

Resistance Induction in Plants by a Brassinosteroid-Containing Extract of *Lychnis viscaria* L.

Udo Roth, Annette Friebe and Heide Schnabl*

Institut für Landwirtschaftliche Botanik, Physiologie und Biotechnologie der Pflanzen,
Universität Bonn, Karlrobert-Kreiten-Str. 13, D-53115 Bonn, Germany.
Fax: 0049 228 731696. E-mail: ilb@uni-bonn.de

* Author for correspondence and reprint requests

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Resistance inducing effects of an extract of *Lychnis viscaria* L. seeds, containing different brassinosteroids (BR), were investigated. Application of aqueous solutions in concentrations from 0.5 to 10 mg/l (dry wt. of extract) resulted in an enhanced resistance of tobacco, cucumber and tomato to viral and fungal pathogens of up to 36% compared with water-treated control plants. No direct anti-fungal effects in mycelium growth assays with *Phytophthora infestans* could be observed. After treatment and inoculation with powdery mildew a stimulation of different PR-proteins (ca. + 20% for peroxidase, + 30% for chitinase and up to + 68% for β -1,3-glucanase) in cucumber was found. A chitinase in gel-electrophoresis assay showed a stronger induction of a distinct isoform under the same conditions.

Time course of peroxidase induction and changes of apoplastic protein patterns revealed by SDS-PAGE indicated an earlier triggering of defence responses after plant-extract treatment and pathogen attack, probably being responsible for the increased resistance. Involvement of the brassinosteroids in the plant extract is discussed to elicit or mediate the activation of defence-mechanisms.

Introduction

For the first time the phenomenon of acquired resistance in plants to pathogens was reported by Chester (1933). In further experiments it was defined as an enhancement of resistance against a broad range of subsequently occurring diseases by prior inoculation with necrosis-producing pathogens (Kúc, 1987; Hammerschmidt, 1993). Over the last years several natural or synthetic agents acting as elicitors of plant defence reactions without any anti-microbial activity were found. Exogenous application of salicylic acid (SA) is known to induce resistance in a variety of plant species (Rasmussen *et al.*, 1991; Summermatter *et al.*, 1995). This compound is also produced endogenously after pathogen infection, and is thus considered as a signal molecule systemic acquired resistance (Malamy *et al.*, 1990; Meuwly *et al.*, 1995). Furthermore 2,6-dichloroisonicotinic acid (DCINA) and benzo-

(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) are two well characterized, synthetic inducers, which probably act along the SA-pathway (Métraux *et al.*, 1991; Uknes *et al.*, 1992; Friedrich *et al.*, 1996; Görlach *et al.*, 1996). In many cases the onset of systemic acquired resistance (SAR) is correlated with the induction of pathogenesis related (PR) proteins (Cohen *et al.*, 1994; Van Loon and Antoniw, 1982; Ward *et al.*, 1991), thus serving as markers for the activation of defence responses.

Besides the compounds mentioned above some naturally occurring substances and amino-acid derivatives are known to activate the defence system in plants. For instance in tobacco and tomato DL-3-aminobutyric acid (BABA) was shown to induce resistance against fungal diseases (Cohen, 1994; Cohen *et al.*, 1994). Chérif *et al.* (1992; 1994) described an enhanced resistance of cucumber plants against root pathogens (*Phytium* spp.) after application of silicon, while extracts of *Reynoutria sachalinensis* were effective, at least partially by induction of defence-related proteins, against fungal diseases in cucumber, wheat, tobacco and other plants (Herger *et al.* 1988; Schneider and Ullrich 1990, 1994).

Abbreviations: BABA, DL-3-aminobutyric acid, BR, brassinosteroids, BTH, benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester, SAR, systemic acquired resistance.

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Schabdach *et al.* (1995) found that sterol compounds, isolated from barley root extracts, induced resistance in barley and wheat against rust fungus infections. Ergosterol, a component of fungal plasma membranes, seems to be the most active steroid compound, since even very low concentrations (10 nM) stimulated H₂O₂ production in cucumber, as a typical early occurring defence reaction (Kauss and Jeblick, 1996). Since this compound is derived from fungi, it should rather be considered as an elicitor, than a resistance inducer. Brassinosteroids (BR) are structurally related to sterols, but show a very high activity regarding plant growth and development (for review see Clouse and Sasse, 1998). Furthermore BRs are reported to improve stress tolerance and also seem to protect plants against various pathogens (Hamada, 1986).

In this paper we report the resistance inducing effects of an extract of *Lychnis viscaria* L. seeds on tobacco, cucumber and tomato plants, which was previously shown to contain the brassinosteroids: 24-*epi*-castasterone and 24-*epi*-secastasterone (Friebe *et al.*, 1999).

Materials and Methods

Plant material

All plant species were grown as seedlings in potting compost ("Spezialmischung", Klamann) in a greenhouse equipped with an additional lighting system (300 µE/m², Philips SGR140) under a 16 h light regime with temperatures between 16° and 25 °C. After repotting, Plantosan (1.2 g per litre soil, Spieß/Urania) was used to fertilize tobacco and tomato seedlings, while cucumber plants were grown without any fertilizer. Cucumber plants were used for experiments after the second true leaf (ca. 3 weeks) expanded. Tobacco plants were cultivated for 6–8 weeks, while leaflets were cut from 5–6 week old tomato plants.

Pathogens

TMV particles were propagated by inoculation of a susceptible tobacco cultivar (*N. tabacum*, cv. Samsun nn). *Sphaerotheca fuliginea* was maintained on cucumber plants of the same cultivar. *Botrytis* was cultivated on potato-dextrose-agar

(PDA) plates (Difco) in a growth chamber in the dark with a temperature of 25 °C.

Phytophthora infestans was cultivated on vegetable-juice agar plates (20% commercially available vegetable juice) in a growth chamber in the dark at 20 °C.

Resistance induction

Whole plants (cucumber and tobacco) were treated with an aqueous solution of an ethanolic extract of *Lychnis viscaria* seeds (plant growth promoter ComCat, supplied by Polus und Partner GmbH, Lindenfels, Germany) using 0.0125% Tween 20 as a detergent. The tomato leaflets were dipped in this solution for a few seconds and then transferred into transparent plastic boxes.

After five days tobacco and cucumber plants were inoculated with the respective pathogen. Tomato leaflets were induced for two days and then inoculated with *Botrytis cinerea*. In order to check antiviral effects, in some experiments tobacco plants were challenged with the pathogen without keeping an induction interval by immediately inoculating the plants after the treatment.

Plant inoculation

For TMV inoculation 2.5 g of frozen leaf material of systemically infected tobacco plants was ground in a mortar in 200 ml sodium phosphate buffer (0.1 M; pH = 6.8). After homogenization, a small amount of carborundum was added to the suspension. This crude virus-preparation was brushed on the second and third leaves of each plant, yielding approximately 50 lesions on leaves of control plants. Disease severity was assayed 6 days after inoculation by measuring the lesion diameter of ten randomly chosen lesions per leaf.

Inoculation with *Sphaerotheca*, the cause of powdery mildew disease, was performed in an inoculation chamber by a ventilator using one heavily infected "donor plant" per four test plants. This led to an average leaf coverage of 60–80% of mildew colonies on control plants. The inoculated plants were placed back into the greenhouse under the conditions described above. Disease development was determined 10 days after inoculation by estimating the ratio between colony-covered and uncovered parts of the leaf surface.

Botrytis was inoculated by application of six 10 µl drops of a spore suspension (10^6 spores per ml) on the leaflets. Disease development was determined by measuring the diameter of the necrotic area around the inoculation site.

Mycelium growth assay

Antifungal activity of the plant extract was examined by measuring the radial mycelial growth of *Phytophthora infestans* in Petri dishes (9 cm diameter) on agar media (vegetable juice, prepared as described above) containing different concentrations of the plant extract. Plates were incubated in the dark at 20 °C for four days. Controls were prepared with distilled water instead of the extract. Three experiments with four replicates were performed.

Protein extraction and enzyme assays

Cucumber leaves were harvested after evaluation of resistance, or for kinetic studies at the times indicated in Figure 6, frozen in liquid nitrogen and ground in a mortar with a pestle. The resulting powders were extracted at 0–4 °C in an acidic buffer system (0.1 M sodium citrate, pH 4.6; 5 mM EDTA; 10 mM sodium ascorbate; 0.1% β-mercaptoethanol) and centrifuged at 10,000×g for 20 min at 4 °C. The supernatants were used as crude extracts for enzyme-activity determination. Protein contents were measured according to Bradford (1976) using BSA as a standard.

Intercellular washing fluid

For SDS-PAGE experiments intercellular washing fluid (IWF) was collected from extract-treated and untreated cucumber leaves, either non-inoculated or inoculated with *Sphaerotheca*. Then the leaves were vacuum-infiltrated with cold (4 °C) sodium phosphate buffer (10 mM; pH = 6.0) for 5 min, blotted dry on paper towels and centrifuged (1000×g; 10 min; 4 °C) in tubes filled with glass beads up to a height of approx. 2 cm. The resulting fluid between the beads was collected and concentrated with a centricon-system (membrane cut-off size 10 kD, Millipore).

Preparations were checked for cytosolic contaminations by malate-dehydrogenase measure-

ments, but showed no significant activity compared to the total activity (data not shown).

Enzyme assays and SDS-gel electrophoresis

Chitinase and β-1,3-glucanase activities were assayed colorimetrically according to Wirth and Wolf (1992) using dye-labelled substrate (carboxymethyl-chitin-Remazol-Brilliant-Violet and carboxymethyl-curdlan-Remazol-Brilliant-Blue, Loewe Biochemica). Peroxidase activity was measured by the guaiacol method (Hammerschmidt *et al.*, 1982).

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 15% SDS polyacrylamide gels with a stacking gel (3%) described by Laemmli (1970) at a constant current of 25 mA. After electrophoresis proteins were stained with Coomassie Brilliant Blue. Molecular weight markers ranging from 14.4 to 97.4 kD (Bio-Rad) were applied on an extra lane to estimate molecular weights of the detected proteins.

In order to investigate the induction of different chitinase-isoforms, SDS-acrylamide gels containing 0.75 mg/ml of dye-labelled chitin (see above) were used. IWF samples were mixed 1:1 with SDS sample buffer without 2-mercaptoethanol. After running the gels, the proteins were renatured in sodium phosphate buffer (0.1 M; pH 6.8) with 1% Triton X 100 for 2 h at 40 °C (modified, according to Trudel and Asselin, 1989). The reaction was stopped with Coomassie-destaining solution (45% H₂O, 45% ethanol, 10% acetic acid) after appearance of lytic zones. Then the gels were dried, scanned with an AGFA-scanning system (Photo look SA 2.08) and activity bands were depicted as negative images.

Results

Bioassays

Treatment of tobacco, cucumber and tomato with extracts of seeds from *Lychnis viscaria* (0.5–10 mg/l) resulted in an enhanced resistance to the respective pathogen, whereas leaf appearance was not altered by the treatment alone (Fig. 1). The intensity of resistance was not strictly dependent on the dose of the extract, but seemed to occur within a broader concentration range. In general, reduction of leaf infestations could be observed up

Table I. Resistance induction in different plants by application of the plant extract five days or three days (tomato) before inoculation with TMV (for details see "material and methods"; effects are presented as reduction of leaf infestation compared with control plants $[(1 - X / Y) * 100]$, X = lesion diameter of treated plants, Y = lesion diameter of control plants]; 7 experiments.

C _{plant extract} [mg/l]	Tobacco		Cucumber		Tomato
	2 nd leaf	3 rd leaf	1 st leaf	2 nd leaf	
0.5	19 ± 2* ^a	0*	16 ± 9	26 ± 13	22 ± 8 ^a
1	20 ± 9 ^a	29 ± 9 ^a	23 ± 10 ^a	19 ± 7 ^a	n.d.
2	27 ± 6 ^a	36 ± 6 ^a	36 ± 8 ⁺	31 ± 25 ⁺	n.d.
5	n.d.	n.d.	n.d.	n.d.	25 ± 6 ^a
10	n.d.	nd.	17 ± 8	17 ± 3 ^a	19 ± 8 ⁺

n = 6 plants or 12 leaflets (tomato), mean ± standard error.

^a = significant according to Student's t-test (p < 0,05).

* = duplicate experiments.

⁺ = three experiments.

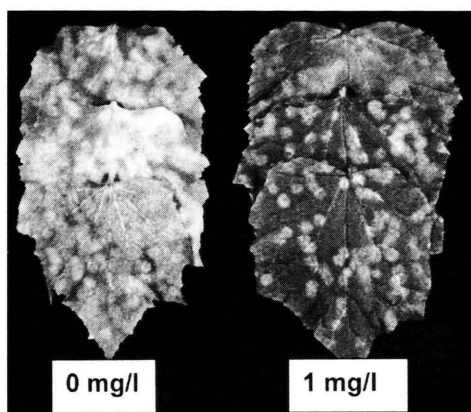


Fig. 1. Effect of the plant extract on the disease development in a cucumber/powdery mildew bioassay. Plants were treated with water or extract (mg/l) containing 0.0125% Tween 20, respectively. After five days the plants were inoculated with *Sphaerotheca fuliginea* and 10 days later assayed for disease intensity.

to 36% in treated plants in comparison to control plants (Table I).

In tobacco, resistance to TMV was increased significantly (between 20 and 36%) using concentrations of 1 and 2 mg/l (Table I). In this system, systemic effects of the plant extract were investigated by inoculating the next leaf above the treated leaves as well, but no reduction of lesion diameters compared with control plants could be measured (data not shown). For applications of 1 mg/l plant extract without keeping a time interval between plant treatment and inoculation no significant reduction of leaf damages were found (Table II).

Table II. Leaf damages of tobacco plants after inoculation with TMV without keeping an induction interval. Treated and non-treated tobacco plants were inoculated with TMV immediately after spraying with the extract; effects are presented as percentage of leaf damage ± standard error in relation to the whole leaf area; 5 experiments.

C _{plant extract} [mg/l]	0	1
2 nd leaf	47.5 ± 9.2	43.0 ± 7.7
3 rd leaf	31.4 ± 5.8	32.8 ± 6.2

n = 6 plants.

In cucumber, significant increase of resistance of the first and the second leaf to powdery mildew was shown to be within the maximum range of 31% and 36% when applying a concentration of 2 mg/l (Table I). However, concentrations of 1 and 10 mg/l resulted in a 19% and 17% reduction of leaf infestation (Fig. 1, Table I), respectively.

For tomato leaflets a ca. 25% decrease of damages by *Botrytis cinerea* was observed for a concentration of 0.5 and 5 mg/l of the plant extract, whereas application of 10 mg/l yielded an increased resistance of 19% (Table I).

Direct effects of plant extract on mycelium growth of Phytophthora infestans

In order to exclude direct effects of the plant extract on pathogens, mycelium growth of *Phytophthora infestans* was determined. Fig. 2 demonstrates that the growth of the fungus was not significantly affected by different concentrations of the plant extract. Even effector concentrations

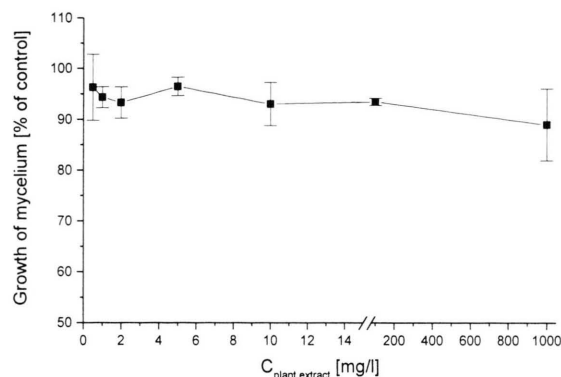


Fig. 2. Effect of different ComCat-concentrations on the mycelial growth of *Phytophthora infestans*. Vegetable juice (20%) agar plates containing different ComCat concentrations were inoculated with fungus' hyphae. After a cultivation period of four days fungus development was determined by measuring the diameter of the respective mycelia ($n =$ four plates per concentration). Values represent mean of three experiments \pm sd.

ten- or hundred-fold higher than applied in the bioassays caused no growth inhibition.

Biochemical assays

For the cucumber / powdery mildew system a stimulation of the three investigated PR-proteins was found after plant extract treatment and inoculation (Fig. 3). Since the specific activity of these enzymes is strongly dependent on the intensity of leaf infestation, the results are presented in% of control.

Chitinase-activity increased by 30% after pathogen inoculation after treatment with 1 mg/l and 10 mg/l of the plant extract (Fig. 3). This increase was coupled with a stronger induction of a distinct isoform shown by an in-gel assay (Fig. 4). In Fig. 3 β -1,3-Glucanase activity was shown to be induced to the highest extend (32% and 68% for 1 and 10 mg/l of the extract, respectively), whereas peroxidase-activity increased only up to ca. 20% using both concentrations.

Studies on time-course of peroxidase-activity showed, that only application of the plant extract did not result in an induction of apoplastic proteins (Fig. 5). In contrast, inoculation with the fungus stimulated peroxidase activities in treated plants by 25% and 257% compared with control plants after 7 and 10 days, respectively (Fig. 5). Activity of control plants after ten days were set

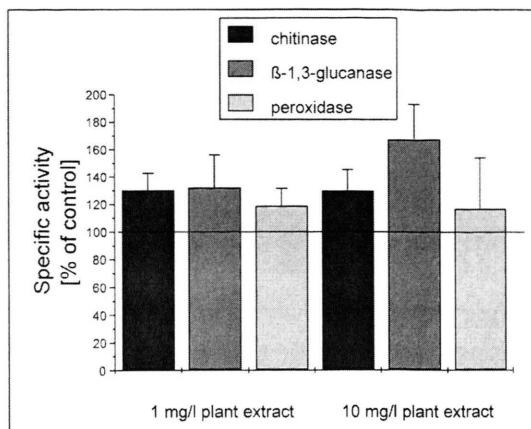


Fig. 3. Effect of ComCat on the induction of PR-proteins in cucumber. Seedlings were treated with ComCat (1 or 10 mg/l), or water containing 0.0125% Tween 20, respectively and inoculated with powdery mildew. After 10 days a crude extract of the first two leaves were prepared and enzyme measurements performed ($n =$ six plants per concentration). Values represent mean of three experiments \pm sd.



Fig. 4. Chitinase-activity bands after renaturation of separated proteins in a SDS-chitin-gel (12.5% acrylamide). 9 μ g of protein of the respective IWF was loaded per lane. IWF = intercellular washing fluid; + pm = inoculated with powdery mildew; C_{ComCat} = 1 mg/l.

as 100%. All other activities were related to this value.

Analysis of the protein pattern of the apoplastic proteins in SDS-acrylamide gels demonstrated the induction of at least two proteins of about 29 and 45 kD after inoculation with powdery mildew in both control and extract-treated plants (1 mg/l) (Fig. 6). The application of 1 mg/l plant extract alone did not lead to an increased synthesis of apoplastic proteins (Fig. 6, lane 1 and 2), but after

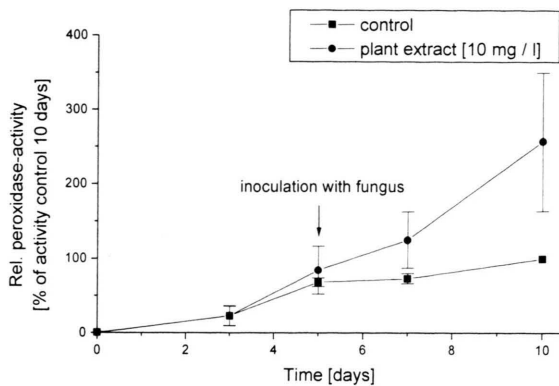


Fig. 5. Time-course of peroxidase-induction in cucumber after treatment with ComCat and inoculation with powdery mildew. Seedlings were treated with water or ComCat (10 mg/l) containing 0.0125% Tween 20. At different times, as shown in the figure, leaf samples were collected and extracted as mentioned in "Material and Methods". The resulting crude extracts were used in order to measure peroxidase activity according to Hammerschmidt *et al.* 1982.

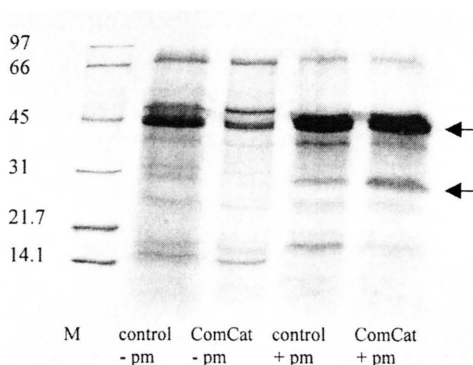


Fig. 6. SDS-PAGE (15% acrylamide). 10 µg of protein of the respective IWF was loaded per lane. After electrophoresis the gel was stained with Coomassie-blue for detection of proteins. IWF = intercellular washing fluid; M = molecular weight marker (from top): 97, 66, 45, 31, 21.7, 14.1 kD, respectively; -/+pm = (non-) inoculated with powdery mildew; C_{ComCat} = 1 mg/l.

infection with the fungus, a stronger induction of the 29 kD protein was observed in comparison to inoculated controls (Fig. 6, lane 3 and 4).

Discussion

Application of very low concentrations of a brassinosteroid-containing extract of *Lychnis viscaria* seeds (Friebe *et al.*, 1999), yielded an increased resistance of tobacco, cucumber and tomato to viral

or fungal pathogens (Table I). The plant extract showed characteristic features of resistance inducers according to Schönbeck *et al.* (1993):

The extract does not possess toxic effects and thus acts indirectly, since no antimicrobial effects could be found (Fig. 2, Table II). A time interval between treatment and inoculation was necessary for induction of resistance in tobacco. Furthermore, the level of induced resistance did not follow a linear dose-effect relationship. The protective effects of the plant extract were not only restricted to one special plant/pathogen interaction, but resulted in a protection against a broad range of pathogens (biotrophic and necrotrophic fungi and a virus).

The degree of protection (max. 36%) we observed, was lower than that achieved by other resistance inducing agents like BABA or BTH, which provided an increase of about 80% (Cohen *et al.*, 1994; Görlach *et al.*, 1996). On the other hand, other natural resistance inducers like culture filtrates of *Stachybotrys chartarum* and SiO_2 showed protective effects similar to our results (Schneider and Ullrich, 1994).

As further evidence for the indirect effect of the plant extract, activation of the plant's defence status was correlated with a stimulation of three PR-proteins, such as chitinase, β -1,3-glucanase and peroxidase, which are described as molecular markers of SAR (Ji and Kuc, 1995; Métraux and Boller, 1986; Rasmussen *et al.*, 1995).

Chitinase and β -1,3-glucanase are thought to be involved in hydrolysis of fungal cell walls, thus inhibiting the growth of pathogens (Mauch *et al.*, 1988), whereas peroxidase was described to be associated with the lignification of the host cell wall (Dean and Kuc, 1987).

Extract treatment alone is not sufficient to trigger defence reactions, but seems to sensitize plants respond earlier to pathogen attack, since peroxidase-activity is increased by extract treatment only after pathogen inoculation (Fig. 5). Furthermore, an enhanced induction of a 29 kD protein in the apoplastic fraction, probably a class III chitinase (Métraux and Boller, 1986), was also found only after extract treatment and subsequent inoculation (Fig. 6). These observations were supported by the results of a renatured chitin-SDS-gel (Fig. 4) showing a stronger induction of one chitinase isoform in extract-treated plants.

The question, which of the compounds of the extract consisting on phytosterols and brassinosteroids (Friebe *et al.*, 1999) are responsible for the resistance inducing effects, cannot be finally answered by the present results. Brassinosteroids (BR), which have been identified as important constituents of the plant extract, might be involved in induced resistance, because they are phytohormones highly active in different physiological processes in nanomolar concentrations (Szekeres *et al.*, 1996; Clouse and Sasse, 1998; Hamada, 1996). Likewise it was shown by our results that resistance in plants can be induced by application of the *Lychnis viscaria* extract in concentrations as low as 1 mg/l. On the other hand, different compounds of the extract could be responsible for inducing effects, as well. In addition, the BR might enhance the activity of other active compounds synergistically.

The idea of BRs being involved in resistance induction is further supported by Szekeres *et al.* (1996), who reported that mutants deficient in expressing a cytochrome P450 gene, which is involved in the biosynthesis of brassinosteroids, showed a remarkably low expression of PR-proteins. Additionally, in mutants overexpressing this gene, a significant induction of PR-proteins was shown.

Koncz (1998) strengthened the idea of the involvement of BR in resistance induction by discussing a "crosstalk between BRs and pathogenic signalling", due to the properties of a plasma membrane-bound BR-receptor, which might dimerize with receptors, encoded by resistance genes. A direct connection of BRs with an enhanced resistance in plants was also reported by Khrupach *et al.* (1999). They showed that application of 0.1 mg/l epi-brassinolide resulted in the protection of potato tubers against *Phytophthora infestans* and cucumber against *Peronospora cubensis*, which in the latter case was coupled with the stimulation of peroxidase and polyphenol-oxidase activity.

In order to clarify the role of the BRs of the plant extract in induced resistance, sufficient amounts of these compounds must either be isolated or synthesized for use in further assays.

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