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Biological Effects of Brassinosteroids

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CONTENTS

I. INTRODUCTION

Plant growth regulators for yield enhancement, quality improvement, and the facilitation of harvesting, together with genetic engineering, represent supplemental technologies in agriculture for increasing crop production and optimizing the use of limited resources. Although plant growth regulators have been used in agriculture for as long as crop protection chemicals, their impact up to now has been relatively small, and their application is limited to some specific cultures.

The discovery of a new naturally occurring plant growth regulator substance, a novel polyhydroxylated steroidal lactone with high growth-promoting activity, termed brassinolide (**1**) (BL), from rape pollen (*Brassica napus L.*) was reported in 1979.[1] Since then, successively new analogs of BL (**1**) were isolated and identified from many kinds of plants[2–4] which indicate the ubiquitous occurrence in the plant kingdom, and constitute a new family of plant growth regulators called brassinosteroids (BRs).

The effect of BRs on plant growth suggests that they play an important hormonal role in plant development. Why BRs can be considered as plant hormones has been pointed out in detail by Sasse,[5] based on a detailed analysis of their physiological action, and there is evidence that they act independently of the other endogenous plant hormones (auxins, gibberellins, cytokinins, abcisic acid, and ethylene).[6–13]

BRs have been evaluated for use in improving crop yield, quality, stress tolerance, and disease resistance and promising results have been reported with several plants.[14] Application of BRs to crops helps to overcome environmental stress such as cold, salt tolerance, and herbicidal injury. The results obtained on large-scale field trials over 6 years by applying 24-epibrassinolide (**2**) (a more economically convenient alternative to BL (**1**)) have successfully demonstrated the suitability of this steroid for the production of crops.[15] Taking into account these characteristics, the use of BRs in agriculture, together with the environmentally safe aspects of these compounds, would mark a major development in the field of plant growth regulators, and would minimize the risks associated with agrochemicals by reducing environmental contamination.

There are only trace amounts of BRs in plants and since their isolation from natural sources is prohibitively expensive and time consuming, synthesis of BRs has been a field of tremendous activity[2,14,16] in order to have a sufficient amount so as to study their effects on growth as well as to evaluate them in different crops for further application in agriculture and horticulture.

This chapter summarizes the present state of the art on the knowledge of BRs in biosynthesis and metabolism, bioactivity, molecular and physiological effects, as well as their application in agriculture. Furthermore, a preliminary quantitative structure-activity relationship as well as a new way to define the structural requirements for BRs to be active is presented.

II. BIOSYNTHESIS AND METABOLISM OF BRASSINOSTEROIDS

Several studies have been done in order to elucidate the metabolism and biosynthetic pathway for BRs. The fact of having detected BRs in a plant with the same 24-alkyl substituent as a phytosterol which is also present in the same plant suggests that phytosterols may be considered as appropriate biogenetic precursors.[17] They could be converted, after several oxidation steps, to the corresponding BRs.

Thus, among others, eight 25-methyldolichosterone derivatives were isolated from immature seeds of *Phaseolus vulgaris*,[3,18,19] 25-methyldolichosterone being one of the major ones.[20] Also, 24-methylene-25-methylcholesterol, which is structurally correlated with 25-methyldolicholide, was identified in the seeds.[21] However, while 88% of the total sterols in *Phaseolus* seeds are those having a 24-ethyl group (sitosterol and stigmasterol) and a 24-ethylidene group (isofucosterol), the corresponding BRs were found in the same seed in quite low contents. This suggests that, in these seeds, the biological transformation leading to BRs is more selective for 24-methyl- or 24-methylenesterols than 24-ethyl- and 24-ethylidenesterols. Another example is found with the green alga *Hydrodyction reticulatum*, where 24-epicastasterone (**3**) and 28-homocastasterone (**4**) have been identified corresponding well with the major sterols present in it, brassicasterol and sitosterol.[22] These findings are in accordance with the hypothesis cited above, although at the moment there is no evidence on the biological conversion of phytosterols into BRs.

A hypothetical biosynthetic pathway for BL (**1**) has been proposed by Yokota et al.[23] (Figure 1) starting from campesterol (**5**) or its analogs. Several oxidation steps of campesterol (**5**) to produce teasterone (TE) (**6**) followed by an isomerization at C_3 to give typhasterol (TY) (**7**), subsequent hydroxylation at C_2 and Baeyer-Villiger-type oxidation could produce BL (**1**) via castasterone (CS) (**8**) intermediate. Because there is some evidence of the possible involvement of 3-dehydroteasterone (**9**) in the conversion of TE (**6**) to TY (**7**), it is included in the proposed biosynthetic pathway for BL (**1**) shown in Figure 1. This pathway has been partially demonstrated in crown gall and nontransformed cells of *Catharanthus roseus*, in which CS (**8**) and BL (**1**) are naturally occurring,[24] using tritiated and/or deuterated TE (**6**),[25] TY (**7**),[25,26] and CS (**8**)[27] as substrates, and the biosynthetic sequence TE (**6**) → TY (**7**) → CS (**8**) → BL (**1**) has been established unequivocally in these cells. Also, a reversible conversion between TE (**6**) and TY (**7**) was observed.[25] However, in mung bean (*Vigna radiata*) cuttings[23] and rice seedlings and etiolated leaf explants,[23,28] in which CS (**8**) is endogenous,[29] tritiated CS (**8**) was never converted to BL (**1**). This corroborated the hypothesis that CS (**8**) itself is biologically active.[28] Feeding experiments with deuterated 3-dehydroteasterone (**9**) to cultured cells of *Catharanthus roseus* provides evidence for the possible involvement of this intermediate in the conversion of TE (**6**) to TY (**7**).[30] The co-occurrence of TE (**6**), TY (**7**), as well as 3-dehydroteasterone (**9**) in wheat (*Triticum aestivum*) grains[31] and in lily cell cultures of *Distylium racemosus*[32] support this hypothesis.

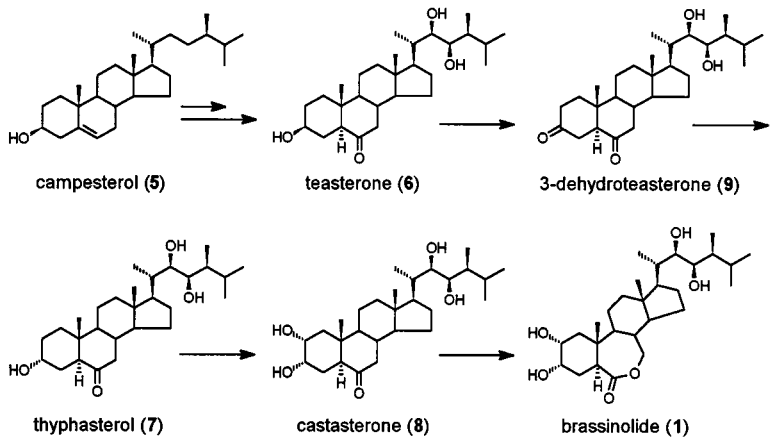


Figure 1 Biosynthetic pathway for brassinolide (**1**).

Few studies have been done to find out how BRs are catabolized in plants, and the results up to now are unclear. Yokota et al.,[33] examining the evolution of CS (**8**) and BL (**1**) in explants of mung bean seedlings, found that while CS (**8**) was converted to unknown water-soluble metabolites in which major

components seemed to be nonglycosidic and minor ones to be glycosidic, BL (**1**) was largely converted to 23-*O*-D-glucopyranoside. The biological activity elicited by this conjugate was nearly comparable to that of BL (**1**), so the authors suggested that this activity may be due to the aglycone (BL (**1**)) liberated in the assay plant tissue or in the assay medium. However, two inactive metabolic products were obtained when ^{3}H-labeled (22*S*,23*S*)-24-epibrassinolide was applied exogenously to the hydroponically grown tomato (*Lycopersicon esculentum*) plants, which remain unidentified.[34] Other experiments have been done in cell suspension cultures of *Lycopersicon esculentum* showing that these media were also able to convert 24-epibrassinolide (**2**) to the corresponding 25-β-D-glucosyloxy and 26β-D-glucosiloxy derivatives.[35] The authors suggest that two cytochromo P-450-dependent mono-oxygenases are involved in the hydroxylation process. Similarly, two 23-O-glucosides, 23-*O*-β-D-glucopyranosyl-25-methyldolichosterone and its epimer at C_2, were isolated from the seeds of *Phaseolus vulgaris*.[3] In contrast, in the case of rice seedling, BL (**1**) and CS (**8**) were converted to nonglycosidic metabolites.[28]

Recently, a new class of BR conjugates, monoglycerides, has been identified in immature seeds of *Cassia tora*: monopalmitin and monoolein of BL (**1**), CS (**8**), TY (**7**), TE (**6**), and 28-norcastasterone,[36] and in *Lilium longiflorum* anthers: teasterone 3-myristate.[37] In spite of all these findings, the following questions remain unanswered: a) why BRs evolve into different compounds, conjugates or not, depending on the seedlings, and b) if these conjugates are really catabolites or are they involved in transport or storage mechanisms? The first catabolic side-chain degradation has been observed in cell suspension cultures of *Ornithopus sativus* where 24-epicastasterone (**3**) and 24-epibrassinolide (**2**) were transformed to 20-keto-pregnan derivatives epimerized at C_3.[38]

III. BIOASSAYS FOR BRASSINOSTEROIDS

Bioassay methods play an essential role in studies of plant growth substances. The response depends both on the structures and the concentration of phytohormones applied. Some of the bioassay developed for auxins, gibberellins, and cytokinins were used to detect BRs in plant tissues, to evaluate the activity of natural and synthetic BRs, and to study their interaction with other hormones and related growth substances. Also, new specific bioassays have been developed. As a consequence, it was found that BRs possess a broad spectrum of biological activities compared to the known plant hormones, including gibberellin-, auxin-, and cytokinin-like activity. This effect qualifies BRs as a sixth group of phytohormones.

Subsequently, a more detailed explanation of the response of BRs in different bioassays, as well as its interactions with other plant growth regulators, is presented to better understand the BRs mode of action, which has been demonstrated to be different from other plant hormones.

Thus, in the bean first internode test,[39] developed for auxins and based on the curvature induced on isolated first internode section from partially etiolated bean plants (*Phaseolus vulgaris*), BL (**1**) showed high activity at a level of 10 ng.[40] Gibberellins caused no bending of the internode but stimulated elongation of the stem section. Furthermore, whereas BL (**1**) stimulated auxin-induced growth in this test, the gibberellin response was inhibited.[41] The sensitivity of this bioassay was improved 100- to 1000-fold by applying the BRs at the apical zone of the section instead of the base.[42]

In the bean second internode bioassay, the BR response is elongation, curvature, swelling, and splitting of the treated bean internodes at hormonal levels of 0.01 to 50 μg. Although this test is sensitive to auxins, gibberellins, and zeatins, only BRs are able to produce splitting of the internode treated.[40] Also this test was improved by omitting nitrate and adding a defined concentration of Ca^{2+} or Mn^{2+} to the solution in which the bean seedlings were grown.[43]

In various bioassay systems, BL (**1**) activity was compared to auxins.[44] Both presented a similar response in bioassays based upon bean hypocotyl hook opening, elongation of maize mesocotyl, pea epicotyl, and azuki bean epicotyl sections (in the last two BL (**1**) was 10 times more active than 3-indoleacetic acid (IAA) at 10 *M*), and fresh weight increase in Jerusalem artichoke and pea epicotyl sections. A powerful synergism between BL (**1**) and IAA was observed in the azuki bean, pea epicotyl, and bean hypocotyl hook bioassays.[44]

Similarly, BL (**1**) was tested in selected gibberellin and cytokinin bioassays.[10] While in the former it was highly active at 0.01 to 10 *M*, in the latter it was less or not effective, which indicated the different mode of action. Unlike IAA, BL (**1**) does not interact synergistically with gibberellic acid (GA_3).

Also, the growth rate of mung bean epicotyls was proved to be adequate to determine the activity of BRs and to study the interaction between BRs and GA_3.[9] A highly significant leaf petiole and epicotyl epinastic curvature was recorded from a range of 10^{-9} to 10^{-7} *M* of BL (**1**) but, at a higher concentration

the activity decreased. An additive but not synergistic effect was detected with GA_3 when they were applied together. Moreover, the independent role of the two promoters in tissue elongation was demonstrated.

A dramatic stimulation of wheat leaf-unrolling was detected with BRs even at 0.5 ng/ml, a result completely opposite to the negative activity found for auxins. In this system, GA_3 and cytokinins produced only slight unrolling at a higher concentration.[7]

The activities of these steroids were also evaluated with intact plants.[45] Lengthening of the hypocotyls, of the cotyledon petioles, and of the whole plant was observed in radish (*Rhapanus sativus*) and tomato (*Lycopersicon esculentum*) shoots at 0.01 ppm. Curvature of the cotyledon petiole and the hypocotyl was also observed even at 0.001 ppm. Auxins and gibberellins are also active in these tests, but only at 10 ppm.

The rice (*Oryza sativa*) lamina inclination test is one of the most widely used to evaluate the activity of BRs because of its high sensitivity to stimulate the lamina inclination of the excised leaf lamina.[46] A similar bending in intact dwarf rice seedlings was also observed.[47] In this test, IAA shows effect but at a higher concentration[46,47] and other phytohormones, like abcisic acid, GA_3, and cytokinins, inhibit the lamina bending.[11] In both rice biotests, the size of the angle between the leaf blades and the stem of rice is used for quantitative evaluation of the biological activity of BRs.

The pea inhibition test[43] is the only bioassay reported on growth inhibition provoked by BRs. It is based on the inhibition of the elongation of etiolated pea stems, probably due to the ethylene production mediated by BRs, and allows for the detection of even 10 fmol of 24-epibrassinolide (**2**). The effect is similar to that produced by IAA.

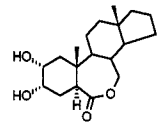
Table 1 summarizes the relative activities of some BRs in various bioassays. It can be seen that although BL (**1**) is the most active in all of them, there are variations in the BR activities in certain cases, depending upon the bioassay systems employed. Moreover, it has been observed that certain tests show different responses depending on the cultivar used.[11,43] Nevertheless, a variation in the response is observed if one compares the activity data described from a defined test using the same cultivars and even by the same authors. For instance, in the rice lamina inclination test, the angle between lamina and sheath elicited by BL (**1**) is described in different papers[11,46,48,49] in a range of more than 30º but the angle is obtained as an average of only ten seedlings and probably in only one experiment. If the large dispersion is due to intrinsic factors not controlled in the experiment, a large number of data might be analyzed to get reproducible results. All these kinds of variations in the activity data (bioassay, cultivar and variability) make it extremely difficult to establish a good structure-activity relationship with the data obtained from literature.

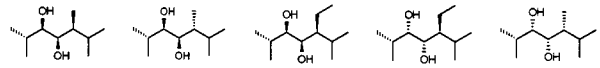
In our aims to gain more knowledge of the structural requirements in order for a BR (brassinosteroid) to be active and to establish quantitative structure-activity relationships, we examined a large number of BR derivatives with different functionalities on the skeleton and side chain. To do this, homogeneous activity data with statistical parameters were necessary, so we decided to test them using intact rice seedlings in a similar manner as described by Takeno and Pharis,[47] who used the cultivar Tan-ginbozu. Because these seeds are not commercially available, four types of rice cultivars (Lemon, Thainato, Bahia, and Senia) were examined with 24-epibrassinolide (**2**) in order to choose the most adequate ones. The rice cultivars were selected by the following criteria: a) high sensitivity in response, b) greater population (number of plants in which the node could be easily distinguished), c) rate of germination, and d) reproducibility. The cultivar Lemon was eliminated because the node where 24-epibrassinolide (**2**) should have been applied was not detected. The cultivar Senia was also discarded after the preliminary experiments (Table 2) due to its low germination rate and the size of its radicle being smaller than the other ones. From the results of this examination the cultivar Bahia was finally selected, due to its higher sensitivity, indicating the usefulness of this cultivar.

Using Bahia rice seedlings, the activity of all the BRs shown in Figure 2 were evaluated by applying 1 μg/plant. All these compounds have been synthesized following different strategies.[50–57] A number of experiments were done for each compound to get statistically significant data. The response of a compound in each experiment is evaluated as the difference between the average of the induced angle produced by the sample and that found for the control. Table 3 and Figure 3 summarize the results obtained.

At doses of 1 μg/plant, the bending of the rice lamina is quite similar for BL (**1**), **2, 4**, **10**, and **11**. Except for **10**, which is surprisingly active in this cultivar, BRs with 22*R*,23*R*-hydroxyls are much more active than those with 22*S*,23*S*-hydroxyls, as has been described in other bioassays.[58] These differences in the response are greater than those observed between lactone- and ketone-type BRs, which indicate that the activity is much more affected by the configuration of the hydroxyl groups in the side chain than the change of lactone to ketone in the B ring. This is in accordance with the results obtained in

Table 1 Relative Activity of Brassinosteroids in Different Bioassays





	1	2	11	12	10
Bean 1st internode[39]	100	36	33	26	34
Bean 2nd internode[39]	100	10	0.1	< 0.1	10
Radish[44]	100	10	10	3	100
Tomato[44]	100	10	1	1	3
Rice (Arborio)[59]	100	10	100	50	5-10
Rice (Bahia)*	100	5	5	< 0.01	1

* Described in this chapter.

Table 2 Biological Activity of 24-Epibrassinolide (**2**) in Different Rice Cultivars

	Senia	Bahia	Thainato
Angle	53.8	74.6	13.3
Repet	9.8	5.1	3.4
Reprod	22	11.8	4.5

Note: Angle, mean of the difference between the average of the induced angle by the BR in each experiment and the control; Repet (repeatability), mean of the normalized standard deviation from all the experiments; Reprod (reproducibility), standard deviation of the averages of the induced angle from all the experiments.

our molecular modeling studies, which will be discussed later. Even more, in some cases, ketone-type BRs have been found to be a little more active than the corresponding lactone-type BRs, opposite to reported data.[58] The significant activity elicited by **16** and **20**, new synthetic brassinosteroid analogs with A/B *cis* junction and 2β,3β diol, is not in accordance with the structural requirements postulated for a BR to be active, indicating the weakness of these requirements, which will be discussed later. Also, it should be taken into account the high bending showed by **23**, another new synthetic BR with a hydroxyl group at C-5.

A dependence of the response on the amount of BRs applied to the seedlings is observed (Figure 4). While BL (**1**) produce high bending of the rice lamina at a level of 10 ng/plant, the same effect is produced by 24-epibrassinolide (**2**), 28-homobrassinolide (**11**), and 28-homocastasterone (**4**), but at a level of 100 ng/plant. A significant bending is also observed for BL (**1**) at a dose of 1 ng/plant. It should be noted that BL (**1**) and (22*S*,23*S*)-24-epibrassinolide (**10**) showed similar bending, using Bahia cultivar as those previously obtained with the cultivar Tan-ginbozu.[47,59]

With respect to auxins, it was observed in our bioassay that BL (**1**) was 100 times more active than IAA and 1000 times more active than α-naphthaleneacetic acid (NAA) (Figure 5). This is in accordance

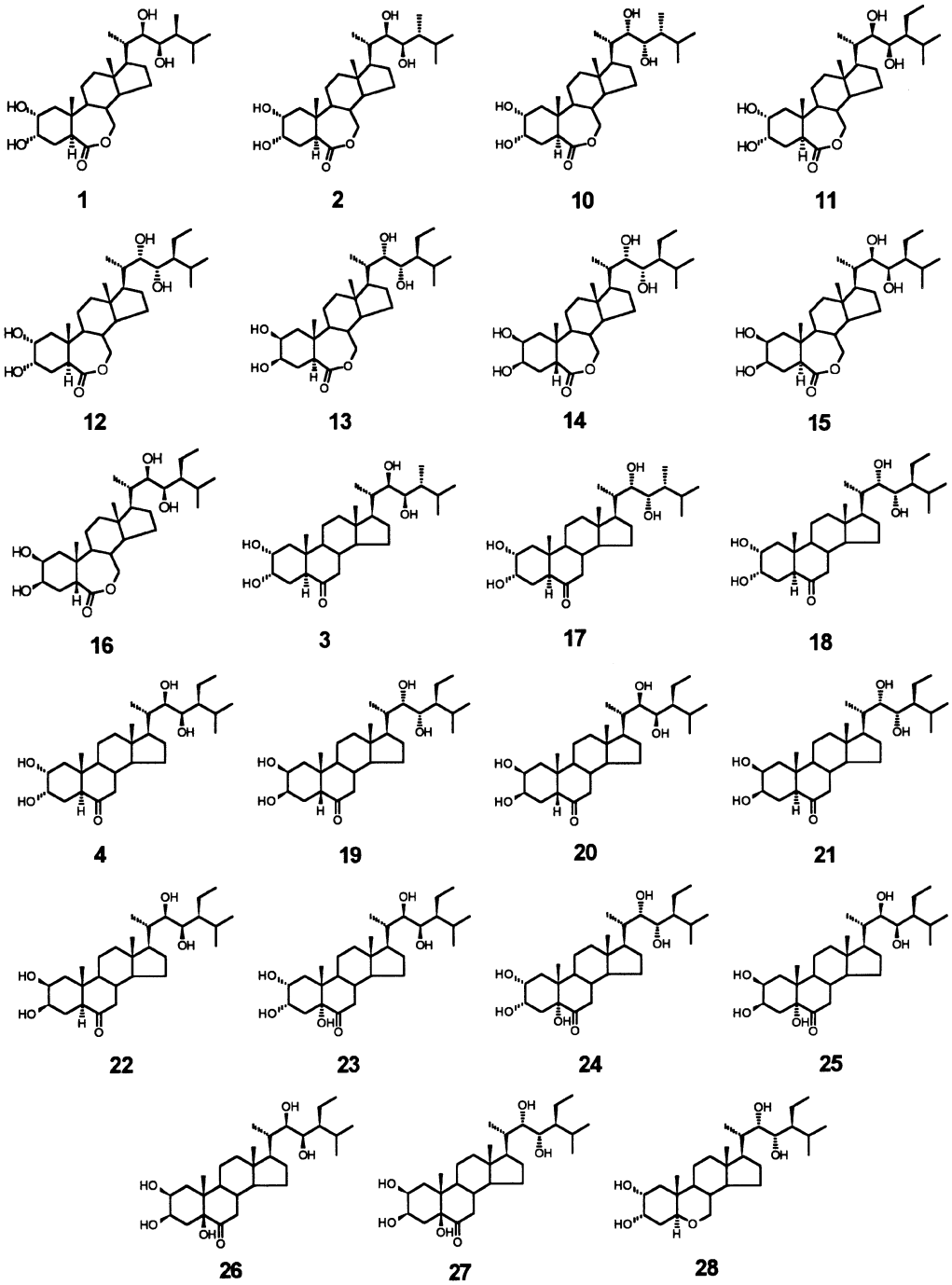


Figure 2 Brassinosteroids evaluated in rice lamina inclination test and used in structure-activity correlation.

with the results described in rice excised leaf lamina[46] and in intact dwarf rice seedlings[47] even with a more significant bending. Also a dose dependence has been detected (Figure 6). Moreover, the minimal effect of **12** and **18** at 1 μg/plant and IAA at 0.1 μg/plant can be enhanced significantly by the co-application of both phytohormones (Figure 7). These findings are encouraging for further application of BRs in agriculture because it would allow for the enhancement of crop yields by the co-application of IAA and BRs with low activity, which are easier to synthesize at lower economic cost.

Table 3 Biological Activity of Brassinosteroids in the Rice Lamina Inclination Test at 1 μg/Plant Using the Cultivar Bahia

	16	**1**	**4**	**10**	**11**	**2**	**3**	**23**	**20**	**22**	**18**	**17**
Angle	83.5	77.2	74.8	74.7	66.7	66.6	55.0	49.3	37.4	17.2	9.4	9.3
Repet	5.1	4.1	4.6	4.2	6.3	4.4	5.3	5.3	6.8	3.9	2.4	2.4
Reprod	6.4	6.9	12.9	17.7	10.0	4.2	11.7	6.4	1.9	4.6	3.1	0.0

	12	**24**	**21**	**25**	**13**	**19**	**28**	**14**	**15**	**27**	**26**
Angle	6.6	4.0	4.0	2.1	0.9	0.9	0.3	0.3	–0.5	–4.5	–4.8
Repet	1.7	2.4	1.4	1.7	1.5	1.3	1.2	1.2	1.5	2.3	1.8
Reprod	3.5	5.3	1.9	4.9	2.1	3.9	2.3	4.1	3.8	4.0	2.5

Note: Angle, mean of the difference between the average of the induced angle by the BRs in each experiments and the control; Repet (repeatability), mean of the normalized standard deviations from all the experiments done for each compound; Reprod (reproducibility), standard deviation of the averages of the induced angle from all the experiments.

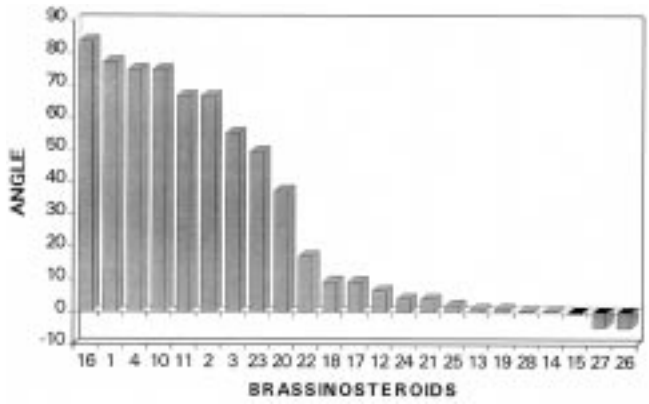


Figure 3 Graphic representation of the activity of brassinosteroids in rice lamina inclination test.

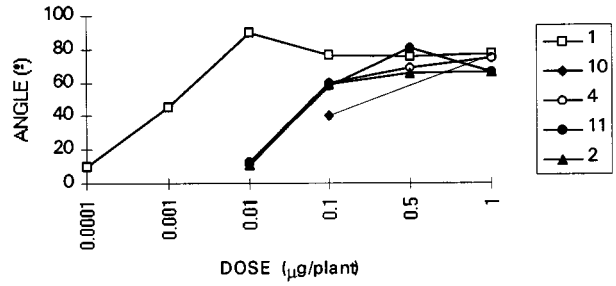


Figure 4 Dose dependence of brassinosteroids.

IV. STRUCTURE-ACTIVITY RELATIONSHIP AND MOLECULAR MODELING OF BRASSINOSTEROIDS

Different qualitative structure-activity relationships have been established taking into account the activity data obtained in special bioassay systems.[40,45,60–62] In general, the structural requirements postulated for a high BR activity are as follows: 2α,3α-diol, 6-ketone or better 7-oxalactone in B ring, A/B *trans* fused ring junction, a *cis* C-22,C-23-diol preferentially with RR configurations, and a C-24 methyl or ethyl substituent. These relationships are more or less stringent depending upon the bioassay. Thus, Takatsuto et al.[45] postulate a 22*R*,23*R*-diol function in radish and tomato tests that were much stricter than those

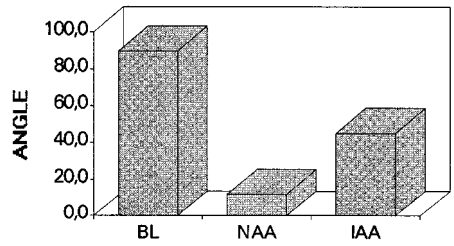


Figure 5 Activity of IAA and NAA at 1 μg/plant compared to BL (**1**) at 0.01 μg/plant.

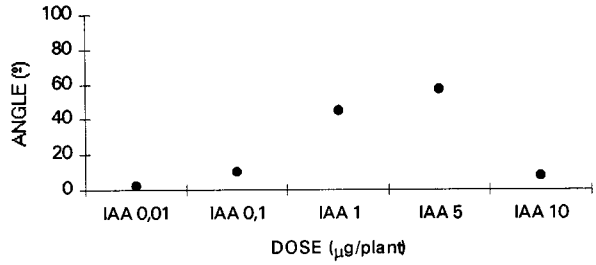


Figure 6 Response of IAA at different doses (μg/plant).

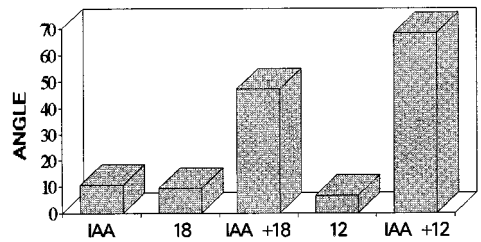


Figure 7 Synergistic effect of (22*S*,23*S*)-28-homocastasterone (**18**) and (22*S*,23*S*)-28-homobrassinolide (**12**) with IAA. Dose: IAA, 0.1 μg/plant; **18** and **12**, 1 μg/plant. Co-application: 0.1 μg/plant of IAA + 1 μg/plant of **18** or **12.**

they had postulated in a rice lamina inclination test where both configurations RR or SS were possible.[60] Likewise, Yokota and Mori report, in an extensive review,[58] that the structural requirements at C-24 are much less rigorous in the rice lamina inclination bioassay. However, the authors include substituents like methyl, ethyl, methylene, or ethylidene at C-24 as well as an additional methyl group at C-25 as active compounds, but they expand a range of 5 to 300 on the activity value. This fact indicates the weakness of this requirement.

Considering that the variations in the requirements postulated depend upon the bioassay employed, a new way to establish the structural requirements should be developed. From our point of view, the active BRs should have a defined three-dimensional structural feature which fits the receptor/s. In fact, the three-dimensional structure between two BRs could be more similar by changing two or more functionalities than by changing only one. Moreover, two of the structural requirements pointed out (2α,3α-diol and A/B *trans* ring junction) are unconvincing because of a lack of data. No BRs with A/B *cis* junction nor with 2β,3β-diol and A/B *trans* or *cis* junction were examined.

An approach to molecular modeling study has been recently reported.[63] However, a more complete study should be performed in order to learn about the BR conformation which interacts with the receptor. This will help us to establish a quantitative structure-activity relationship (QSAR) to predict the activity of new analogs and to design the BR with the best synthetic cost-activity ratio for further application in agriculture. It will also help us to gain more knowledge about the binding site of BRs receptors.

Regarding this, we undertook molecular modeling studies which allowed us to establish a preliminary QSAR correlation following the typical scheme shown in Figure 8. Thus, a broad number of BR analogs were selected for which strictly homogeneous activity data were required. The compounds chosen and their activity data are summarized in Figures 2 and 3, respectively. The relative activity data correspond to those obtained following the procedure cited above by applying 1 μg/plant.

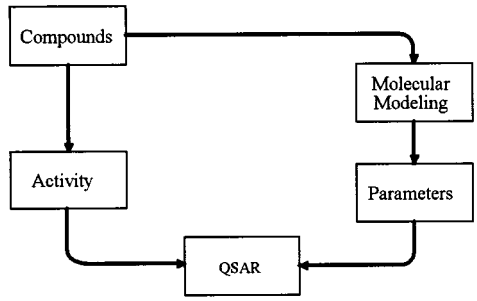


Figure 8 Quantitative structure-activity relationship (QSAR) scheme.

Concerning these compounds, a systematic molecular modeling study has been developed to find the conformation of each BR, named "active conformation", which might interact with the receptor binding site.[56] On it, the atoms involved in the BR-receptor interaction ought to have the same spatial situation in all molecules.

A conformational analysis has been done to find the possible conformation(s) within a range of 3 kcal/mol taking into account two structural points with higher conformational movement in BR molecules: the seven-member B ring and the side chain. A different number of possible conformers has been found: 7 conformers for the side chain of 22*R*,23*R*-diol-24*S*-methyl, 6 for 22*R*,23*R*-diol-24*R*-methyl, 9 for 22*S*,23*S*-diol-24*R*-methyl, 12 for 22*R*,23*R*-diol-24*S*-ethyl, and a big range of possible orientations for the side chain 22*S*,23*S*-diol-24*S*-ethyl. In each side chain, although the energy conformation encountered for each conformer is similar, the relative position of each atom differs, in some cases significantly.[56]

Also a Free-Wilson analysis[64] was done indicating that the RR configuration for the side chain hydroxyls groups and αα for A ring diol participate with 35% and 25%, respectively, to the total activity. This corresponds to the two binding subsites in the receptor postulated by Takatsuto et al.[45] but, on the contrary, they considered the affinity of the A ring diol stronger than that of the side chain. Although they did not mention any interaction of the B ring with the receptor, it should influence in some way. In fact, the activity diminishes when there is a 6-ketone, or practically disappears when there is a 6-oxalactone [65] or no functionality[18] instead of 7-oxalactone present in BL (**1**). One can consider that the influence of this B ring could be due to two factors: a) an additional binding interaction of the B ring or b) the need for an optimal distance between the two moieties of the substrate interacting with the binding subsites cited above which should change according to the B ring functionalities. Thus, the B ring has been considered for a complete description of the activity although its contribution has been found to be less important in the Free-Wilson analysis (11%).

By considering both analyses, the *active conformation* for each compound was found by comparing the spatial position of the oxygen atoms at C-2, C-3, C-22, C-23, and C-6 in all possible conformers encountered. One could consider these atoms as the ones which are probably involved in the receptor/ligand interaction. The conformer of BL (**1**) which presented more similarity to the other ones was taken as its *active conformation* as well as the reference for the other compounds.

Several parameters for each compound *active conformation* have been calculated and correlated with the activity.[56] It has been found in this preliminary QSAR that the activity correlates with the oxygen atoms at C-2, C-3, C-6, C-22, and C-23 because they are involved in the parameters X_1, X_2, and X_3 (Figure 9). This is in accordance with the model we previously proposed.

We conclude that the activity of BRs depends on the spatial position of the oxygen atoms. This could be the reason for the relatively high activity of **20** (with A/B *cis* junction and 2β,3β diol) where its spatial distribution is similar to those of **4** (with A/B *trans* junction and 2α,3α diol) which presents high activity (Figure 10). This is an example of how two modifications can be better than only one. Therefore, the structural requirements should be indicated as distances or angles between them.

$$-\log A = 0{,}135\ X_1 - 0{,}296\ X_2 + 0{,}13\ X_3 + 0{,}1775\ X_4 - 0{,}085\ X_5 - 1{,}295$$

Statistics parameters: $r = 0{,}922$; $s = 0{,}212$; $F = 15{,}96$ ($F_{95\%} = 3$) ; $r(cv)^2 = 0{,}652$

■

Where:

$$X_i = \sum_{j=1}^{n} Y_j\ Y_j$$

X_1 : Y_j = absolute atomic charges
n = atoms pairs between 7 and 8 Å
X_2 : Y_j = negative atomic charges
n = atoms pairs between 6 and 7 Å
X_3 : Y_j = negative atomic charges
n = atoms pairs between 8 and 9 Å.

X_4 : Y_j = Van der Waals Radios
n = atoms pairs between 12 and 13 Å.
X_5 : Y_j = Van der Waals Radios
n = atoms pairs between 14 and 15 Å.

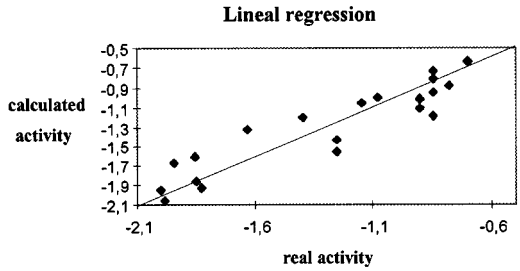


Figure 9 Structure-activity correlation.

dist.	O-2	O-3	O_{carb}	O-22	O-23
O-2	-	2.7	6.3	11.3	12.8
O-3	2.8	-	4.9	11.0	12.9
O_{carb}	6.6	4.9	-	10.0	11.7
O-22	10.2	11.2	9.9	-	2.6
O-23	11.8	13.2	11.8	2.7	-

20

4

Figure 10 Distances between oxygen atoms at C-2, C-3, C-6, C-22, and C-23 for **20** (white area) and for 28-homocastasterone (**4**) (shadow area).

Taking into account these results, we propose a new way to define the structural requirements shown in Figure 11. Thus, a compound will be more active the closer its values in the white triangle are to the ones for BL (**1**) in the shadowed area. In Figure 11, two BRs are shown as examples: one presenting high activity, 24-epibrassinolide (**2**), and another one with low response, (22*S*,23*S*)-28-homocastasterone (**18**). While the first has values quite similar to those of BL (**1**), the second differs significantly. Furthermore, Figure 10 shows the spatial similarity between **20** and **4** in agreement with its similar and high activity. Moreover, there is a relationship between the number of possible conformations and the activity. Thus, compounds with 22*S*,23*S*-diol-24-ethyl side chain, which possess the lowest activities, have a much larger number of possible conformers. In these compounds the *active conformation* could be diluted into the other ones. So, we found two independent factors to explain the activity: an entropic one that is related to the number of conformers, and an enthalpic one which is related to the spatial location of the oxygen atoms.

In another context, a first BL-inhibitor named KM-01 has been isolated from the fungus *Drechslera avenae* and its structure elucidated, corresponding to a fatty acid ester of bipolaroxin. It inhibited the hypocotyl elongation of radish and bending angle of rice seedlings.[66] Its inclusion in QSAR calculations may help to gain more knowledge about the receptor structure.

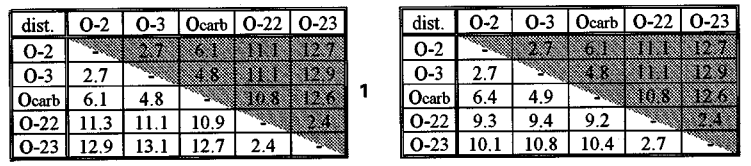


Figure 11 Distances between oxygen atoms at C-2, C-3, C-6, C-22, and C-23 for **2** and **18** (white area) and for brassinolide (**1**) (shadow area).

V. EFFECTS OF BRASSINOSTEROIDS AT THE MOLECULAR LEVEL

In the early years of the discovery of BRs a great deal of effort was dedicated to the synthesis of such compounds and the evaluation of their physiological action, but few studies were done in order to elucidate the mechanism of BR action at the molecular level. The reason was the lack of radiolabeled BRs and a model system with properties suitable for the development of these studies.

In spite of the response of BRs at the physiological level being similar to the other plant growth regulators, as has been discussed above, the questions to answer were a) if they were really plant hormones, b) if the mode of action differed from the others (some evidence has been pointed out before), and, if so, c) what would the mechanism at the molecular level be? Recently, promising results have been obtained which allow us to better understand how BRs regulate growth and corroborate that the mode of action of BRs differs from that of the other plant growth regulators.

It has been speculated from structural considerations that BRs may act through a mechanism similar to that of animal steroid hormones, via a receptor/ligand complex which binds to nuclear or cytoplasmatic sites to regulate the expression of specific genes. Tissue growth depends upon the synthesis of nucleic acids and proteins and the latter is essential for continued cell enlargement in plants.[67] On the other hand, total RNA polymerase activity increases in plants treated with auxins, gibberellins, and cytokinins and furthermore, the application of the latter two produce an increase in RNA content.[68,69] Also, selective enhancement on peroxidase, polyphenol oxidase, and ATPase activity were detected on BL treatment of mung bean.[70] In addition, the enhancement of the RNA and DNA polymerase activities as well as the levels of RNA, DNA, and protein were detected in pinto bean and mung bean when they were treated with BL (**1**).[67,71] This enhancement was inhibited in the presence of selected inhibitors of RNA and protein synthesis, affecting cell elongation and cell division of the tissues.[72] These findings provided additional evidence that BRs act as plant growth regulators. By making an analogy to steroid-regulated gene expression in animals, Kalinich et al.[67,71] postulated a cytoplasmic receptor which may then transport them to the nucleus and have an effect on transcription and replication, but immunocytochemistry of germinated pollen from *Brassica napus* showed significant label in nuclei, suggesting that there is specific binding of BRs to nuclear components.[73]

Two model systems have been used by Clouse et al. to study the effects of BRs on plant gene expression: *Arabidopsis thaliana* stem and root elongation[6] and soybean stem elongation systems.[74,75] In both systems, BRs affect the pattern of gene expression in elongating tissues by increasing the abundance of some specific *in vitro* translatable mRNAs and decreasing others. Exogenous hormones, such as auxins, cytokinins, and ethylene, inhibit root elongation in wild-type *Arabidopsis* seedlings, and the ability of specific mutant plants from a mutagenized population to elongate their roots in the presence of these compounds allows for the isolation of mutants insensitive to each of these growth regulators. By random chemical mutagenesis of *Arabidopsis thaliana* followed by screening for BR-insensitive phenotypes based on their ability to elongate roots in the presence of 24-epibrassinolide (**2**), several likely BR-insensitive mutants are being currently characterized.[6] The same authors[6] have shown that 24-epibrassinolide (**2**) inhibited root elongation in the *axr1* auxin-insensitive mutant of *Arabidopsis* as well as auxin did in the putative BL-insensitive mutant obtained following the procedure cited above, corroborating the independent mode of action for both hormones. These findings are in accordance with the fact that the antiauxin 2-(*p*-chlorophenoxy) isobutyric acid did not affect BR-induced elongation in pea stems and BL-induced elongation can be markedly retarded by an inhibitor of cellulose biosynthesis.[76] However, DCC, an inhibitor of membrane-bound ATPase, results in a strong inhibition of both IAA- and BR-induced elongation, while it has no effect on GA induction.[8]

Recently Zurek and Clouse[77] constructed a cDNA library from BR-treated soybean epicotyl sections. Using differential hybridation, a single cDNA corresponding to an mRNA of approximately 1050 nucleotides whose abundance was increased by BR treatment was cloned and partially characterized. This represented the first gene isolated that is specifically regulated by active BRs. The presence of a signal peptide which would be required for export to the cell wall and the sequence homology (48% identity and 62% similarity) with a xyloglucan endotransglycosylase,[78] which has catalytic activity on cell wall polymers, suggest that this gene encodes an enzyme that has some type of cell wall activity. Moreover, Zurek and Clouse[77] have evidence that regulation of expression by BL (**1**) is at the posttranscriptional level. Recently, the same authors[79] have isolated a second unrelated clone which may be regulated by BL (**1**) at the transcriptional level. Using the *small auxin up RNA* (SAUR) gene and the auxin-insensitive diageotropica (*DGT*) mutant of tomato (*Lycopersicon esculentum* Mill.), Zurek et al.[80] conclude that, although both endogenous hormones affect wall relaxation processes, the mechanism through which BL (**1**) promotes elongation does not proceed through the auxin signal transduction pathway. These findings represent significant progress in the study of the mechanism of gene regulation by BRs.

In other contexts, it has been shown that BRs stimulate ethylene production in etiolated mung bean hypocotyl segments and a synergistic stimulation was observed when BR was applied together with IAA.[81] Moreover, it has been demonstrated that BRs and IAA stimulate the ethylene biosynthetic pathway between *S*-adenosylmethionine and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC)[82] by enhancing ACC synthase.[83] These findings were supported by the fact of having observed epinasty, due to the endogenous production of ethylene,[84] on hydroponically grown tomato plants when they were submitted to root treatment with BRs.[12] In this experiment, the greatest amount of ACC appeared to accumulate in the youngest petioles and the lack of ACC in the xylem sap suggested that there was a signal transport from the roots which stimulated ACC synthesis in the leaf tissue. Nevertheless, although both BRs and IAA stimulate ethylene production, it appears that the two mechanisms of action are different. Thus, the stimulation of ethylene production was induced in young as well as in mature tissues of bean even though mature tissues were not able to grow with auxins or BL (**1**).[2] Furthermore, the stimulation of ACC synthase by BRs and IAA decreased in the presence of ethylene biosynthesis inhibitors such as $CoCl_2$,[12] aminooxyacetic acid (AOA),[12] 2,3,5-triiodobenzoic acid (TIBA),[13] and 2-(*p*-chlorophenoxy)-2-methyl propionic acid (CMPA).[13] The effect observed with these inhibitors was different for both hormones with respect to the production rate, response, and optimal concentration for the inhibitor.

VI. PHYSIOLOGICAL EFFECTS OF BRASSINOSTEROIDS

Even though the physiological effect of BRs is similar to that of other endogenous plant growth hormones such as auxins, gibberellins, and ethylene, much evidence indicates important differences in the mechanism of action. In fact, most of the physiological effects shown by BRs have already been discussed extensively, specifically how they induce elongation. Other significant physiological effects will be mentioned in this section.

Stimulatory effects of BRs on elongation are among the most documented physiological effects. It has been observed in many assay systems such as radish,[45] tomato,[45] sunflower and cucumber hypocotyls,[10] normal and dwarf pea,[10] mung bean,[9] and azuki bean epicotyls, maize mesocotyls,[44] *Arabidopsis* peduncle,[6] and wheat coleoptiles.[7] How BRs induce elongation has been a field of tremendous study, but the mechanism remains unknown. In pakchoi (*Brassica chinensis*) it was shown that (22*S*,23*S*)-24-epibrassinolide (**10**) stimulated hypocotyl elongation by increasing wall relaxation but, unlike auxin action, without inducing a large change in wall mechanical properties.[85] However, while a similar stimulation of elongation was observed in soybean when applying BL (**1**), a change in wall mechanical properties was also detected. Moreover the mechanism involved does not proceed through the auxin signal transduction pathway.[80]

In general, cell elongation is associated with an increase of acid secretion. Some BRs have been shown to stimulate the secretion of H^+ and to hyperpolarize the plasmalemma in different plant tissues such as maize root,[86] azuki bean epicotyls,[87] radish cotyledons,[88] and *Egeria densa*[89,90] and *Vicia fava*[90] leaves. These effects were completely suppressed by inhibitors of RNA and protein synthesis. However, stigmasterol, ergosterol, and cholesterol, which were ineffective on maize root elongation, also stimulated H^+ secretion,[86] so it seems there is no correlation between growth and proton extrusion. Also, it has been shown by adding erythrosine B, a plasma membrane ATPase inhibitor, in *Vicia fava* leaf discs, that the

BR-induced H^+ extrusion is strongly reduced, indicating that the ATP-driven proton pump is involved in the effect.[90]

BRs are also able to modify the development process in roots at low concentration. Different root systems, such as adventitious cutting roots, intact seedling roots, excised root segments, and excised cultured roots were used to evaluate the effect of BRs. The results obtained are, in some cases, contradictory, and have been clearly reviewed by Roddick and Guan.[91] Although promotion of root growth has been observed after treatment with BRs in some systems, inhibition has been the predominant effect observed. Recently, an increase of adventitious roots has been shown in cuttings taken from mature Norway spruce plants[92] suggesting that BRs may act as antistress agents enhancing the ability of the cutting to resist the stress imposed by planting. Likewise, increased root regeneration has been found in *Pinus radiata* seedlings after transplantation.[93]

The fact that BR application to crops helps to overcome unfavorable cultural and weather conditions has been observed in other cultivars. Therefore, the effect of (22*S*,23*S*)-24-epibrassinolide (**10**) on barley, bean, or lettuce plants was more prominent in soil deficient in fertilizer than in fertilized soil.[94] Also, application of the same BR to gram (*Cicer arietinum)* significantly increased the plant growth under water stress,[95] as did (22*S*,23*S*)-28-homobrassinolide (**12**) in sugar-beet plants[96] and counteracted the inhibitory effects of NaCl and herbicides in rice even at low temperature.[97] It was reported that BL (**1**) induced resistance to chilling in rice, cucumber, corn,[16] and maize.[98] Furthermore, 24-epibrassinolide (**2**) and (22*S*,23*S*)-28-homobrassinolide (**12**) protected wheat leaf cells from heat shock and saline stress.[99]

VII. APPLICATION OF BRASSINOSTEROIDS IN AGRICULTURE

A considerable effort has been made in practical application of BRs and promising results have been obtained in several crops that have been reviewed extensively.[15,100] Although these findings are encouraging, most of them result from preliminary experiments in greenhouse and limited field plots, with a major exception the work done by Ikekawa and Zhao,[15] who, by applying 24-epibrassinolide (**2**) in large-scale field trials of wheat, corn, tobacco, and other plants over 6 years, obtained significant increases in crop yield. It must, however, be mentioned that laboratory biotests do not give a complete idea of the growth capacity of BRs when they are applied in the field. In some cases, the responses in greenhouse and field conditions do not correspond with those obtained in the bioassay system. The reasons could be different. For instance, the variability shown in the growth stage at which BR should be applied is greater than those obtained in a bioassay. Furthermore, the time spent to measure the effect in a bioassay might not be long enough to elicit the maximum activity, but it may be in the field. In this sense, one example of this is found with (22*R*,23*R*)-2α,3α-isopropylidenedioxy-22,23-epoxy-7-oxa-B-homo-5α-stigmastan-6-one, which did not exhibit activity in the rice lamina inclination test but, under field conditions, a greater increase in yield was obtained than in BL (**1**).[101] Another example is (22*S*,23*S*)-28-homobrassinolide (**12**), which has a surprisingly high response in several plants in the field with respect to results obtained in bioassays.[91,96] Moreover, some of the poor reproducibility of the results observed in field trials could surely be due to the lack of knowledge on how to apply these compounds in the field. BR application in crops can be realized by spraying the seedlings once or repeatedly at different stages of growth (leaves, flowers or stems), or by soaking the seeds in a solution of BR before planting them. However, the poor absorption detected in the plant could be the reason behind the variability observed in some field trials. Clearly, further detailed studies on formulation and application are required.

Another important characteristic which enhances the interest of BRs is the antistress effect, where promising results have already been achieved with unfavorable environmental factors such as drought,[95] extreme temperatures (chill, warmth),[8,98,99] salinity,[99] and herbicidal injury.[97] Also, the effect of BRs was more pronounced in plants grown in soil deficient in fertilizer than in plants grown in fertilized soil.[94]

Taking into account all these considerations, several parameters need further investigation, such as formulation, and time and method of application, before the full potential of BRs for improving the yield and quality of crops, and controlling diseases and environmental stress can be realized.

VIII. CONCLUSIONS

This chapter has attempted to review the state of the art in the field of BRs, paying special attention to the mode of action of these compounds in the plant kingdom, an essential requirement in profiting from their application in agriculture.

The practical application of BRs is now almost a reality in Japan, where they are being used in large agricultural areas to improve the yield of crops such as wheat, rice etc., where an increase in crop yield in the range of 20% is expected, depending on the cultivar conditions and climatology. Nevertheless, due to the synthetic difficulty which involves a high cost of such compounds, the use of BRs is not, at present, economical for extensive cultivars such as cereals, potatoes, vegetables, etc. One needs to establish which kind of crops will be more suitable and to what extent adverse cultural and environmental conditions will be relieved by BRs.

In my opinion, the most interesting application lies in cultivars with a high added value, whether it's for the intrinsic value of the crop yield or the value of the products obtained from it: drugs, colorings, and other products of industrial use.

Another interesting point that should be mentioned is the ecological factors. The fact that BRs are natural, nontoxic products which are applied in extremely low doses, and capable of improving the crop yields even in nonfertilized fields, makes BRs suitable candidates for their application in agriculture, especially in adverse climatic conditions, reducing the use of fertilizers and agrochemicals, which are serious contaminants of soils and water. The improvement of synthetic strategies to produce BRs, a more precise knowledge of the mode of action at physiological and molecular levels, as well as how to apply them in agriculture (formulation, doses, time and periodicity of the application, etc.) will help to gain more benefits from these potent plant growth regulators in agriculture.

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NOTES ADDED IN PROOF

A more precise knowledge of the biosynthetic pathway for BL (**1**) has been obtained (Figure 12). Thus, feeding experiments with [$^{13}C_5$]campesterol and [$^{2}H_6$]6α-hydroxycampestanol the biosynthetic sequence campesterol (**5**) → campestanol (**29**) (CP) → 6α-hydroxyCP (**30**) → 6-oxoCP (**31**) was confirmed in cultured cells of *Catharantus roseus*.[102] Moreover, there is evidence of the conversion of cathasterone (**32**), which was found to be endogenous in these cells, to TE (**6**).[103] Therefore, it could be involved in the transformation of 6-oxoCP (**31**) to TE (**6**) by stepwise hydroxylation of the side chain. The biological activity (rice lamina inclination and wheat-leaf unrolling tests) of the biosynthetic intermediates of BL (**1**) increased according to the order in the biosynthetic pathway.[104] 3-Dehydroteasterone (**9**) has been demonstrated to be the biosynthetic intermediate from TE (**6**) to TY (**7**) in cultured cells of *Lilium longiflorum*[105] and *Catharantus roseus*.[106] (Figure 1). The same pathway from TE (**6**) to CS (**8**) has also been observed in seedlings of some higher plants such as *Nicotiana tabacum* and *Oryza sativa* where no conversion to BL (**1**) is found. In the last three cells, CS (**8**) was found to be the biosynthetic precursor of 3-epicastasterone.[106] Similar biosynthetic sequence has been demonstrated from 6-deoxoTE (**33**) to 6-deoxoCS (**36**) through the corresponding 6-deoxo analogs **34** and **35** in cultured cells of *Catharantus roseus* in which most of them are endogenous. Moreover, 6-deoxoCS (**36**) was transformed to CS (**8**) and BL (**1**) suggesting that two possible pathways, namely "early C6-oxidation pathway" and "late C6-oxidation pathway", are involved in the biosynthesis of BL (**1**).[107] Also 6-deoxoTY (**35**) and 3-dehydro-6-deoxoTE (**34**) have been found in the pollen of *Cupressus arizonica*.[108] Teasterone 3-myristate was metabolized to BL (**1**) via TE (**6**) and TY (**7**) in cultured cells of *Lilium longiflorum*. Also the interconversion between TE ((**6**) and the corresponding esters 3-myristate and 3-laurate was achieved supporting the idea of the involvement of these conjugates in the storage mechanisms of BL (**1**).[109] 3-Laurate, 3-myristate and 3-palmitate esters of 3,24-bisepi-brassinolide and 3,24-bisepi-castasterone were identified in cell suspension culture of *Ornithopus sativus* after exogenously applied 24-epibrassinolide (**2**) and 24-epicastasterone (**3**) suggesting that the epimerization at C3 is a prerequisite for the enzymatic acyl transfer.[110]

BR inhibitor KM-01 was evaluated in several bioassays indicating that no activity was observed with auxins, cytokinins and ethylene bioassays although a synergistic effect with GA_3 and an inhibitory effect on ABA were observed. Therefore, KM-01 is the first selective BR inhibitor found in natural sources.[111]

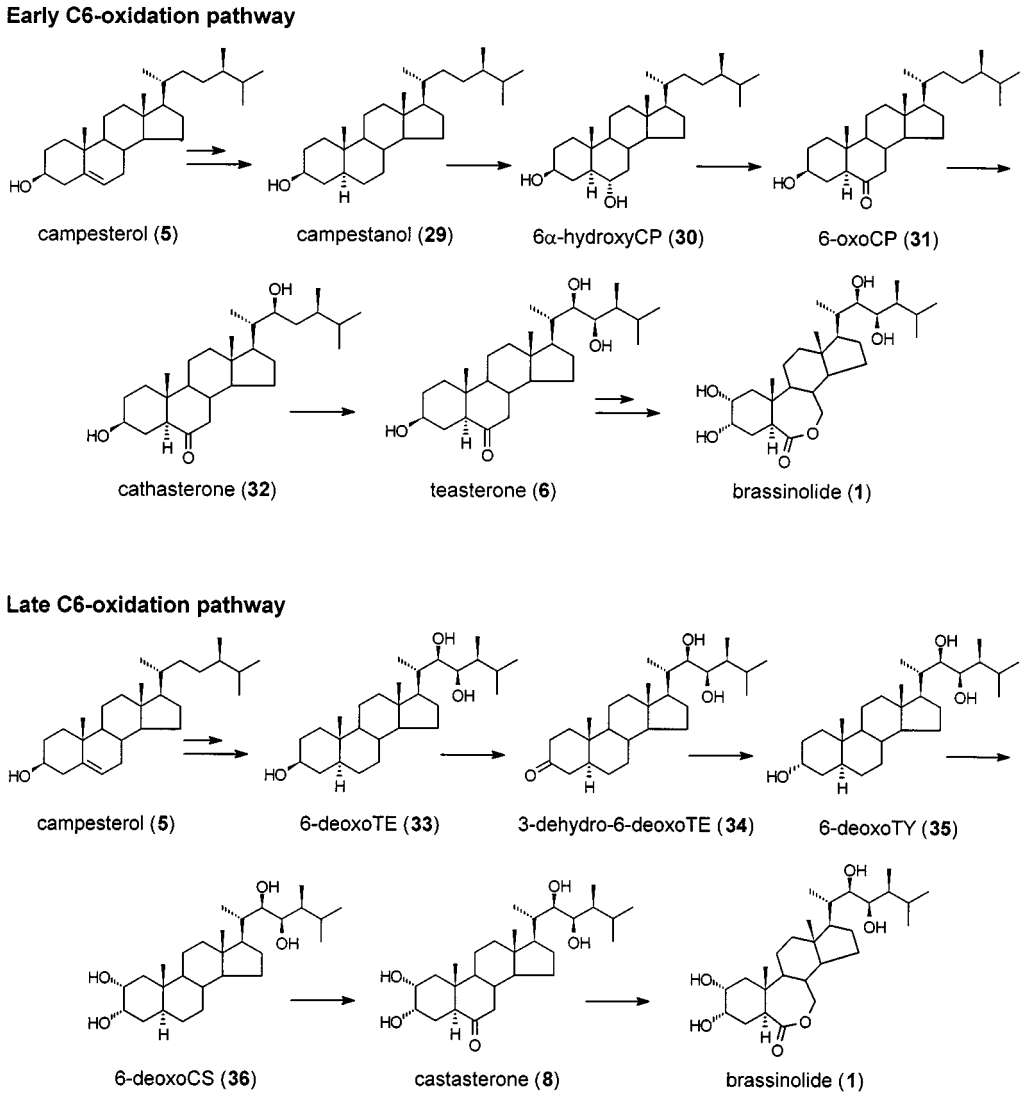

Figure 12 Biosynthetic pathways for brassinolide (**1**): early C6 oxidation and late C6 oxidation.

These findings represent new evidence of the independent action of BRs with respect to other plant growth regulators.

The effect of 24-epibrassinolide (**2**) on ATPase activity was evaluated in different buckwheat genotypes increasing the total ATPase activity by 35% in tetraploid and decreasing it twice in diploid buckwheat genotypes.[112]

A better structure–activity relationship has been obtained considering not only the oxygen atoms spatial position but also the type of oxygen functionality involved (hydroxyl, ketone, lactone, etc.).[113,114] This has been achieved by performing the molecular electrostatic potential map for all the active conformers. The overlay of all of them was carried out by means of the electrostatic Carbó index calculation.[115] This molecular similarity index correlated better with the activity than the previous one and allowed the design of new BRs with predictable good activity which were confirmed experimentally. Thus, the methodology developed to find a QSAR opens a new way to explain the activity of different BRs from the structural point of view and seems to be appropriate to predict the activity of new analogs suitable for further application in agriculture.

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