huyou 15 (Li *et al*., 2005). Analysis of the polar auxin transport (PAT) activities of *Brassica napus* seedlings using [^{14}C]IAA showed that BL treatment strongly promoted shoot basipetal IAA transport and exogenous BL treatment (0.01–0.1 μM) changed the IAA concentrations in different organs.

In roots of pea (*Pisum sativum* L. cv. Alaska) seedlings, the average lag-time required for initiation of the gravitropic response was reduced proportionally to the concentration of 24-EBL added to the root solution (10^{-13}–10^{-8} M) (Amzallag and Vaisman, 2006). A treatment with clotrimazole, an inhibitor of steroid synthesis, prevents the initiation of gravitropic response and this effect was partly reverted by EBR application. They suggested that BR stimulates the root curvature through a gravitropic-induced change in sensitivity to the hormones regulating cell elongation.

Whippo and Hangarter (2005) suggested that brassinosteroids, which are hormonal repressors of photomorphogenesis, are involved in the repression of very-low-light phototropism, given by hypocotyl curvature, and the enhancement of high-light phototropism as addition of BL (1.0 nM) resulted in a strongly enhanced high-light response in *Arabidopsis thaliana* plants.

Studies with *Arabidopsis thaliana* ecotype Wassilewskija and mutants were used to test the effect of HBL (20 μM) in circadian clock (Hanano *et al*., 2006). These authors showed that cytokinins delayed circadian phase, auxins regulated clock precision and brassinosteroid and abscisic acid modulated circadian periodicity. As a result of HBL application, circadian periodicity was shortened for CCR2, CAB2 and CCA1 mutants rhythms (1.0–2.7 hours) under both constant-light or constant darkness conditions.

3. BRASSINOSTEROID-APPLIED AMELIORATIVE EFFECTS TO A WIDE RANGE OF PLANT STRESS

Since the discovery and isolation of brassinosteroid, there is a continuous effort to discover how this compound acts and how it can be used in plants. The most related articles focus on the role to diminish toxic or non-desirable effects caused by biotical and abiotical stress. In the next topics, some information has been collected about the positive action in protecting plants from reactive oxygen species, pigments destruction and the ability to help plants to synthesize protective substances and expression of genes involved in defense responses as well as biosynthesis of other plant hormones.

3.1 Disease stress

Recent studies try to elucidate the possible role of BR-applied on plant tolerance and resistance to pathogen attack (Table 5).

BL induced resistance in rice to rice blast and bacterial blight diseases caused by *Magnaporthe grisea* and *Xanthomonas oryzae* pv. oryzae, respectively (Nakashita *et al.*, 2003). They observed that the application of BL (100 or 10 μg/pot) reduced disease symptoms caused by infection with the virulent pathogen *Xanthomonas oryzae* pv. oryzae race 003. These authors also verified that wild-type tobacco treated with BL exhibited enhanced resistance to the viral pathogen tobacco mosaic virus (TMV), to the bacterial pathogen *Pseudomonas syringae* pv. tabaci (Pst) and

Table 5. Effects of brassinosteroids on disease stress

Species	Pathogen	Hormone and concentrations applied	Organ which received BR	Mode of application	Physiological effects analyzed	Author
Oryza sativa	*Magnaporthe grisea*	BL (2, 20 and 100 µg/pot)	Root	Soil drench application (pre-treatment)	Reduction disease symptoms	Nakashita, *et al.* (2003)
Oryza sativa	*Xanthomonas oryzae* pv. oryzae	BL (2, 20 and 100 µg/pot)	Root	Soil drench application (pre-treatment)	Reduction disease symptoms	Nakashita *et al.* (2003)
Nicotiana tabacum	Tobacco Mosaic Virus (TMV)	BL (20, 40 and 200 µM)	Selected leaves	Foliar spraying (pre-treatment)	Enhanced resistance	Nakashita *et al.* (2003)
Nicotiana tabacum	*Pseudomonas syringae* pv. tabaci	BL (20 µM)	Whole plant (5-week-old)	Foliar spraying (pre-treatment)	Enhanced resistance	Nakashita *et al.* (2003)
Nicotiana tabacum	*Oidium* sp.	BL (20 µM)	Whole plant (5-week-old)	Foliar spraying (pre-treatment)	Enhanced resistance	Nakashita *et al.* (2003)
Cucumis sativus	*Fusarium*	24-EBL (0.1 µM) 24-EBL (0.2 µM–10 mL plant)	Root and leaves	Addition to nutrient solution Spraying on leaves	Reduction in pathogen-induced accumulation of reactive oxygen species, reduction disease severity	Ding *et al.* (2009a, b)
Cucumis sativus	Cucumber mosaic virus (CMV)	24-EBL (0.1 µM)	Seedling	Whole plant spraying	Expression of genes involved in defense response	Xia *et al.* (2009)

to the fungal pathogen *Oidium* sp (Nakashita *et al.*, 2003). BL-treatment did not induce either acidic or basic pathogenesis-related (PR) gene expression, suggesting that BL-induced resistance is distinct from systemic acquired resistance (SAR) and wound inducible disease resistance. They suggested that BL functions as one of the common signaling molecules in the innate immunity system of higher plants. It seems that BR is also involved in plant defense, by regulating thionin protein which is a low-molecular-weight, basic cysteine-rich antimicrobial protein and is expressed in a broad range of plant species. Transcripts of thionin genes encoding antimicrobial peptides were present at a high level in rice coleoptiles just after germination and decreased to an undetectable level after about 3 days but this decline was suppressed by co-treatment with gibberellic acid and brassinosteroid (Kitanaga *et al.*, 2006). Rice plants (*Oryza sativa* L. cv Nipponbare) were treated with a 1 µl solution containing 10 ng of GA_3 or 10 ng of BL at the

base of the coleoptiles in the light. The results indicate an action of sequential regulation between the biosynthesis of GA/BR and JA jasmonic acid in a light dependent manner, mediated by a kind of collaborative cross-signaling process from GA and BR, leading to control of thionin transcript levels.

Root and foliar applications of 24-EBL were evaluated for their effects on reducing *Fusarium* wilt and their influence on antioxidant and phenolic metabolism in roots of cucumber plants (*Cucumis sativus* L. cv. Jinyan No. 4) (Ding *et al.*, 2009a, b). EBR treatments significantly reduced pathogen-induced accumulation of reactive oxygen species (ROS), flavonoids and phenolic compounds, activities of defense-related and ROS-scavenging enzymes (superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase, catalase as well as phenylalanine ammonia-lyase and polyphenoloxidase). Xia *et al.* (2009) verified the effects of 24-EBL (0.1 μM) in cucumber (*Cucumis sativus* L. cv. Jinyan No. 4) seedlings submitted to cucumber mosaic virus (CMV) inoculation. Water-treated plants developed typical CMV symptoms 10 days post-inoculation. CMV-disease severity and malondialdehyde content in EBR-treated plants were lower than those of water-treated plants, the authors showed that EBR treatment induced expression of genes involved in defense response.

3.2 Water Stress

In plants submitted to stresses, one of the first symptoms is the synthesis of enzymes whose functions are to maintain the cell membrane integrity, the antioxidant enzymes like catalase, peroxidase and superoxide dismutase, the ABA content increases and, as a consequence, stomata close in order to diminish loss of water by transpiration (Table 6).

In soybean (*Glycine max* L.), BL (0.1 $mg.L^{-1}$) was applied by foliar spray at the beginning of bloom in plants submitted to two levels of soil moisture (80% field capacity for well watered control and 35% for drought stress treatment) (Zhang *et al.*, 2008). BR increased biomass accumulation and seed yield for both treatments. Drought stress inhibited translocation of assimilated ^{14}C from the labeled leaf, but BR increased the translocation for both treatments. BR treatment increased maximum quantum yield of PSII, the activity of ribulose-1,5-bisphosphate carboxylase and the leaf water potential of drought stressed plants. BR also increased the concentration of soluble sugars and proline and the activities of peroxidase and superoxide dismutase of soybean leaves when drought-stressed. However, it decreased the malondialdehyde concentrations and electrical conductivity of leaves under drought stress. This study shows that BR can be used as a plant growth regulator to enhance drought tolerance and minimize the yield loss of soybean caused by water deficits.

Table 6. Effects of brassinosteroids on water stress

Species	Hormone and concentrations applied	Organ which received BR	Mode of application*	Physiological effects analyzed	Author
Glycine max	BL (0.1 mg/L)	Leaves	Foliar Spraying at the beginning of bloom	Biomass accumulation, grain yield, quantum yield of PSII, enzymes activities, soluble sugar and proline content	Zhang *et al.* (2008)
Robinia pseudoacacia	BL (0 to 0.4 mg/L) BL (0 to 0.4 mg/L)	Roots and leaves of seedlings (1-y-old)	Root soaking before planting Foliar spraying	Soluble sugar and proline content, antioxidant enzymes activities, gas exchanges, seedling survival	Li *et al.* (2008)
Sorghum vulgare	28-HBL (2 and 3 µM) 24-EBL (2 and 3 µM)	Seeds	Petriplates provided with filter paper supplied with BRs	Antioxidant enzymes activities, soluble proteins and proline content	Vardhini and Rao (2003)
Phaseolus vulgaris	28-HBL (1 and 5 µM) 24-EBL (1 and 5 µM)	Seedling at flowering stage	Foliar spraying	Root nodulation, endogenous ABA and cytokinin, nitrogenase activity	Upreti and Murti (2004)
Arabidopsis thaliana *Brassica napus*	24-EBL (1 µM)	Seed	Addition to nutrient solution	Growth, morphological changes	Kagale *et al.* (2007)

* All the BR-treatments were done before water stress

In *Robinia pseudoacacia* seedlings, soaking roots in BL (0–0.4 $mg.L^{-1}$) prior to planting increased the activities of POD, SOD and catalase in water stressed plants when compared to the control plants and the survival and growth of seedlings (Li *et al.*, 2008). The best results were in 0.2 $mg.L^{-1}$ BL treatment, because plants decreased the transpiration rate, stomatal conductance and malondialdehyde content. In sorghum (*Sorghum vulgare* Pers), 28-HBL and 24-EBL application in susceptible and resistant varieties reduced peroxidase and ascorbic acid oxidase activities while catalase activity was increased in plants submitted to osmotic stress (Vardhini and Rao, 2003). The higher activity of catalase in brassinosteroid treated seedlings of sorghum might have resulted in increased oxidation of harmful substrates, leading to increased seedling growth. A crucial mechanism in the adaptation process of plants to water stress is the osmotic adjustment because it might support the tissue metabolic activity and provide *de novo* growth after rewatering. The osmotic compounds include proteins and amino acids. Under osmotic stress (polyethylene glycol alone), there was increase in the levels of free proline in seedlings as compared to unstressed control seedlings. The application of brassinosteroids further increased the levels of free proline

under osmotic stressed conditions. The free proline levels were higher in 3 μM brassinosteroid treatments, where the stress alleviation was also found to be maximum.

Application of 1 and 5 μM EBL or HBL prior to water stress induction in the nodulated roots of *Phaseolus vulgaris* L. cv Arka Suvidha was studied (Upreti and Murti, 2004). Brassinosteroids in the unstressed plants increased root nodulation, zeatin contents and nitrogenase activity and also ameliorated their stress-induced decline in the nodulated roots, the response was more prominent at 5 μM concentration in 4-days stressed plants. Among the brassinosteroids, EBL was relatively more effective than HBL (Upreti and Murti, 2004).

Arabidopsis thaliana and *Brassica napus* seedlings grown on nutrient solution containing 1 μM 24-EBL and then transplanted to sand were subjected to drought stress by withholding water for 96 or 60 hours (Kagale *et al.*, 2007). Visible morphological changes in response to drought stress, such as leaf wilting, reduction in growth and complete drying of some seedlings were frequently observed in untreated but were considerably reduced in EBR-treated seedlings.

3.3 Saline Stress

Proline accumulation is an indicator of saline stress; this compound is accumulated in order to maintain plant water relations. BR application enhances proline accumulation (Table 7).

The application of 24-EBL and 28-HBL to rice seeds (*Oryza sativa* L.) reduced the impact of salt stress on growth, prevented photosynthetic pigment loss and increased nitrate reductase activity (Anuradha and Rao, 2003). The seeds treated with 3.0 μM of brassinosteroid solution considerably reduced the growth inhibitory effect of salt stress as reflected in the growth of the plants.

Osmotic stress-induced accumulation of proline, an important protective osmolyte in higher plants, is dependent on the expression of Δ^1-pyrroline-5-carboxylate synthase (P5CS) and proline dehydrogenase (PDH) enzymes that catalyze the rate-limiting steps of proline biosynthesis and degradation, respectively. Stimulation of proline synthesis by abscisic acid and salt stress correlates with a striking activation of P5CS1 expression in *Arabidopsis* (Ábrahám *et al.*, 2003). By contrast, P5CS2 is weakly induced whereas PDH is inhibited to different extent by ABA and salt stress in shoots and roots of light-grown plants. Proline accumulation and light-dependent induction of P5CS1 by abscisic acid and salt stress is inhibited in dark-adapted plants. During dark adaptation P5CS2 is also down-regulated, whereas PDH expression is significantly enhanced in shoots. The inhibitory effect of dark

Table 7. Effects of brassinosteroids on saline stress

Species	Hormone and some concentrations applied	Organ which received BL	Mode of application	Physiological effects analyzed	Author
Oryza sativa	24-EBL (3 μM) 28-HBL (3 μM)	Seed	Soaking seeds (pre-treatment)	Photosynthetic pigments, nitrate reductase activity	Anuradha and Rao (2003)
Arabidopsis thaliana	24-EBL (0.1 μM)	Seedling	Medium constituent (Pre-treatment)	Proline content, proline metabolism enzymes	Ábrahám *et al.* (2003)
Oryza sativa	BB-16 (0.001, 0.01 mg/dm^3)	Seedling	Medium constituent (pre-treatment)	Antioxidant enzymes	Núñez *et al.* (2003)
Oryza sativa	24-EBL (3 μM)	Seed	Soaking seeds (pre-treatment)	Growth, antioxidative system, lipid peroxidation, proline and soluble protein content	Özdemir *et al.* (2004)
Brassica napus	24-EBL (1 and 2 μM)	Seed	Medium constituent Co-application (EBL and NaCl)	Germination and growth	Kagale *et al.* (2007)
Cicer arietinum	28-HBL (10^{-10} and 10^{-8} M)	Seed	Soaking seeds (applied before and after NaCl application)	nitrate reductase and carbonic anhydrase activities, nodule number	Ali *et al.* (2007)
Brassica junceae	28-HBL (10^{-10}, 10^{-8} and 10^{-6} M)	Seedling	HBL solution added to soil (after salt stress)	Nitrate reductase and carbonic anhydrase activities, chlorophyll content and photosynthetic rate, proline content	Hayat *et al.* (2007a)
Medicago sativa	BL (5 μM/L)	Seed	Seed soaking (pre-treatment to salt stress)	Germination and seedling growth, lipid peroxidation, antioxidant enzymes	Zhang *et al.* (2007)
Triticum aestivum	24-EBL (0.052, 0.104 and 0.156 μM)	Seed and seedling	Growth medium (co-application with salt stress)	Growth and grain yield	Ali *et al.* (2008a)
Triticum aestivum	24-EBL (0.0125, 0.025 and 0.0375 mg.l^{-1}/25 mL per pot)	Whole plant (~40-d-old)	Foliar spray	Growth and photosynthesis, enzymes	Shahbaz *et al.* (2008)
Zea mays	28-HBL (10^{-7}, 10^{-9} and 10^{-11} M	Seed	Seed soaking (Co-application with NaCl)	Growth, lipid peroxidation and antioxidative enzyme activities	Arora *et al.* (2008)
Spirulina platensis	24-EBL(0.5, 1.0 and 3.0 μM)	Culture	Constituent medium (Co-application with NaCl)	Growth and proline content	Saygideger and Deniz (2008)
Hordeum vulgare	24-EBL (3.0 μM)	Root meristem cells	Seed soaking	Mitotic activity	Tabur and Demir (2009)
Sorghum bicolor	24-EBL			Leaf development	Amzallag (2004)

adaptation on P5CS1 is mimicked by the application of 24-EBL (0.1 μM) for 3 days before the addition of either ABA or NaCl. The fact that both ABA and salt induction of P5CS1 transcription is inhibited by BL in light-grown plants suggests that steroid hormones may negatively regulate this common salt and ABA response pathway. Alternatively, BL may inhibit the light or sugar (or both) regulated maintenance of basal P5CS1 transcription which is essential for further induction by salt and ABA. Núñez *et al.*, (2003) studied the effects of a polyhydroxilated spirostanic analogue of brassinosteroid (BB-16) on the activities of antioxidant enzymes in rice seedlings (*Oryza sativa* L. cv. J-104), susceptible to saline stress, grown *in vitro* supplemented with NaCl. Seedlings exposed to 0.01 $mg.dm^{-3}$ for 16 days showed significant increase in the activities of catalase, superoxide dismutase and glutathione reductase and a slight increase in ascorbate peroxidase. On the other hand, 4 days exposure to BB-16 only increased superoxide dismutase and catalase activities at concentration 0.001 $mg.dm^{-3}$ BB-16. These results indicate that BB-16, which is structurally modified in the lateral chain in relation to natural brassinosteroids, changes the activity of key antioxidant enzymes, which might confer tolerance to saline stress. The effects of 24-EBL on seedling growth, antioxidative system, lipid peroxidation, proline and soluble protein content were investigated in seedlings of the salt-sensitive rice (*Oryza sativa* L.) cultivar IR-28 (Özdemir *et al.*, 2004). Seed application of 24-EBL (3 μM) improved seedling growth, alleviated the lipid damage and decreased proline accumulation caused by salt stress (120 mM) in a salt-sensitive rice variety. However, except for ascorbate peroxidase, it did not increase the activities of peroxidase, catalase and glutathione reductase under salinity stress. To determine the influence of 24-EBL salt-stress induced inhibition of *Brassica napus*, seed germination were allowed to germinate on a nutrient medium containing 1 or 2 μM of 24-EBL and different concentrations of NaCl (Kagale *et al.*, 2007). Presence of 24-EBL in the medium in particular at a concentration of 2 μM, considerably reduced the inhibitory effect of high salt on seed germination as evidenced by increase in germination and early seedling growth. Seeds of chickpea (*Cicer arietinum* L. cv. KPG-59) imbibed in aqueous solution of 10^{-10} or 10^{-8} M of 28-HBL and NaCl (1 or 10 mM) were evaluated (Ali *et al.*, 2007). The plants resulting from the seeds soaked in HBR (10^{-8} M) possessed higher leaf nitrate reductase and carbonic anhydrase activities, more dry mass, higher nodule number and more nodule fresh and dry mass, compared with water soaked, control. These values declined significantly in plants raised from the seeds soaked in NaCl. This effect was overcome, if NaCl treatment was given before or after HBR treatment. Other study verified the effect of 28-HBL (10^{-10}, 10^{-8}, 10^{-6} M) on salinity-induced changes in *Brassica juncea* Czern. and Coss cv.Varuna (Hayat *et al.*, 2007a). Plants that received only NaCl (50, 100 or 150 mM)

treatment exhibited a decrease in nitrate reductase and carbonic anhydrase activities, chlorophyll content and photosynthetic rate 60 days after sowing. Subsequent treatment with HBR significantly increased all of these parameters. The 10^{-8} M concentration of HBR generated the best response and also overcame the detrimental effects when NaCl concentration was 50 mM. The HBR concentration of 10^{-8} M along with the NaCl concentration of 150 mM resulted in the increased concentration of tissue proline concentration compared to the other treatments.

Zhang *et al.* (2007) tested the seeds of three lucerne (*Medicago sativa* L.) varieties (cv. Victor, Victoria and Golden Empress) to investigate the effects of seed priming with 5 $\mu M.L^{-1}$ BL on germination and seedling growth under a high level of salt stress. Seed priming with BL improved the salt tolerance of lucerne seedlings. This was supported by increasing germination ability, root length, root vigour, root dry weight and shoot fresh and dry weight under a high level salt stress (13.6 dSm^{-1} NaCl solution). It was also demonstrated by the increase in peroxidase, catalase and superoxide dismutase activities and the lower malondialdehyde, reflecting the level of lipid peroxidation in lucerne seedlings.

Ali *et al.* (2008a) verified that root applied 24-EBL improved growth and yield of two wheat (*Triticum aestivum*) cultivars (S-24, salt tolerant and MH-97, moderately salt-sensitive). Plants were grown at 0 or 120 mM NaCl in continuously aerated Hoagland's nutrient solution. Different concentrations of 24-EBL (0.052, 0.104, 0.156 μM) were also maintained in the nutrient solution. Exogenous application of 24-EBL counteracted the salt-stress induced growth and grain yield inhibition of both wheat cultivars. The most effective concentrations for promoting growth were 0.104 and 0.052 μM under normal and saline conditions. However, root applied 0.052 μM 24-EBL enhanced the total grain yield and 100 grain weight of salt stressed plants of both cultivars and suggested that total grain yield was mainly increased by an increase in grain size which might have been due to 24-EBL induced increase in translocation of more photoassimilates towards grain. Growth improvement in both cultivars due to root-applied 24-EBL was found to be associated with improved photosynthetic capacity. Shahbaz *et al.* (2008) using the same wheat cultivars (S-24 and MH-97) under salinity stress (150 mM NaCl), verified that foliar spray of 24-EBL (0.0125, 0.025 and 0.0375 $mg.L^{-1}$) increased plant biomass and leaf area per plant of both cultivars under non-saline conditions. However, under saline conditions, improvement in growth due to exogenous EBR was observed only in S-24 (salt tolerant cultivar). Photosynthetic rate was reduced due to salt stress in both cultivars, but this inhibitory effect was ameliorated significantly by the exogenous application of EBR. The most effective dose in improving growth of both cultivars due to EBR spray under

non-saline or saline conditions was found to be 0.025 mg.L^{-1}. EBR induced increase in growth was associated with improved photosynthetic capacity.

Arora *et al.* (2008) studied the effects of 28-HBL on seedling growth, lipid peroxidation and antioxidative enzyme activities in the seedlings of *Zea mays* L. var. Partap-1 under salt stress. The seeds were germinated in recipients containing different concentrations of NaCl (25, 50, 75 and 100 mM) only, 28-HBL (10^{-7}, 10^{-9} and 10^{-11} M) only and NaCl supplemented with 28-HBL for 7 days (Arora *et al.*, 2008). It was observed that 28-HBL treatments reduced the toxicity of salt on seedling growth considerably, 10^{-9} M concentration being the most effective. Lipid peroxidation level was significantly increased under saline stress, but lowered with HBR applications revealing less oxidative damage. Further HBR treatments to the seedlings showed an enhancement in activities of superoxide dismutase, guaiacol peroxidase, catalase and ascorbate peroxidase. The activities of all antioxidative enzymes were further increased in seedlings treated with solution containing HBR and salt together as compared to seedlings treated with different concentrations of salt solution only. The concentration of malondialdehyde (MDA) got increased by NaCl treatments but decreased with HBR supplementations. The MDA content of seedlings treated with different concentrations of salt in combination with various concentrations of HBR showed maximum decrease in 10^{-9} M concentration of HBR.

The biomass, growth and free proline concentration were investigated in *Spirulina platensis* treated with different concentrations of NaCl (50, 100, 150 and 200 mM) and 24-EBL (0.5, 1.0 and 3.0 μM) over 5 days (Saygideger and Deniz, 2008). Among the cultures supplied with different combinations of NaCl and EBR, growth rate was maximal for the culture containing 150 mM NaCl and 1.0 μM combination. Free proline concentration also increased in *S. platensis* under salinity stress, but EBR showed no notable effect on proline. Cytogenetic response of 24-EBL was evaluated under different NaCl conditions (0.3, 0.35 and 0.4M NaCl) on root meristem cells of barley (*Hordeum vulgare* L. cv. Bülbül 89) seeds (Tabur and Demir, 2009). EBR pretreatment in higher concentrations of salt (0.4 M NaCl) caused total inhibition of mitotic activity in root tip cells. However, comparison of all concentrations of salt and control revealed to have a successful performance in ameliorating the detrimental effects of salinity on chromosomal abnormalities. Leaf development of salt-treated *Sorghum bicolor* L. Moench plants are influenced by treatment with 24-EBL but only during a short period in development (Amzallag, 2004). The effects of EBR on leaf malformations during their unfolding in plants exposed to 150mM NaCl showed that treatments with 24-EBL enable modification of initiation, duration and intensity of this critical period of reorganization. It is suggested that BR, at

specific concentrations and time in development may induce changes in cellular sensitivity to many growth regulators.

3.4 Thermal Stress

Plant chilling and plant heat injury inhibit growth by an effect on some essential metabolic enzymes from photosynthesis and respiration. BRs application induces the synthesis of heat-shock proteins, antioxidant enzymes and the expression of cold-related genes (Table 8).

Table 8. Effects of brassinosteroids on thermal stress (heat stress (HS) and cold stress (CS)

Species	Type of thermal stress	BR and concentrations applied	Organ which received BR	Mode of application	Physiological effects analyzed	Author
Lycopersicon esculentum	Heat stress	24-EBL (1, 10 and 20 µM)	Leaves (4 weeks old)	Spray (before HS)	Plant survival, CO_2 gas exchange	Singh and Shono (2005)
		24-EBL (1, 10 and 50 µM)	Pollen grain (in vitro)	Addition to medium (before HS)	Mitochondrial small heat shock proteins	
Lycopersicon esculentum	Heat stress	24-EBL (2.12 and 10.6 nM) MH5 (2.12 and 10.6 nM)	Leaf discs	Incubation in Petri dishes (before HS)	Antioxidant enzymes activities Pollen viability	Mazorra *et al.* (2002)
Lycopersicon esculentum	Heat stress	24-EBL (0.01, 0.1 and 1.0 mg/L)	Whole plant	Spray (before HS)	CO_2 gas exchange	Ogweno *et al.* (2008)
Brassica napus	Heat stress	BL (10^{-6} M)	Seedling	Spray (before HS)	Endogenous ABA content	Kurepin *et al.* (2008)
Arabidopsis thaliana	Heat stress	24-EBL (1 µM)	Seed	Addition to medium (before HS)	Bleaching	Kagale *et al.* (2007)
Chorispora bungeana	Cold stress	24-EBL (0.05 mg/L)	Cultured cells	Medium constituent	Reactive oxygen species, lipid peroxidation, antioxidant enzymes	Liu *et al.* (2009)
Vigna radiata	Cold stress	EBL (3 mM)	Seedling (5-day-old)	Spray (applied after cold stress)	Proteins	Huang *et al.* (2006)
Brassica napus	Cold stress	24-EBL (0.05, 1 µM) 24-EBL (1 µM)	Seedling: Cotyledons Primary leaves	Injection into the apoplast by gentle pressure (applied before CS)	Membrane permeability, pigment content	Janeckzo *et al.* (2007)
Brassica napus	Cold stress	24-EBL (1 µM)	Seed	Addition to medium (before CS)	Transcripts of cold-related genes	Kagale *et al*. (2007)
Arabidopsis thaliana	Cold stress	24-EBL (1 µM)	Seed	Addition to medium (before CS)	Transcripts of cold-related genes	Kagale *et al.* (2007)
Cucumis sativus	Cold stress	24-EBL (0.1 µM)	Seedling	Spraying (before CS)	Electron transport rate	Xia *et al.* (2009)

3.1.1 Heat Stress

Tomato plants (*Lycopersicon esculentum* Mill.) treated with 24-EBL are more tolerant to high temperature stress than untreated plants (Singh and Shono, 2005). When it was analyzed the mitochondrial small heat shock proteins (Mt-sHSP), the authors verified that these proteins did not accumulate in EBR treated plants (1 μM) at 25°C, although treatment of plants at 38°C induced much more accumulation of Mt-sHSP proteins in EBR treated than in untreated plants. EBR possibly induced thermotolerance in tomato plants and these plants had better photosynthetic efficiency. Exposure to 45°C for 3 hours completely killed more than 90% untreated plants, while 1 μM EBR application was found to be most effective for survival of tomato plants at lethal temperature. Besides, *in vitro* pollen germination at high temperature showed a varied response to different EBR concentrations. About 50 μM EBR totally inhibited pollen germination; however, we observed a significant increase in *in vitro* pollen germination with the control at high temperature. Other effects of 1 μM EBR on pollen viability included enhanced pollen tube growth and reduced pollen bursting during heat stress. In other study with tomato discs (*Lycopersicon esculentum* Mill.) cv. Amalia, it was verified that the effects of 24-EBL (10.6 and 2.12 nM) and MH-5 (10.6 and 2.12 nM), a polyhydroxylated spirostanic analogue, in the activity of the enzymes catalase, peroxidase and superoxide dismutase at 25 and 40°C (Mazorra *et al.*, 2002). Both concentrations of EBR and MH-5 stimulated the activity of SOD at 25 and 40°C, the MH-5-stimulated increase of this enzyme was greater. Superoxide dismutase is a key enzyme in the detoxification of superoxide radicals. The increased superoxide dismutase activity after EBR treatment at 25°C suggests that EBR-promoted activation of SOD might decrease the possible toxic concentrations of O_2^- radicals. Peroxidase activity was unaffected at 25°C, while at 40°C the activity was enhanced by both compounds. The changes in catalase activity markedly depended on the structure of BRs, doses and temperature. The results suggest a possible role of EBR and MH-5 in the reduction of cell damage produced by heat stress due to induction of enzymatic antioxidants.

When BL (0.1% aqueous ethanol plus BL at 10^{-6} M) was applied to canola seedlings (*Brassica napus* L. cv Westar) at 20°C and 45°C (heat stress), Kurepin *et al.* (2008) verified that endogenous abscisic acid concentration was not affected in plants maintained at normal temperatures. However abscisic acid concentration was significantly elevated by heat stress alone and doubled by heat stress plus BL. These results suggest that the well-known enhancement of tolerance to high temperature stress that can be obtained by brassinosteroid applications may be caused by a brassinosteroid-induced elevation in endogenous abscisic acid concentration. When exogenously 24-EBL

concentrations (0.01, 0.1 and 1.0 $mg.L^{-1}$) were applied in tomato (*Lycopersicon esculentum* Mill. cv. 9021) exposed to high temperature (40/30°C), the net photosynthetic rate, stomatal conductance and maximum carboxylation rate of Rubisco (ribulose 1,5-bisphosphate carboxylase oxygenase) were decreased (Ogweno *et al.*, 2008). The activities of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and catalase increased during heat treatments and these increases proved to be more significant in EBR-treated plants (0.1 $mg.L^{-1}$ EBR). EBR application also reduced total hydrogen peroxide (H_2O_2) and malonaldehyde (MDA) contents, while significantly increased shoot weight following heat stress. It was concluded that EBR could alleviate the detrimental effects of high temperatures on plant growth by increasing carboxylation efficiency and enhancing antioxidant enzyme systems in leaves. EBR showed a concentration-dependent effect on net CO_2 assimilation at high temperatures (14.8%, 26.6% and 10.2% for 0.01, 0.1 and 1.0 $mg.L^{-1}$ EBR treatments, respectively). The results indicated that the rate-limiting enzyme Rubisco in the Calvin Cycle and other enzymes involved in RuBP regeneration were protected by EBR pretreatment and functioned well under heat stress. It is clear that heat stress increased lipid peroxidation in plants and this was significantly alleviated by EBR pretreatment.

Arabidopsis thaliana seedlings were exposed to 43°C in the presence and absence of 1μM 24-EBL for 1, 2, 3 or 4 hours and then allowing them to recover at 22°C for 7 days (Kagale *et al.*, 2007). Untreated seedlings exposed to 2, 3 and 4 hours of heat stress exhibited increasing levels of bleaching, whereas in EBR-treated seedlings, mild to severe bleaching was observed only with 4 hours of heat stress.

3.1.2 Cold Stress

Suspension cultured cells of *Chorispora bungeana* with or without 24-EBL (EBR) application were exposed to 4 and 0°C for 5 days (Liu *et al.*, 2009). The 24-EBL (0.05 $mg.L^{-1}$) treated cells exhibited higher viability after exposure to low temperatures compared with the control. Under chilling stress, reactive oxygen species (ROS) levels and lipid peroxidation were increased in the cultured cells which were significantly inhibited by EBR treatment. The activities of antioxidant enzymes such as ascorbate peroxidase, catalase, peroxidase and superoxide dismutase were increased during chilling treatments and these increases were more significant in the EBR-applied suspension cells. The EBR treatment also greatly enhanced contents of ascorbic acid and reduced gluthatione under chilling stress. From these results, it can be concluded that EBR could play the positive roles in the alleviation of oxidative damage caused by ROS (reactive oxygen species) overproduction

through enhancing antioxidant defense system, resulting in improving the tolerance of *C. bungeana* suspension cultures to chilling stress. BRs may reduce chilling injury of plant cell membranes due to lipid peroxidation, therefore protecting the structural integrity of the membranes and resulting in the enhancement of chilling tolerance.

Mung bean epycotils (*Vigna radiata* L.) whose growth was initially suppressed by chilling partly recovered their ability to elongate after treatment with 24-EBL (10 μM); 17 proteins down-regulated by this chilling were re-up-regulated and these up-regulated proteins are involved in methionine assimilation, ATP synthesis, cell wall construction and the stress response (Huang *et al.*, 2006). Other experiment using 24-EBL to investigate the effect on cold resistance of rape seedlings (*Brassica napus* L. cv. Lycosmos), verified that at 2°C, BR injection into cotyledons (0.05 and 1.00 μM) or primary leaves (1.0 μM) abolished the effect of cold on permeability as plants submitted to cold treatment without BR injection elevated the membrane permeability (Janeckzo *et al.*, 2007). BR solutions strongly elevated the membrane permeability at 20°C. In seedlings exposed to 2°C, pigments content was significantly higher in BR-treated leaves as compared to control. There were no differences between pigment contents of leaves injected with BR solutions or only water/ethanol at 20°C. The increase in membrane permeability at 20°C is probably due to the hormonal effect of 24-EBL on cell elongation. On the contrary, the elevated ion leakage induced by infiltration and cold stress at 2°C can be alleviated or abolished by BR treatments, which shows the stress protecting effect of BR.

Brassica napus and *Arabidopsis thaliana* grown on a nutrient solution containing 1 μM 24-EBL were exposed to cold stress by transferring seedlings to a growth chamber set at 2°C for a maximum of 3 days, the transcripts of cold-related genes analyzed clearly accumulated to higher levels in 24-EBL treated plants (Kagale *et al.*, 2007).

Xia *et al.* (2009) verified the effects of 24-EBL (0.1 μM) in cucumber (*Cucumis sativus* L.) cv.Jinyan No.4 seedlings submitted to chilling stress. Chilling stress (8°C) caused significant reduction in electron transport rate. EBR treatment alleviated chilling stress and enhanced the electron transport rate.

3.5 Pesticide stress

Pesticides induce destruction of pigments, hormonal imbalance and other harmful effects, recent studies have verified the potential to alleviate stress caused by pesticides (Table 9).

Table 9. Effects of brassinosteroids on pesticides stress

Species	Type of pesticide	BRs concentrations applied	Organ which received BR	Mode of application	Physiological effects analyzed	Author
Eucalyptus grandis	Imazapyr Glyphosate	BB-16 (0.08 and 0.16 mg/L)	Plants (3-month-old)	Spraying (before and after pesticide application)	Photosynthesis	Silva *et al.* (2009)
Cucumis sativus	Paraquat, fluazifop-ρ-butyl, haloxyfop, flusilazole, cuproxat, cyazofamid, imidacloprid, chlorpyrifos and abamectin	24-EBL (0.1 mg/L)	One half of the seedling	Spraying 1 day before pesticide treatment	gas exchange and chlorophyll fluorescence measurements	Xia *et al.* (2006)
Cucumis sativus	Paraquat	24-EBL (0.1 μM)	Seedling	Spraying before pesticide treatment	chlorophyll fluorescence measurements	Xia *et al.* (2009)

The effects of Imazapyr (0.750 $kg.ha^{-1}$) and Glyphosate (1.440 $kg.ha^{-1}$), two types of herbicides, and their interactions with a spirostanic analogue of castasterone (BB-16) (0.08 $mg.L^{-1}$ and 0.16 $mg.L^{-1}$) on the growth of seedlings clones of *Eucalyptus grandis* were evaluated (Silva *et al.*, 2009). A treatment verified BB-16 12 hours before herbicides application and other treatment verified the application of BB-16 after herbicide application. The seedlings that received the glyphosate application associated or not to BB-16, independently of concentration and time, exhibited necrosis before the seventh day, while the seedlings that received Imazapyr associated to BB-16 showed only necrotic lesion at the extremity of the lateral branches. The interaction of Glyphosate and BB-16 increased the potential to lead the plant to death while Imazapyr and BB-16 showed the potential to alleviate stress during a short time after the beginning of treatments when compared to the application of Imazapyr only. These finding suggests that the tolerance found in plants submitted to Imazapyr versus BB-16 may be related to the receptors of BB-16 and Imazapyr in plasma membrane.

The phytotoxicities of nine pesticides (paraquat, fluazifop-ρ-butyl, haloxyfop, flusilazole, cuproxat, cyazofamid, imidacloprid, chlorpyrifos and abamectin) at practical dosages on photosynthesis were investigated in cucumber (*Cucumis sativus* L. cv. Jynian No.4) at four leaf-stage by gas exchange and chlorophyll fluorescence measurements (Xia *et al.*, 2006). Inhibition of net photosynthetic rate (P_N) were alleviated by 24-EBL (0.1 $mg.L^{-1}$ 1 day before pesticide treatment) for the pesticides examined except paraquat and flusilazole. EBR pretreatment also increased quantum efficiency

of photosystem II and photochemical quenching coefficient (qP). It is likely that EBR enhanced the resistance of cucumber seedlings to pesticides by increasing CO_2 assimilation capacity and activities of antioxidant enzymes. Ultrastructural studies showed that 22(S),23(S)-HBL (1.0 μM) applied to potato leaves (*Solanum tuberosum* L. cv. Désirée) significantly reduced H_2O_2 negative effects on cellular sub-structures, allowing better recovery of affected structures and reducing the macroscopic injury symptoms on leaves (Almeida *et al.*, 2005).

Xia *et al.* (2009) verified the effects of 24-EBL (0.1 μM) on cucumber (*Cucumis sativus* L.) plant sensitivity to paraquat which causes photo-oxidative stress. They compared the effects on photosynthetic efficiency by comparing the maximum photochemical efficiency of PSII in the dark adapted state (F_v/F_m). Fluorescence images showed that paraquat treatment resulted in a significant decrease in F_v/F_m in control plants and Paraquat-induced reduction in F_v/F_m was less in EBR-treated plants.

3.6 Heavy metals stress

BRs have ability to regulate the uptake of ions into the plant cells and they can be used to reduce the accumulation of heavy metals and radioactive elements in plants (Table 10).

Among pollutants of agricultural soils, Cu has become increasingly hazardous due to its involvement in fungicides, fertilizers and pesticides. However, Cu at high levels become strongly phytotoxic and cause inhibition of plant growth or even death. When *Brassica juncea* L. cv. PBR91 seeds were treated before germination with 24-EBL (10^{-7}, 10^{-9}, 10^{-11} M) and submitted to copper stress, there was an improvement in the shoot emergence and plant biomass production (Sharma and Bhardwaj, 2007). This compound at 10^{-7} M concentration was the most effective for lowering the Cu uptake and accumulation of ions. The fresh weight of the whole plant was increased in all the concentrations as compared to the control.

Although nickel is an essential element, required at low concentrations for urease metabolism, nickel at high concentration is toxic because it inhibits photosynthesis, respiration, enzymes activities and protein. Plants of *Brassica juncea* L. cv. T-59 were supplied with 50 or 100μM nickel at 10 days after sowing and sprayed with 28-HBL (10^{-8} M) at 20 days after sowing (Alam *et al.*, 2007). The plants treated with nickel alone exhibited reduced growth, net photosynthetic rate, content of chlorophyll and the activities of nitrate reductase and carbonic anhydrase, observed 40 days after sowing, whereas the contents of peroxidase, catalase and proline were increased. The spray of HBR partially neutralized the toxic effect of nickel on most of the parameters. Sharma *et al.* (2008) using *Brassica juncea* L. cv.

Table 10. Effects of brassinosteroids on heavy metals stress

Species	Heavy metal	BR and concentrations applied	Organ which received BL	Mode of application	Physiological effects analyzed	Author
Brassica junceae	Cu	24-EBL (10^{-7}, 10^{-9}, 10^{-11} M)	Seed	Seed soaking	Shoot emergence and plant biomass production	Sharma and Bhardwaj (2007)
Brassica junceae	Ni	28-HBL (10^{-8} M)	Leaf	Spraying	Growth, photosynthesis, enzymes activities	Alam *et al.* (2007)
Brassica junceae	Ni	28-HBL (0.01, 1.0 and 100 nM)	Seed	Seed soaking	Growth, protein content and antioxidative enzymes activities	Sharma *et al.* (2008)
Brassica napus	Cd	24-EBL (100 nM)	Seedling	Medium constituent *in vitro* culture	Chlorophyll fluorescence, photosynthetic pigments	Janeckzo *et al.* (2005)
Brassica junceae	Cd	28-HBL (0.01 µM)	Leaves	Spraying	Growth, chlorophyll pigments, enzymes, proline content	Hayat *et al*. (2007b)
Raphanus sativus	Cd	24-EBL (3 µM) 28-HBL (3 µM)	Seed	Seed soaking	Seed germination, seedling growth, proline content, enzymes, lipid peroxidation	Anuradha and Rao (2007a)
Cicer arietinum	Cd	28-HBL (0.01 µM)	Seedling	Spraying	number of nodules, leghemoglobin content, nitrogen and carbohydrate content, chlorophyll content, enzymes	Hasan *et al.* (2008b)
Vigna radiata	Al	24-EBL (10^{-8} M) 24-EBL (10^{-8} M)	Seedling	Foliar spray	Growth, photosynthesis, antioxidant enzymes	Ali *et al.* (2008b)
Phaseolus aureus	Al	BL (0.1, 10, 100 and 100,000 ng/L)	Seedling	Growth solution	Growth, chlorophyll content	Abdullahi (2003)
Chlorella vulgaris	Pb	20-hydroxyecdysone (10^{-10}–10^{-8} M)	Culture cells	Medium constituent	Growth, chlorophyll, sugar and protein content and phytochelatins synthesis	Bajguz and Godlewska-Zylkiewicz (2004)
Raphanus sativus	Pb	24-EBL			Antioxidant enzymes	Anuradha and Rao (2007b)

PBR91 seeds soaked for 8 hours in different concentrations of 28-HBL (0.01, 1.0 and 100nM) and submitted to nickel concentrations (25, 50 and 100 mg.dm^{-3}) verified, 7 days after germination, that the growth of seedlings was inhibited by Ni, however, less after HBL pre-treatment. The protein content and antioxidative enzymes activities (catalase, glutathione reductase, ascorbate peroxidase, superoxide dismutase, guaiacol peroxidase) were also

increased by HBL treatment. The seed germination and seedling growth was significantly reduced by the Ni treatment but the HBL alone enhanced the germination percentage as well as shoot and root length (maximum germination observed with 100 mg.dm^{-3} Ni and 1.0 nM HBL).

Cadmium is extremely toxic to plants. It retards biosynthesis of chlorophyll, alters water balance, decreases activity of various enzymes, favors stomatal closure, induces oxidative stresses in plants and slows down the rate of photosynthesis. Cadmium inhibits both the "light" and "dark" reactions of photosynthesis, but the Calvin cycle is more sensitive to its activity. The inhibition of photochemical processes by Cd may result from the limitation in the use of ATP and NADPH by the Calvin cycle and accompanying increase of pH gradient across the thylakoid membranes. Seedlings of winter rape (*Brassica napus* L.) cv. Górczánsky were cultured *in vitro* on media containing 24-EBL (100nM) and cadmium (300μM) (Janeckzo *et al.*, 2005). After 14 days of growth, fast fluorescence kinetics of chlorophyll a (Chl a) and contents of photosynthetic pigments and Cd in cotyledons were measured. Cd was strongly accumulated but its content in cotyledons was 14.7% smaller in presence of EBR. EBR reduces the toxic effect of Cd on photochemical processes by diminishing the damage of photochemical active reaction centers and the activity of O_2 evolving centers as well as maintaining efficient photosynthetic electron transport. The change in *Brassica juncea* plant growth and photosynthesis submitted to Cd (100 or 150 μM) and 28-HBL (0.01 μM) application was also verified by Hayat *et al.* (2007b). These authors observed that the plants fed with cadmium alone exhibited a decline in growth, in the levels of carbonic anhydrase and chlorophyll pigments and net photosynthetic rate. Nitrate content, the activity of nitrate reductase and the level of carbohydrate both in the leaves and roots decreased as the concentration of Cd increased. The toxic effect generated by Cd was overcome if the stressed plants were sprayed with HBL. The activities of antioxidant enzymes (catalase, peroxidase and superoxide dismutase) and the contents of proline increased over the control, irrespective of the treatments. Their level increased further, if the plants supplied with Cd were also supplemented with HBL. The effect of 24-EBL and 28-HBL on seed germination and seedling growth of radish (*Raphanus sativus* L.) was studied under Cd toxicity (Anuradha and Rao, 2007a). Both BRs at 3 μM concentration caused a considerable increase in seedling growth even under stress and restored the growth to the level of unstressed control seedlings. Besides, in response to Cd stress, radish seedlings accumulated proline (BR enhanced proline content), decreased catalase activity (BR increased) and peroxidase activity (BR reduced). However, Cd stress increased the activities of ascorbic peroxidase (BR enhanced), guaiacol peroxidase (BR enhanced), ascorbic acid oxidase (BR decreased), and superoxide dismutase

(BR enhanced). Lipid peroxidation induced by Cd was found reduced with the supplementation of BRs. Brassinosteroids strongly protect radish seedling from Cd induced oxidative stress by minimizing the impact of reactive oxygen species by increasing antioxidant enzyme activity, which may represent a secondary defensive mechanism against oxidative stress. The seedlings of *Cicer arietinum* L. cv. Uday were supplied with Cd (50, 100 and 150 μM) and sprayed with 0.01 μM of 28-HBL at 30-day stage (Hasan *et al.*, 2008). Plant fresh and dry mass, number of nodules, leghemoglobin content, nitrogen and carbohydrate content in the nodules, leaf chlorophyll content, nitrate reductase and carbonic anhydrase activities decreased proportionately with the increasing concentrations of Cd but the content of proline and the activities of catalase, peroxidase and superoxide dismutase increased. These effects were overcome if the stressed plants were sprayed with 28-HBL.

The aluminum toxicity is the major growth-limiting factor for crop cultivation on acidic soil. Seedlings of mung bean (*Vigna radiata* L. Wilczec) were subjected to aluminium (1 or 10 mM) stress at one week old stage and sprayed with 10^{-8} M of 24-EBL or 28-HBL at 14-day stage (Ali *et al.*, 2008b). The authors revealed that the level of antioxidant system (superoxide dismutase, catalase, peroxidase and proline) increased in response to Al stress that was further improved by brassinosteroid treatment (HBL and EBR). Therefore, it may be suggested that the ameliorated level of antioxidant system, at least in part, was responsible for the development of resistance against Al stress in mung bean seedlings. The increase in the degree of resistance due to the applications of BR was reflected in the improvement of plant growth, photosynthesis and related processes, in the presence of aluminium. It was also noticed that EBL was more effective than HBL. The difference between the effectiveness of these BR analogues is due to their structure and stability, because EBL is more stable than HBL under field conditions. Other study verified that BL (0.1, 10, 100, 100,000 $ng.L^{-1}$) promoted growth of mung bean (*Phaseolus aureus* Roxb.) seedlings under aluminium stress (2 and 5 mM) (Abdullahi, 2003). BL significantly increased fresh weights of shoots and roots and chlorophyll content under Al stress.

Lead is a heavy metal that accumulates in plant cell wall and causes growth inhibition. When *Chlorella vulgaris* cultures were inoculated with 10^{-6}–10^{-4} M lead, their growth and chemical composition decreased during the first 48 hours of cultivation. Application of 20-hydroxyecdysone (10^{-10}–10^{-8} M), considered a brassinosteroid-related compound, restored the decreased growth and composition of *Chlorella vulgaris* cells treated with lead (Bajguz and Godlewska-Zylkiewicz, 2004). This compound reduced the impact of lead stress on growth, prevented chlorophyll, sugar and protein loss and increased phytochelatins synthesis. Concentration-dependent stimulation was observed with increasing concentration of 20-hydroxyecdysone (20E) and

decreasing concentration of lead. The supplementation of 24-EBL to radish seedlings (*Raphanus sativus* L.) reduced lead toxicity and enhanced the growth (Anuradha and Rao, 2007b). The activities of antioxidant enzymes (catalase, ascorbate peroxidase, guaiacol peroxidase, superoxide dismutase) showed an increase in brassinosteroid treated Pb-stressed seedlings when compared to control and a reduced peroxidase activity and an increase in the total glutathione content.

3.7 Hypoxia Stress

Kang *et al.* (2009) verified the effects of 24-EBL (1 $\mu g.L^{-1}$) added to nutrient solution on growth of cucumber (*Cucumis sativus* L.) under root-zone hypoxia, seedlings were hydroponically grown for 8 days in normoxic and hypoxic nutrient solutions with and without EBR. EBR added to hypoxic nutrient solution caused an increase in the concentration of fructose, sucrose and total soluble sugars in the roots but not in the leaves. EBR exerted little influence on plant performance in the nutrient solution, while EBR alleviated root-zone hypoxia-induced inhibition of root and shoot growth and net photosynthetic rate (P_N). EBR enhanced alcohol dehydrogenase activity but lowered lactate dehydrogenase activity in hypoxic roots. These results suggest that EBR may stimulate the photosynthate allocation down to roots and the shift from lactate fermentation to alcohol fermentation in hypoxic roots, resulting in the increase in ATP production through glycolysis and the avoidance of cytosolic acidosis and eventually enhanced tolerance of cucumber plants to root-zone hypoxia.

4. BRASSINOSTEROID POTENTIAL USE IN PLANT TISSUE CULTURE

As BR can affect plant elongation, cell division and vascular development influencing morphogenesis, the use of this plant regulator in plant biotechnology is promising as supplementation to medium (Table 11).

Spirostane analogues of brassinosteroids (BB-6 and MH-5) were tested for callus induction and plant regeneration in lettuce (*Lactuca sativa*) (Núñez *et al.*, 2004). The analogues enhanced both callus formation and shoot regeneration from cotyledons in lettuce when added at determined concentrations (0.001 or 0.01 $mg.L^{-1}$) with 0.1 $mg.L^{-1}$ 6-BA (benzyladenine, a cytokinin) in the culture medium. However, there was no callus induction when 6-BA was substituted by these analogues. These results showed that BB-6 and MH-5 stimulated callus formation in the presence of cytokinin.

Table 11. Effects of brassinosteroids on plant tissue culture as a constituent medium

Species	BR and concentrations applied	Physiological effects analyzed	Author
Lactuca sativa	BB-6 (0.001 mg/L or 0.01 mg/L) MH5 (0.001 mg/L or 0.01 mg/L)	Callus induction, shoot regeneration from cotyledons	Núñez *et al.* (2004)
Saccharum spp.	BB-6 (0.001 mg/L) MH5 (0.01 mg/L)	Protein metabolism	Nieves *et al.* (2007)
Nicotiana tabacum	BL (10^{-10}–10^{-6}M)	Cell division, cell-cycle related gene expression, organellar DNA content	Miyazawa *et al.* (2003)
Onosma paniculatum	BL (10, 10^3, 10^5 and 10^7 pg/L)	Growth and secondary metabolism (shikonin formation)	Yang *et al.* (2003)
Pinus taeda *Pseudotsuga menziesii* *Picea abies* *Oryza sativa*	BL (0.1 µM)	Somatic embryogenesis	Pullman *et al.* (2003, 2005, 2009)
Pinus wallichiana	24-EBL (2.0 µM)	Somatic embryogenesis	Malabadi and Nataraja (2007)
Spartina patens	BL (0.005–0.05 mg/L)	Callus growth and regeneration	Lu *et al.* (2003)
Arabidopsis thaliana	BL (1 µM)	Tracheary element differentiation	Oda *et al.* (2005)
Arabidopsis thaliana	28-HBL (1 mg/ml) 28-homocastasterone (1 mg/ml)	Cell expansion, membrane hyperpolarization	Zhang *et al.* (2005)
Gossypium hirsutum	BL (0.1, 0.5 and 1.0 µM)	Fiber development	Sun *et al.* (2005)
Gossypium hirsutum	BL (0.1, 0.5 and 1.0 µM)	Somatic embryogenesis	Aydin *et al.* (2006)
Brassica spp.	24-EBL (10^{-6} M) BL (10^{-7} M)	Microspore embryogenesis	Ferrie *et al.* (2005)
Hybrid (Eucalyptus grandis x *Eucalyptus urophylla)*	28-HCTS (4, 10, 25 and 62.5 mg/L) 5F-HCTS (4, 10, 25 and 62.5 mg/L)	Elongation and formation of new main shoots	Pereira-Netto *et al.* (2006a)
Malus prunifolia	5F-HCTS (0.5, 1.0, 5.0, and 10.0 µg/explants)	Branch elongation	Pereira-Netto *et al.* (2006b)
Nicotiana tabacum	BL (10^{-10}–10^{-8} M)	Shoot formation	Kim *et al.* (2008)
Cocos nucifera	22(S)23(S)-HBL (0.01, 0.1, 1.0, 2.0, 4 µM)	Initial callus formation, somatic embryogenesis	Azpeitia *et al.* (2003)

Taking into account the synergism reported between auxin and brassinosteroids, the presence of BB-6 or MH-5 in the culture medium may have increased the auxin/cytokinin ratio making it necessary to include 6-BA for callus formation. However, the brassinosteroid analogues did not show any effect on callus fresh weight nor shoot number per callus formation evaluated 25 days after culture initiation.

These two analogues, BB-6 and MH-5, were used in concentrations of 0.001–0.01 $mg.L^{-1}$, respectively, to evaluate the protein metabolism in sugarcane (*Saccharum* spp.) somatic embryogenesis (Nieves *et al.*, 2007). It was verified that BRs analogue treatments at high concentration did not

differ from control (without analogues and one with NAA-naphthaleneacetic acid) regarding to somatic embryos production. Both BRs influenced total soluble proteins, storage proteins (albumins, globulins, prolamins and glutelins) and free-proline levels. Some storage proteins such as prolamins and glutelins showed decreases in their content in relation to control treatment. These results imply that the BRs studied were involved in differentiation and maturation of sugarcane somatic embryos caused by a decrease in proline synthesis. The authors suggest that BB-6 and MH-5 influenced proteins metabolism, in particular storage proteins, which are considered as an important nitrogen reserve in somatic and zygotic embryos.

To evaluate the BRs effects in cell division, the tobacco (*Nicotiana tabacum*) Bright Yellow 2 (BY-2) cell line, which is a widely-used model system in plant cell biology was used (Miyazawa *et al.*, 2003). BL (10^{-10}–10^{-6}M) promoted cell division only during the early phase of culture and in the absence of auxin (2,4D). At later stages in the culturing periods of BL-supplied and 2,4D-supplied BY-2 cells, differences in cell multiplication and cell-cycle related gene expression were observed. Moreover, the BL treated BY2 cells had morphological differences from the 2,4D treated cells. To determine whether suppressed organellar DNA replication limited this promotion of cell division during the early culture phase, this replication was examined and it was found that BL treatment had no effect on activating organellar (plastid and mitochondrial) DNA synthesis. These results suggest that the mechanism of the promotion of cell division by BL treatment is distinct from that regulated by the balance of auxin and cytokinin.

BL interact with IAA and 6-benzylaminopurine (BAP) to influence cell growth and secondary metabolism of cultured *Onosma paniculatum* cells (Yang *et al.*, 2003). In a BL and IAA interaction experiment, the optimal BL concentration for cell growth increased with IAA concentration. IAA concentrations of 0.05, 0.1, 1.0 and 10 $mg.L^{-1}$ in the growth medium, the optimal BL concentrations for cell growth were 10, 10^3, 10^5 and 10^7 $pg.L^{-1}$, respectively. In a BL and BAP interaction experiment, cell growth decreased with increasing concentration of BL at any given concentration of BAP. The optimal concentrations of BL and IAA for cell growth were 10 $pg.L^{-1}$ and 0.05 $mg.L^{-1}$, respectively. The optimal concentrations of BL and BAP for cell growth were 10 $pg.L^{-1}$ and 0.5 $mg.L^{-1}$, respectively. The optimal concentrations of BL and IAA for enhanced shikonin (a compound resulted from the secondary plant metabolism with pharmaceutical potential) production were 10^7 $pg.L^{-1}$ and 0.05 $mg.L^{-1}$, respectively and in BL and BAP combination the concentrations were 10^5 $pg.L^{-1}$ (BL) and 0.5 $mg.L^{-1}$ (BAP). BL increased phenylalanine ammonia-lyase (PAL) and p-hidroxybenzoic acid geranyltransferase (PHB-geraniltransferase) activities but decreased the

activity of PHB-O-glucosyltransferase. These results suggest that enhanced shikonin formation induced by BL involves regulation of these key enzymes.

Somatic embryogenesis in rice and conifers can be improved by BL application (Pullman *et al.*, 2003). Using BL supplemented (0.1 μM) medium improved initiation percentages in loblolly (*Pinus taeda* L.) (15.0–30.1%), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (16.1–36.3%), Norway spruce (*Picea abies* L. Karst) (34.6–47.4%) and rice (*Oryza sativa* L.) (10%). BL (0.1 μM) increased the weight of loblolly pine embryogenic tissue by 66% and stimulated initiation in the more recalcitrant families of loblolly pine and Douglas-fir, thus compensating somewhat for genotypic differences in initiation. BL is part of the culture medium for embryogenic tissue initiation of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Pullman *et al.*, 2009) and is a constitutive of the culture medium for somatic embryogenesis of *Pinus taeda* and *Pseudotsuga menziesii* (Pullman *et al.*, 2005). *Pinus wallichiana* is one of the most recalcitrant species to *in vitro* propagation via somatic embryogenesis among all the Indian pines. The use of 24-EBL (2.0 μM) with 9.0 μM 2,4D in three genotypes of *Pinus wallichiana* enhanced the formation of embryogenic tissue from mature zygotic embryos on half-strength MSG basal medium. However the frequency of somatic embryogenesis was not similar in all the three genotypes tested (Malabadi and Nataraja, 2007).

The effect of BL on cultured calluses of *Spartina patens* (Ait.) Muhl., a halophyte monocot, was studied (Lu *et al.*, 2003). BL at 0.005 $m.L^{-1}$ (with benzyladenine at 0.2 $mg.L^{-1}$) and IAA (3 $mg.L^{-1}$) promoted regenerated shoot growth most significantly, increasing the shoot height increasing ratio by 395% after a 40-day culture. The authors suggested that BL at 0.03 $mg.L^{-1}$ is suitable for the callus growth and shoot regeneration, while BL at 0.005 $mg.L^{-1}$ effectively enhanced the regenerated shoot growth.

Brassinosteroids are thought to be related to tracheary element differentiation. In transgenic *Arabidopsis thaliana* cell suspension, BL applied exogenously at 1.0 μM promoted tracheary element differentiation in a dose dependent manner (Oda *et al.*, 2005). Zhang *et al.* (2005) verified that 28-HBL and its direct precursor 28-homocastasterone promoted cell expansion of *Arabidopsis thaliana* suspension cells and this cell expansion induced by HBL and HCS was correlated with the amplitude of the plasma membrane hyperpolarization they elicited. They observed that membrane hyperpolarization and cell expansion were partially inhibited by the proton pump inhibitor erythrosin B, suggesting that proton pumps were not the only ion transport system modulated by the two BRs. The authors also verified that anion currents were inhibited by HBL and HCS while outward rectifying K+ currents were increased by HBL but inhibited by HCS. The different electrophysiological

behavior shown by these BRs indicates that small changes in the BR skeleton might be responsible for changes in bioactivity.

BRs regulate fiber development on cultured cotton (*Gossypium hirsutum* cv. Coker 312) ovules (Sun *et al.*, 2005). The application of BL (0.1 μM) stimulated fiber elongation while brassinazole (Brz2001) inhibited fiber development. Besides, treatment of cotton floral buds with brassinazole results in the complete absence of fiber differentiation, indicating that BR is required for fiber initiation as well as elongation. BL (0.1, 0.5 and 1 μM) was used to examine the potential effect on cotton somatic embryogenesis in cotton (*Gossypium hirsutum*) calli pieces (Aydin *et al.*, 2006). Somatic embryogenesis was stimulated especially for transition to cotiledonary phase at 0.5 $mg.L^{-1}$ BR. Histological preparations from embryogenic calli and somatic embryos at different stages of development revealed the spontaneous polyploidisation during early somatic embryogenesis on BR-treated calli. These results suggest that BR negatively affected calli growth, however, had a stimulating role in maturation of somatic embryos.

An increase in embryogenesis was observed in all *Brassica napus* lines evaluated including Topas 4079 and several recalcitrant cultivars. Garrisson, Westar and Allons treated with 24-EBL (10^{-6} M) and BL (10^{-7} M) (Ferrie *et al.*, 2005). The microspore embryogenesis, calculated as the number of embryos at 21 days of culture, was increased in the recalcitrant cultivars up to 12 times that of control. An increase in microspore embryogenesis was also observed for *Brassica juncea* when EBL or BL was added to the culture medium. In contrast, no significant increase in embryogenesis was observed for several other *Brassica* species evaluated (*Brassica nigra*, *Brassica carinata* and *Brassica rapa*). The addition of brassinosteroids to the induction media did not affect the subsequent conversion of the embryos to plantlets, but did appear to influence chromosome doubling.

28-Homocastasterone (28-HCTS) was used to treat *in vitro*-grown shoots of a hybrid between *Eucalyptus grandis* and *Eucalyptus urophylla* (Pereira-Netto *et al.*, 2006a). Treated shoots showed enhanced elongation and formation of new main shoots (the shoots originating directly from the initial explant) at low doses. Coincidently there was reduced elongation and formation of primary lateral shoots (shoots originating from the main shoot). However, a 5α-monofluoro derivative of 28-HCTS (5F-HCTS) was unable to either stimulate elongation or formation of new main shoots, although it did stimulate elongation of primary lateral shoots. The differential responses seen for these compounds on shoots of *Eucalyptus* suggest different BR biosynthetic routes, differential chemical stability or perhaps different receptor sites for each compound. Although auxin and cytokinin were used in the culture medium, it was quite apparent that exogenously supplied brassinosteroids are able to change shoot patterns (apical dominance) in *Eucalyptus* and it

seems likely that shooting in *Eucalyptus* might be influenced by the endogenous pool of bioactive brassinosteroids. Pereira-Netto *et al.* (2006b) demonstrated that 5F-HCTS (0.5, 5.0, 1.0 and 10.0 μg/explants) stimulated branch elongation in *in vitro*-grown shoots of *Malus prunifolia* (Wild.) Borkh, the marubakaido apple rootstock. The authors showed that this BR-stimulated branch elongation is paralleled by an increase in ethylene release.

When several concentrations of BL were added to a shoot induction medium that contained only benzyladenine, redifferentiation of adventious shoots from tobacco (*Nicotiana tabacum* L. cv. NT1) leaf discs was unaffected at low BL levels (10^{-10}–10^{-8} M), but was inhibited at higher concentrations (Kim *et al.*, 2008). When BL was applied without BA, only cell expansion occurred and no shoots formed. The determination time for shoot formation was shortened at low BL concentrations, but their formation was postponed at higher concentrations. In conclusion, at low concentrations, BL has no effect on shoot formation. However, it inhibits their formation at high concentrations when cytokinin is included in the media.

Explants from coconut (*Cocos nucifera* L.) were exposed to different concentrations of 22(S),23(S)-HBL (0.01, 0.1, 1.0, 2.0, 4 μM) and these explants responded favorably to the brassinosteroid, increasing their capacity to form initial callus, embryogenic callus and somatic embryos (Azpeitia *et al.*, 2003). The largest amount of somatic embryos formed was obtained exposing the explants for 3 days to the concentrations of 0.01 or 0.1 μM HBL.

5. CONCLUSION

Developing stage of the plant, the concentration and the time the brassinosteroid is applied as well as the types of brassinosteroid that are used are very important parameters to study the effects of exogenous BRs in plants. More studies should still be carried out in a great number of plants and other compounds with brassinosteroid-like responses should be tested. The great majority of works use brasinolides and EBLs but there are brassinosteroid analogues that are not commonly used and still have to be exploited. The beneficial effects of brassinosteroid application are incontestable mainly when plant stress conditions are evaluated. They enhance antioxidant enzymes activities and plant yield by improving the photosynthesis process. It seems that BRs effects are more detectable on seedlings and suspended cells than in older plants. The concentrations used vary a lot and the effects that were observed vary from plant to plant and from organ to organ.

Anyway, it is quite evident that BRs influence plant growth and development whether they are used alone or with other plant hormones such as auxins, gibberellins, ethylene or cytokinins or by interactions with other

substances (phytochromes, salycilic acid, jasmonates and others). There really is a great expectation that BRs can elucidate other plant metabolic processes that have not been solved so far.

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