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PHYTOPHTHORA MEGASPERMA CULTURE FILTRATE AND CELL WALL PREPARATION STIMULATE GLYCEOLLIN PRODUCTION AND REDUCE CELL VIABILITY IN SUSPENSION CULTURES OF SOYBEAN

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Key Word Index—*Glycine max*; Fabaceae; *Phytophthora megasperma*; glyceollin; suspension cultures; cell viability.

Abstract—Cultivation of soybean cells in a medium containing fungal culture filtrate induced glyceollin biosynthesis and loss of cell viability. Most of the glyceollin was released into the medium. Purified fungal cell walls (100 μg/ml) and × 2 diluted fungal culture filtrate caused similar levels of glyceollin synthesis in soybean cell suspensions. Fungal culture filtrate treated cells, however, were dead after four days of culture, whereas, loss of cell viability in the case of 100 μg/ml fungal cell wall treated cells was gradual. Culture filtrate-induced glyceollin production was not lethal to all cells if the cells were rinsed 24 hr after treatment. Toxic fungal products may be involved in this cell death. Addition of glyceollin to cell cultures, at amounts comparable to that produced in these studies, caused loss of cell viability but the surviving cells gradually regrew. Live cells caused the disappearance of glyceollin from the medium but autoclaved, glyceollin-treated cells had no effect on the added glyceollin even after 24 hr. Evidently, glyceollin was catabolized or bound only by living cells.

INTRODUCTION

Phytophthora root rot disease causes important economic losses in soybean cultivation. Resistance in soybean to *Phytophthora megasperma* f. sp. *glycinea* Kwan and Erwin (causal agent of soybean *Phytophthora* root rot) is determined by a single dominant gene; however, the gene product is still elusive [1]. Glyceollin, a soybean phytoalexin, is thought to be important in determining resistance to the fungus [2, 3]. Unconfirmed reports on both race specific elicitors [4] and suppressors [5] of glyceollin production are in the literature.

Soybean cell suspension cultures have been used to study glyceollin elicitation by *Phytophthora megasperma* cell walls [6]. Schmelzer *et al.* (7) have reported a similar time course of *m*RNA induction in *Phytophthora* treated soybean hypocotyls and in cell cultures treated with fungal elicitors. Cell suspension cultures, therefore, can serve as a useful tool in studies of glyceollin elicitation as well as its metabolism. Since plant regeneration in soybean tissue cultures is feasible [8], it is desirable to investigate glyceollin biosynthesis *vis a vis* its effect on cell growth, to help devise *in vitro* selection systems. Behnke [9–11], using crude fungal culture filtrates of the potato pathogens, *Phytophthora infestans* and *Fusarium oxysporum*, was able to select resistant callus. Interestingly, selected callus was resistant to four *Phytophthora infestans* pathotypes and the trait was also expressed in regenerated plants. Similarly, callus and embryogenic cultures of *Brassica napus* has been selected for growth in the presence of a crude culture filtrate of *Phoma lingam*. Some regenerated plants showed increased resistance to the pathogen and preliminary studies of the progeny suggests a genetic basis for the acquired resistance [12]. Here, we report the effect of fungal culture filtrates and cell wall preparations on glyceollin biosynthesis, cell viability and growth of soybean cells in suspension cultures. Glyceollin also was added to the cell cultures to study its phytotoxicity.

RESULTS AND DISCUSSION

Fungal growth of race 1 and 3 in a liquid medium was comparable (3.1 g and 3.04 g fresh wt. respectively) and the culture filtrate prepared from these cultures induced glyceollin biosynthesis in soybean cell suspension cultures (Table 1). Some of the glyceollin produced was present in the medium (Table 1). In the cotyledon bioassay of glyceollin elicitation, most of the glyceollin is found in the diffusion drop at the wound site [13]. It is difficult to speculate about the mechanism of glyceollin release, but under *in vivo* conditions it may be beneficial to the host for containing the pathogen. The amount of glyceollin induced by dilutions of culture filtrate from race 1 (incompatible) was higher than race 3 (compatible) at the lower dilutions. Using purified fungal cell wall elicitor, Ebel *et al.* [6] observed similar amounts of glyceollin from race 1 or race 3 elicitor-treated Harosoy 63 cell suspensions. Whether the different response of race 1 or race 3 culture filtrate-treated soybean cells is due to the presence of specific elicitors or specific suppressors of glyceollin production is not known because the presence of such elicitors or suppressors in fungal culture filtrates has not been confirmed (1).

Treatment of cultured cells with fungal culture filtrate

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Table 1. Glyceollin production in soybean cells treated with *Phytophthora megasperma* f. sp. *glycinea* race 1 or 3 culture filtrate, cells were harvested after 24 hr of treatment and glyceollin was determined both in the cells and culture medium [cells (1.5 g) were seeded into 50 ml of medium]

Culture filtrate dilutions		Glyceollin μg/gm f wt. Cells	Culture medium
Race 1	*× 1/10	13.1	2.2
	× 1/4	29.0	3.2
	× 1/2	38.1	2.9
Race 3	× 1/10	17.5	2.1
	× 1/4	31.5	2.1
	× 1/2	25.6	1.4
	Control	0.0	0.0

*Represent dilution of the culture filtrate.
Data represent average of 3 replicates.

caused a loss of soybean cell viability. After 4 days there was 100% cell death except when × 10 diluted culture filtrate was used (Table 2). In the latter case the viable cells showed regrowth, as was evident from the fresh weight of cells measured after 10 days of cultivation (Table 2). Even when lower dilutions of fungal culture filtrate were used, the living cells resumed growth after a lag phase of 24 hr if the fungal culture medium was removed and the cells were washed and resuspended in fresh medium without the fungal culture filtrate (Fig. 1 data only from × 2 diluted filtrate). This indicates that glyceollin production can be induced and yet all cells are not killed if the cells are rinsed after 24 hr induction. No differences were evident in terms of cell viability loss in race 1 or 3 culture filtrate-treated cells. Thus soybean cells, could possibly be selected for resistance to the fungal culture filtrate both by one step [14] or gradual selection procedures [15]. Similar methods have been successfully employed for selection of potato and flax cultures resistant to *Phytophthora infestans* and *Phoma lingam* [9–12] and the resistance trait was expressed in plants regenerated from the resistant cells.

In order to find the cause of the cell viability loss, we also studied the effect of purified fungal cell wall preparations on glyceollin production and cell viability. There was no significant difference in the amount of glyceollin produced by soybean cells treated with either × 2 diluted race 3 fungal culture filtrate or 100 μg/ml purified race 3 fungal cell walls, yet the loss of cell viability was only gradual in the later treatment (Table 3). Even after 10 days of culturing in the presence of 100 μg/ml fungal cell walls, soybean cells showed 17% viability. Purified glyceollin, when added to the cells at 10 μg/ml or 15 μg/ml (concentrations lower than produced in the cell cultures in our experiment), caused an initial loss of viability (Table 4). Exogenous glyceollin was catabolized/sequestered by the cells within 24 hr as no detectable glyceollin was evident, either in the culture medium or in the cells, when 10 μg/ml

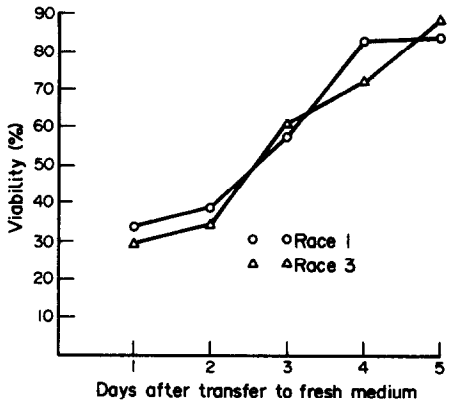

Fig. 1. Increase in the soybean cell viability after removal of the fungal culture filtrate (× 2 diluted) from the medium. After 24 hr of incubation the cells were washed × 2 with B_5CA medium and resuspended in B_5CA medium. O–O, race 1 culture filtrate treated cultures; Δ–Δ, race 3 culture filtrate treated cultures.

Table 2. Viability (%) of soybean cultivar H63 cell suspensions cultured in medium containing *Phytophthora megasperma* race 1 or race 3 culture filtrate

Culture filtrate dilutions		% viability after days in culture 1	2	3	4	5	10
Race 1	× 10	54	33	29	51	73	83 (7.9)
	× 4	51	43	12	0	0	0
	× 2	39	31	5	0	0	0
Race 3	× 10	57	37	30	45	67	84 (7.5)
	× 4	54	31	13	0	0	0
	× 2	46	27	3	0	0	0
Control 1*		71	77	83	81	87	74 (10.8)
Control 2*		79	81	85	87	85	80 (11.3)

*Controls 1 and 2 represent growth of cells in presence of 2 × diluted fungal culture medium in the B_5CA medium and B_5CA medium respectively.

Data in parenthesis gives fresh weight of cells (g) obtained after 10 days of culture. Cells (1.5 g) were seeded 50 ml^{-1} of the medium.

Table 3. Viability (%) of soybean cells (cultivar H63) cultured in medium containing *Phytophthora megasperma* race 3 cell wall preparation

	% viability after days in culture					
Treatment	1	2	3	4	5	10
50 μg/ml	64	33	43	51	57	64 (3.80)*
100 μg/ml	61	44	51	41	37	17 (1.64)
Control	71	80	92	91	87	81 (11.72)

* Data in parentheses represent fresh weight (g) of cells obtained after 10 days of culture. 1.5 g of cells were seeded in 50 ml of the medium.

Table 4. Effect of glyceollin on % viability of soybean cells (cultivar H63)

	% viability after days of glyceollin treatment					
Treatment	1	2	3	4	5	10
Control	73	77	84	81	83	74 (1.30)*
Glyceollin 10 μg/ml	61	57	43	31	47	63 (0.87)
Glyceollin 15 μg/ml	51	37	31	33	41	43 (0.50)

* Data in parentheses fresh weight of cells (g) at the end of the 10 day treatment. Cells (150 mg) were incubated in 5 ml of the medium.

Table 5. Amount of glyceollin extracted from cells and culture medium treated with 10 μg glyceollin/ml of the medium containing 150 mg cells

	Glyceollin* (μg)			
	2 hr		24 hr	
Cell state	Cells	Medium†	Cells	Medium†
Viable cells (83%)	0.00	1.5	0.00	0.00
Autoclaved cells	14.5	18.1	13.9	20.9

* Data represent average from 3 replicates.
† 5 ml of medium.

glyceollin-treated cells were analysed for glyceollin (Table 5). In contrast, glyceollin-treated autoclaved cells yielded the same amount of glyceollin from cells or culture medium after 2 or 24 hr of treatment (Table 5). Cell cultures, therefore, might serve as a good system to study glyceollin metabolism. Metabolism of exogenously added phaseollin by cell cultures of *Phaseolus vulgaris* and tobacco has been reported [16, 17].

Based on our data, it was evident that glyceollin was toxic to the cells when added to the medium or when induced by culture filtrates or cell wall preparations. Kaplan *et al.* [18] and Boydston *et al.* [19] had reported the inhibition of respiration in isolated soybean and beet mitochondria by glyceollin. Glyceollin was suggested to block electron flow between NADH dehydrogenase and coenzyme Q, i.e. site 1 of mitochondrial electron transport [19]. Toxic effects of phaseollin, a *Phaseolus*, phytoalexin, in terms of reduced respiration and growth, and enhanced cell death also has been reported in bean and tobacco cell cultures [16, 17]. In our experiments, rapid loss of cell viability by fungal culture filtrate treatment compared to the gradual viability loss caused by fungal cell walls, suggests involvement of some other toxic fungal product in this response.

EXPERIMENTAL

Raising cell suspensions. Sterilized seeds of soybean (*Glycine max* L. Merr) cv Harosoy 63 were grown on Murashige and Skoog [20] medium without hormones, to raise seedlings under aseptic conditions. Callus was induced from the root explants on B_5 medium [21]. Callus pieces were dispersed in liquid B_5 medium containing 2 g/l casein hydrolysate (B_5CA medium) to obtain a cell suspension which was passed through a 105 μm mesh sieve after 10 days and used to raise the subsequent cultures. Cell cultures were maintained by five-fold dilutions into fresh B_5CA medium every 7th day.

Fungal cultures. Races 1 and 3 of *Phytophthora megasperma* f sp. *glycinea* were inoculated on lima bean agar medium (Difco). After 5 days of growth, 0.5 cm diameter discs were punched out with a sterile cork borer and 25 discs were inoculated into 500 ml of sucrose–asparagine medium [22]. After 15 days of growth in this medium on a rotary shaker at 100 rpm at room temp. (24 ± 2°) the fungus was harvested by filtering through Whatman No. 1 filter paper. Race 3 of *Phytophthora megasperma* was cultured in a similar way to obtain the fungal cell walls, prepared by the procedure of ref. [5] except that the last Sephadex column step was omitted.

Preparation of culture medium with fungal culture filtrate. Constituents of B_5CA medium were added to flasks containing 100, 250 or 500 ml of fungal culture filtrate. Final volume was then adjusted to 1 l with distilled H_2O. The fungal culture filtrate dilutions, therefore, were × 10, 4 and 2 respectively, without change in the composition of B_5CA medium. After adjusting the pH to 5.8 the medium was autoclaved. Cells (1.5 g fr. wt) were seeded in 50 ml of this medium to study glyceollin production and cell viability. In another experiment, an autoclaved fungal cell wall preparation from race 3 (50 μg or 100 μg/ml) was added to 50 ml of B_5CA medium containing 1.5 g (fr. wt) cells.

Determination of cell viability. Cell viability was determined by the phenosafranin dye exclusion method [22] from 15 sample observations from three replicates.

Extraction of glyceollin from cells and medium. Cells were harvested by filtering through Whatman No. 1 filter paper on a Buchner funnel by applying house vacuum for 1 min. Cells were suspended in 10 ml of methanol and left overnight in a refrigerator. After homogenization, with a pestle and mortar, the resulting slurry was centrifuged at 5000 *g* for 15 min. The pellet was extracted × 2 with 10 ml of MeOH, the combined supernatants was evaporated to dryness in a rotary evaporator at 50° and redissolved in 1 ml of MeOH. The sample (20 μl) was analysed on an Altex ODS Column (25 cm × 4.6 mm ID; Beckman Instruments, Berkeley, CA), using a flow rate of 1 ml/min of 65% MeOH in H_2O. Absorbance was measured at 287 nm (AUFS 0.05) and the concentration of glyceollin within each sample was calculated by an Altex C-RIA integrator using an ext. stand.

The cell culture medium was extracted × 3 with equal vols of

ethyl acetate. The ethyl acetate extract was evapd to dryness in a rotary evaporator and redissolved in 1 ml of MeOH and glyceollin was estimated as described above.

Preparation of glyceollin. Soybean cotyledons were surface sterilized with 3% H_2O_2 for 10 min. A portion of the lower epidermis was removed with a sterile razor blade and the cotyledons were placed on a moist Whatman No. 1 filter paper in a petri dish. The cut surface was inoculated with a drop of fungal culture filtrate. After 24 hr the cotyledons were dried overnight in an oven at 60°C. The dried cotyledons were extracted with × 10 (by weight) of extraction mixture (1 part 95% EtOH and 1 part 0.01 M K_2HPO_4) overnight in a refrigerator. The extract was evaporated in a rotary evaporator to dryness and redissolved in MeOH. The crude extract was run on a silica gel (Kieselgel 60 GF 254, E. Merck, Germany) thin layer plate in hexane–EtOAc MeOH (30:20:1). The glyceollin band, corresponding to the authentic standard and showing quenching in 254 nm UV light, was scraped off and extracted from the silica with MeOH. The sample was run on a Whatman Partisil M 9 ODS Column (10 mm × 25 cm) at a flow rate of 4 ml/min of 65% MeOH in H_2O. Glyceollin fractions were collected, pooled, evapd to dryness and redissolved in H_2O. The H_2O extract was again evaporated in a second flask to remove traces of MeOH. Glyceollin was finally dissolved in H_2O. This glyceollin preparation was used at a concentration of 10 and 15 μg/ml to study its effect on cell viability. Cell suspensions containing 150 mg of cells in 5 ml medium were used. Ten μg/ml glyceollin treated cells were also harvested after 2 and 24 hr of glyceollin exposure. Glyceollin was extracted from the medium and the cells as described above. Autoclaved cells (killed) were used as a control.

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