Induction of plant defense responses by *Ocimum gratissimum* L. (Lamiaceae) leaf extracts

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ABSTRACT


Aqueous extracts of the leaves of *Ocimum gratissimum* at 10, 25, 40 and 50% (w/v) concentrations induced the production of phytoalexins in soybean cotyledons and sorghum mesocotyls. The aqueous extracts also induced systemic resistance in cucumber to *Colletotrichum lagenarium*, reflected by reduction in disease incidence and an increase in chitinase production. Modes of action and the existence of possible elicitors of defense response in *O. gratissimum* leaf extracts are discussed.

Keywords: resistance induced sorghum, soybean, cucumber.

RESUMO


Extratos aquosos de folhas da planta medicinal *Ocimum gratissimum* em concentrações de 10, 25, 40 e 50% (p/v) induziram a produção de gliceolina em cotilédones de soja e de deoxiantocianidinas mesocótilos de sorgo. Os extratos aquosos também induziram resistência sistêmica em pepino contra *Colletotrichum lagenarium*, refletido na redução da incidência da doença e no aumento da produção de quitinase. O modo de ação e a existência de possíveis elicitores de resposta de defesa nos extratos de folhas de *O. gratissimum* são discutidos.

Palavras-chave adicionais: resistência induzida sorghum, soja, pepino.

In agriculture, the active search for bioactive molecules in plants, insects and microorganisms could represent an alternative for disease control, since our continuously selected varieties of plants rapidly become vulnerable to phytopathogenic agents. The most practical and economically viable method for achieving this goal is protecting plants against phytopathogens through effective complete resistance (20).

Induced resistance has been studied in several plant species, and involves the activation of latent resistance mechanisms through various agents, the so called elicitors, resulting in local or systemic responses, such as SAR, a broad, physiological immunity that results from infection with a necrotrophic pathogen (18; 17).

Medicinal plants are potential sources of microbiocide compounds, which could be used in the management of plant diseases (5). *Ocimum gratissimum* is one such species, and its leaf extracts have been successfully tested for the control of several phytopathogenic fungi in vivo and in vitro, including *Rhizopus* sp., *Curvularia lunata*, *Ustilago maydis* and *Phytophthora palmivora* (3), *Aspergillus flavus*, *A. niger* and *R. solani* (2) and *Colletotrichum lindemuthianum* (1).

A chemical compound will be considered an activator of the SAR response if it induces resistance to the same spectrum of pathogens and the expression of the same biochemical markers as in the biological model and, in addition, has no direct antimicrobial activity (18). In order to investigate if *O. gratissimum* leaf extracts may lead directly to defense response induction in plants, this work reports the production of phytoalexins in soybean and sorghum, and the induction of resistance in cucumber against *Colletotrichum lagenarium*.

MATERIALS AND METHODS

Extract preparation

Aqueous extracts of *O. gratissimum*, were prepared from 50 g of fresh leaves collected from young plants in spring at Universidade Estadual de Maringá. Leaves were ground in 100 mL of distilled water in a blender for 1 min, and the homogenate was filtered subsequently through cheesecloth and Whatman filter papers n° 40 and n° 1. The filtrate (50%; w/v) was diluted to 10, 25 and 40% (w/v).

Phytoalexin determination

Glyceollins were determined according to the methodology proposed by Ayers et al. (1976) in soybean (*Glycine max* L. Merr.) cotyledons. Cotyledons were detached from 10-day-old seedlings.
Sections (0.5 cm thick and 0.5 cm in diameter) were cut from the upper surface of each cotyledon. Aliquots of 20 mL of each aqueous extract (10, 25, 40 or 50%; w/v) were applied on the wounded cotyledons. Distilled water was used as control. The treated cotyledons were incubated in moist filter paper disks in covered Petri plates at 25 °C. After 20 h, each set of cotyledons was transferred to 15 mL of distilled water in Erlenmeyer flasks, and kept under agitation for 1 h to rinse off the droplets of sample fluid retained on the wound surfaces. Measurements were presented in terms of this wound-droplet solution, with the elicitor activity expressed in terms of absorbance at 285 nm.

The 3-deoxyanthocyanidins in sorghum (Sorghum bicolor L.) mesocotyls were measured as suggested by Nicholson et al. (1987). Seeds were surface sterilized by immersion in an aqueous solution of 10% sodium hypochlorite for 10 min and then repeatedly washed to remove residues. Seedlings were grown in the dark for 3 to 4 days between layers of moist germination paper to allow the uniform elongation of mesocotyls. The seedlings were subjected to 4 h of light to stop elongation. Mesocotyls were excised and transferred to 1.5 mL tubes (3 mesocotyls per tube) containing 1 mL of each of the aqueous extracts (10, 25, 40 or 50%; w/v) or distilled water (control). Tubes were kept in moist environment at 27°C. After 60 h, mesocotyls were weighed, cut in three segments and transferred to tubes containing 80% methanol to extract the phytoalexins. The 3-deoxyanthocyanidins content of the methanolic solution was determined spectrophotometrically at 480 nm (29). For both systems, a regression analysis was performed using the Program SAEG, with concentration as the independent variable (with five levels: 0, 10, 25, 40 and 50%) and absorbance as the dependent variable.

Inoculum’s production
Colletotrichum lagenarium was previously isolated from cucumber leaves showing anthracnose symptoms, and cultured in oat-agar medium in Pirex® dishes kept under constant light to induce sporulation. An aqueous suspension was prepared by dissolving a spore suspension of the mucilage from a sporulating colony in distilled water. Spores were counted with the aid of a hemocytometer and adjusted to 1x10^7 conidia.mL^1 (6).

Resistance induction experiment
Cucumber (Cucumis sativus L.) cv. Safira seeds were planted in 1.5 L plastic pots (3 seeds per pot) containing sterilized soil, and kept in a greenhouse. The first true leaves were sprayed until run-off with O. gratissimum aqueous solutions (10, 25, 40 or 50%; w/v), 7, 3, 1 and 0 days before inoculation with a suspension of C. lagenarium on the first and second true leaves. Controls consisted of inoculated seedlings, without pretreatment. After 10 days, the number and size of lesions were measured on both leaves, and 2.5 cm² leaf discs were collected from both leaves for chitinase enzyme analysis. A factorial ANOVA was performed considering extract concentration (with four levels: 10, 25, 40 and 50%; w/v) and time before inoculation (four levels: 7, 3, 1 and 0 days), followed by a regression analysis.

Chitinase activity was determined according to Wirth and Wolff (1990). Leaf discs were obtained from leaves at the time symptoms were measured (10 days after inoculation), homogenized with 4 mL of 100 mM acetate buffer (pH 5) and filtered through Whatman filter paper no 1 (enzyme solution) (26). CM-chitin-RBV in aqueous solution (0.1 mL, 2 mg.mL^-1), buffer (0.1 mL; 0.2 M sodium acetate, pH 5) and enzyme solution (0.2 mL) were incubated in reaction caps for 30 min at 40 °C. The reaction was terminated by the addition of 1 N HCl (0.1 mL), causing precipitation of the non-degraded substrate.

The reaction caps were cooled on ice (10 min) and centrifuged (5000 g, 5 min, 4 °C). The supernatants were transferred to glass cuvettes and absorbance was measured spectrophotometrically at 550 nm against a blank prepared similarly but without the addition of enzyme solution during incubation. The specific enzyme activity (EA) was calculated for each repetition according to the equation: EA = EU x mg protein^-1, where EU is the enzymatic unit, given by the spectrophotometrical readings divided by 30 (minutes of incubation), and proteins were determined by Bradford’s method (7), with bovine serum albumine as a standard.

**RESULTS AND DISCUSSION**

In sorghum, all extracts of O. gratissimum induced the production of deoxyanthocyanidins showing high values when 40% concentration was used (Figure 1A). Similarly, in soybean all extracts induced phytoalexins compared with the control (Figure 1B). It was observed that the induction of phytoalexins in soybean was almost 6 times higher than the control when the concentration of 25% of O. gratissimum extracts was used. For both plant species, there was an almost increase in the production of phytoalexins with the less concentrated extracts. Regression analysis showed a theoretical minimum around the 30% concentration.

For both glyceollin and deoxyanthocyanidin, there was an initial increase in phytoalexins production. Such production decreased at the highest extract concentrations possibly because of phytoalexin inactivation, since at least for deoxyanthocyanidins (especially luteolinid) there can be irreversible binding to cell wall debris (23), which cannot be removed or detected. Besides, exceedingly concentrated extracts could be phytoxic. The small induction observed in the controls could be due to the injury caused during excision (30; 29).

For soybean, elicitors have been shown to be predominantly glycosidic. Glyceollins have been traditionally shown to increase in response to preparations obtained from Phytophthora spp. (16), M. ramossimus (25) and Diaporthe phaseolorum f.sp. meridionalis (21), as well as to plant extracts, such as Alibertia myrcifolia and Rudgea jasminoide (Rubiaceae) (8) and Eucalyptus citriodora (Myrtaceae) (6).

Extracts prepared with O. gratissimum leaves were rich in proteins and formed by distinct fractions containing several polar compounds and/or essential oils, such as geraniol and nerol (10). Since composition suggests different molecules or mixtures of molecules may trigger defense responses in sorghum and soybean. Yet, cell walls may contain cellulose, hemicelluloses, pectic compounds, lignin, suberin and proteins, and their proportions may vary among species and between primary and secondary cell walls in the same species (11). Therefore these substances could also be released when cell walls are injured during extract preparation.

For cucumbers grown in the greenhouse, typical anthracnose symptoms developed after C. lagenarium inoculation, with lesions being mainly small chlorotic spots and necrosis being observed at leaf margins. Extracts of O. gratissimum led to a systemic response, since the second leaves had a smaller number (Table 1), especially when the first leaves were treated 3 days before inoculation. Chitinase activity at the second true leaves was significantly affected by both time and concentration, with maximum production following treatment with the 25% extract, 1 day before inoculation (Table 2).

In the cucumber seedlings, the number of lesions was significantly
reduced on the treated inoculated plants probably due to systemic induction, but the answer was more results dependent on time of application than on extract concentration. For other plant extracts, including cucumber, a dose response study indicated that increasing concentrations generally produce increasing levels of systemic resistance in cucumber to *Colletotrichum lagenarium* and that some are phytotoxic at higher concentrations (14). We did not observe a dose response, but there were no signs of phytoxicity in response to the concentration tested of the extract either.

Many products have been shown to induce resistance responses in cucumber, especially following inoculation with *Sphaerotheca fuliginea*, *Cladosporium cucumerinum* and *Colletotrichum lagenarium*. Examples include Acibenzolar-S-Metyl (22), salicylic acid and its derivatives (13). Among elicitors of plant origin, the best studied example is *Reynoutria sachalinensis* leaf extracts (12). Resistance induction in cucumber has also been obtained by the application of PGPR preparations, especially from *Pseudomonas* sp. and *Bacillus* sp. (24).

Cucumber defence responses include decreases in disease incidence and/or severity and have been frequently associated with the induction of peroxidases and the PR-proteins b-1,3-glucanases and chitinases production (17). In particular, chitinase production has been associated to several other defense responses following various treatments (22). In fact, induced resistance in plants is a multi component system and also includes the ability of a plant to delay or avoid the entrance and/or the subsequent activity of a pathogen in its tissues (15). In our study, maximum induction of plant defense response did not coincide with a smaller number of lesions, which indicates that chitinase may not be the sole determinants in the resistance reaction in this system. However increases of induced plant defense were observed following almost all treatments compared to the control, suggesting that may be other mechanisms and operating along with the chitinase production.

Table 1. Number of lesions caused by *Colletotrichum lagenarium* on the first and second true leaves of cucumber seedlings treated with *Ocimum gratissimum* leaf extracts. Aqueous extracts were applied on the first true leaves 7, 3, 1 and 0 days before inoculation of both leaves with a suspension of 1x10⁶ conidia.mL⁻¹. Symptoms were measured on the first and second true leaves 10 days after inoculation. Concentration effects were not statistically significant. Values are means of four repetitions, followed by standard deviations.

<table>
<thead>
<tr>
<th>Time (dbi)</th>
<th>First Leaves</th>
<th>Second Leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.56±2.41</td>
<td>11.25±1.09</td>
</tr>
<tr>
<td>1</td>
<td>11.13±2.98</td>
<td>4.38±1.74</td>
</tr>
<tr>
<td>3</td>
<td>6.38±1.76</td>
<td>2.44±1.01</td>
</tr>
<tr>
<td>7</td>
<td>8.19±4.88</td>
<td>4.38±2.20</td>
</tr>
<tr>
<td>Control</td>
<td>1.33±1.53</td>
<td>2.17±0.65</td>
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</tbody>
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Table 2. Chitinase activity on the second true leaves of cucumber seedlings treated with *Ocimum gratissimum* leaf extracts following inoculation with *Colletotrichum lagenarium*. Aqueous extracts at 10, 25, 40 and 50% (w/v) were applied on the first true leaves 7, 3, 1 and 0 days before inoculation of both leaves with a suspension of 1x10⁶ conidia.mL⁻¹. Activity was determined from 2.5 cm² leaf discs collected 10 days after inoculation. CM-chitin-RBV was used as substrate. Activity was expressed as the enzyme specific activity (EA), given by the equation \( EA = EU \times mg protein^{-1} \). Values are means of three repetitions, followed by standard deviations.

<table>
<thead>
<tr>
<th>Time (dbi)</th>
<th>Concentration (% w/v)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>0</td>
<td>4.28±0.34</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>3</td>
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</tr>
<tr>
<td>7</td>
<td>2.45±0.67</td>
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</table>

Figure 1. Phytoalexin production in soybean and sorghum following the treatment with *Ocimum gratissimum* aqueous extracts. Distilled water was used as control. Values are means of three repetitions. (A): 3-deoxyanthocyanidins were extracted from sorghum mesocotyls incubated with the extracts at 25 °C for 60 h, using methanol 80%. Phytoalexins were detected in the methanolic solutions and expressed as absorbance units at 480 nm per gram of fresh weight. (B): Glyceollins were extracted from soybean cotyledons incubated with the extracts at 25 °C for 20 h in the dark. Phytoalexins were detected in the aqueous solution obtained by the agitation of cotyledons in water and expressed as absorbance units at 285 nm.
cucumber hypocotyls (10), which suggests that penetration and/or initial development of the pathogen might be affected.

Many different substances seem to trigger responses in cucumber, with no apparent common structural features among a compound that does or does not induce systemic resistance (14). Not surprisingly, in most in previous studies plants parts, of *O. gratissimum* including the leaves, showed a very complex chemical composition. Considering only distilled oils, three groups could be distinguished based on high contents of eugenol, thymol or geraniol (9). Other components have been cited in essential oils, such as *p*-cimene, a-copene, g-selinene and g-terpinene and spatulenol and myrcene (27). *O. gratissimum* is also frequently cited for the flavonoids cirsimaritine, isotimusine, xantomicrol and luteolin (27) and for high contents of calcium (19).

In conclusion, the application of *O. gratissimum* leaf extracts can lead to certain defense responses in soybean, sorghum and cucumber, which may result from the combination of a direct antimicrobial activity of essential oils and the elicitation of defense responses induced by the extracts components. Further experiments are necessary to determine the best times of application and/or extract concentrations. Of equal importance is the elucidation of the extract components. Still, the potential of *O. gratissimum* extract an elicitors of resistance in these plants should not be discarded.

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