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Phytoalexins in *Phaseolus vulgaris* and *Glycine max* induced by chemical treatment, microbial contamination and fungal infection

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Summary. Bean seeds, treated with $AgNO_3$ or exposed to the naturally occurring microflora, accumulated phaseollin, phaseollinisoflavan and kievitone in constant relative quantities. In seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli*, relative quantities of these phytoalexins were changed. In contrast, proportions of glyceollin isomers in soybean hypocotyls were not affected by the phytoalexin-inducing agent.

Attack by nematodes, fungi, bacteria and viruses, and treatment with chemicals, UV-light or temperature shock can induce phytoalexin production in plants. Phytoalexin accumulation seems to be a general response to stress. In plants that synthesize several phytoalexins, the quantitative relationship between these phytoalexins may vary significantly depending on the elicitor[2-5].

The present paper describes a) phytoalexin accumulation in bean seeds after chemical treatment, contamination by the naturally occurring microflora or inoculation with *Fusarium oxysporum* f. sp. *phaseoli*, and b) accumulation of glyceollin isomers in soybeans following chemical treatment or inoculation with *Phytophthora megasperma* f. sp. *glycinea*.

Materials and methods. Seeds of *Phaseolus vulgaris* L. (red kidney) were imbibed in distilled water for 2 h in the dark. Subsequently, the seeds were imbibed in 1 mM $AgNO_3$ for 2 h, rinsed with sterile distilled water and incubated in the dark at 25 °C and 90% relative humidity for 5 days. Seeds imbibed in distilled water for 4 h, but not treated with $AgNO_3$, were either incubated likewise and, thus, allowed to be contaminated by the naturally occurring microflora, or surface sterilized in 2.5% Na-tetraborate for 10 min, rinsed with sterile distilled water, and inoculated with a thick mycelial suspension of *Fusarium oxysporum* f. sp. *phaseoli* Kendrick and Snyder (CMI 141119). *F. oxysporum* was grown in potato broth containing 30 g glucose/l distilled water at 25 °C for 10 days.

Phytoalexins were extracted with ethanol. The solvent was evaporated to dryness at 40 °C under vacuum. The residue was resuspended in ethyl acetate and chromatographed on Merck Silica gel F_{254} TLC-plates with chloroform-methanol (25:1; v/v) as solvent[3]. Phaseollin from beans inoculated with *F. oxysporum* was purified further on Sephadex LH-20 (bed vol. 40 ml, flow rate 0.1 ml/min) eluted with 95% ethanol[6]. Phytoalexins were identified by their R_f-values and absorbance spectra[3], and concentrations were calculated from their absorbance at 280 nm (phaseollin, phaseollinisoflavan) and 293 nm (kievitone), respectively, and their extinction coefficients[7].

Seeds of *Glycine max* (L.) Merr. cv Altona were placed between sheets of moist filter paper for 24 h in the dark. Seed coats were removed. The cotyledons were separated, rinsed with 1 mM $AgNO_3$, and put into Petri dishes with the flat side upward. Prior to incubation in the dark at 25 °C, 90% relative humidity for 48 h, a 50 µl drop of 1 mM $AgNO_3$ was placed on each cotyledon. Soybean seedlings (cv Altona and Maple Arrow) were grown in Vermiculite (previously soaked in water) in the dark at 25 °C, 90% relative humidity for 5 and 10 days, respectively. *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin *(Pmg)* race 4 (avirulent on cv Altona) was grown on solidified bean broth at 25 °C. Droplets, 10 µl each, of either a suspension of *Pmg* zoospores[8] (10^5/ml) or 1 mM $AgNO_3$ were placed on hypocotyls from the cotyledons to the roots[9]. The seedlings were incubated in the dark at 25 °C, 90% relative humidity for 48 h. Seedling age always refers to the age prior to glyceollin-inducing treatments.

Glyceollin was extracted[10] and chromatographed on Whatman LK6DF TLC-plates with toluene-methanol (95:8; v/v). Glyceollin was located by reference to a co-chromatographed standard, eluted, and the concentration was calculated from its absorbance at 285 nm and its extinction coefficient[11]. Isomers were separated with HPLC, using a

Table 1. Phaseollin, phaseollinisoflavan and kievitone in bean seeds following treatment with 1 mM $AgNO_3$, exposure to the naturally occurring microflora or inoculation with *Fusarium oxysporum* f. sp. *phaseoli*

Treatment	Phaseollin[a]		Phaseollinisoflavan[a]		Kievitone[a]	
	µg[b]	%	µg[b]	%	µg[b]	%
1 mM $AgNO_3$	5.78±1.21	27.8±8.0	5.11±0.63	24.6±4.9	9.90±4.85	47.6±10.8
Natural microflora	2.87±0.74	28.8±7.6	1.90±0.16	19.1±2.4	5.18±1.36	52.1± 8.4
F. oxysporum	0.19±0.05	18.6±1.0	0.45±0.13	45.0±1.6	0.37±0.14	36.4± 2.5

[a]Means and SD were calculated from 4 experiments. [b]Data indicate µg phytoalexin/g seed.

Table 2. Glyceollin isomers in soybean hypocotyls and cotyledons 48 h after treatment with 1 mM $AgNO_3$ or inoculation with zoospores of *Phytophthora megasperma* f. sp. *glycinea*

Cultivar		Seedling age (days)	Treatment	Glyceollin (µg/mg dry wt of extracted tissues)[a]	Isomeric proportions of glyceollin I : II : III
Altona	Hypocotyls	5	$AgNO_3$	3.72±0.93	24.3±4.7 : 1.4±0.3 : 1
Altona	Hypocotyls	5	*Pmg*	0.95±0.39	20.0±5.6 : 1.3±0.2 : 1
Altona	Hypocotyls	10	$AgNO_3$	1.64±0.34	13.7±0.6 : 1.2±0.2 : 1
Maple Arrow	Hypocotyls	5	$AgNO_3$	3.03±1.52	15.9±3.0 : 1.3±0.2 : 1
Altona	Cotyledons		$AgNO_3$	0.01	6.3±2.2 : 1.2±0.2 : 1

[a]Means and SD were calculated from triplicate experiments.

0.46×25 cm Partisil (5 µm, Whatman PXS) column eluted with hexane-propane-2-ol (95:5; v/v) and monitored at 288 nm. The flow rate was 0.8 ml/min. Three glyceollin isomers with retention times of 16.2, 17.7 and 19 min, respectively, were detected and identified as isomers I, II and III by means of the UV-absorbance spectra[12].

Results and discussion. Bean seeds exposed to the naturally occurring microflora were soft and overgrown with various fungi and bacteria, whereas the seeds imbibed in $AgNO_3$ showed no contamination. Seeds inoculated with *F. oxysporum* f. sp. *phaseoli* were completely overgrown with mycelium.

Following treatment with $AgNO_3$, bean seeds accumulated more phaseollin, phaseollinisoflavan and kievitone than seeds exposed to the naturally occurring microflora (table 1). The relative quantities of the phytoalexins, however, were similar (table 1). Seeds inoculated with *F. oxysporum* accumulated very little phaseollin, phaseollinisoflavan and kievitone; the relative quantities differed from those found in seeds treated with $AgNO_3$ and in seeds exposed to the naturally occurring microflora (table 1). The chemical agent and the mixed population of microorganisms thus appear to induce a reaction different from that caused by a specific parasite. This is consistent with results from other laboratories[3,5] and with the hypothesis that the phytoalexin response varies, indicating a possible specificity to various stimuli[13]. One has to keep in mind, however, that fungi imperfecti such as *Fusarium* species may metabolize certain phytoalexins[14] and, thus, change the relative quantities of phytoalexins.

$AgNO_3$ induced larger quantities of glyceollin in soybean hypocotyls than an avirulent race of *Pmg* (table 2). Hypocotyls of 5-day-old seedlings treated with 1 mM $AgNO_3$ accumulated more glyceollin than hypocotyls of 10-day-old seedlings (table 2).

As already reported[12,15], glyceollin isomers, I, II and III were found in extracts from soybean hypocotyls and cotyledons (table 2). The nature of the phytoalexin-eliciting treatment had no significant effect on the isomeric proportions of glyceollin in hypocotyls of 5-day-old seedlings (table 2). Hypocotyl age, however, significantly affected isomeric proportions of glyceollin (table 2). Furthermore, isomeric proportions in hypocotyls of cv Maple Arrow treated with $AgNO_3$ differed from those in hypocotyls of cv Altona treated similarly (table 2); ratios between glyceollin isomers thus appear to be cultivar-specific. Isomeric proportions of glyceollin in hypocotyls also differed distinctly from those in cotyledons of the same cultivar (table 2).

In conclusion one can say that there appears to be some specificity in the phytoalexin response of beans, i.e. a change in the relative quantities of the accumulated phytoalexins due to the inducing treatment used, but possibly caused by fungal phytoalexin metabolism. In contrast, isomeric proportions of the soybean phytoalexin glyceollin were not affected by the treatments used, but appear to be cultivar-specific and depend on age and source of tissues.

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