Plant Physiol. (1981) 67, 1032–1035
0032-0889/81/67/1032/04/$00.50/0

Release of a Soluble Phytoalexin Elicitor from Mycelial Walls of *Phytophthora megasperma* var. *sojae* by Soybean Tissues[1]

Received for publication August 26, 1980 and in revised form November 20, 1980

MASAAKI YOSHIKAWA, MASAYOSHI MATAMA, AND HAJIME MASAGO
Laboratory of Plant Pathology, Faculty of Agriculture, Kyoto Prefectural University, Kyoto 606, Japan

ABSTRACT

A soluble elicitor of glyceollin accumulation was released from insoluble mycelial walls of *Phytophthora megasperma* var. *sojae* after incubation with soybean cotyledon tissue for as little as 2 minutes. Various enzymic and chemical treatments of the released elicitor indicated that the activity resided in a carbohydrate moiety, and gel filtration disclosed the presence of at least two active molecular species. Cell-free extracts from soybean cotyledons or hypocotyls also released soluble elicitors from fungal cell walls that were similar to those released by living cotyledon tissue. These results may suggest that contact of fungal pathogens with host tissues is required to release fungal wall elicitors which then initiate phytoalexin accumulation in the plant.

Phytoalexins are low molecular weight antibiotic compounds that are inducibly formed in higher plants after infection by microorganisms and which confer natural disease resistance (4, 11, 12). Phytoalexins also accumulate in plants in response to substances of pathogen origin termed elicitors, and these have been implicated in the induction of phytoalexin production in infected plants (1, 11). Initial events in the interaction between elicitors and plant tissues, however, are largely not understood.

The soybean (*Glycine max* [L.] (Merr.)—a fungal pathogen *Phytophthora megasperma* var. *sojae* A. A. Hildb. interaction is one of the best understood gene-for-gene plant-pathogen systems in which the processes leading to resistance expression have been analyzed (11, 15, 17, 18). The evidence strongly supports a role for accumulation of the phytoalexin glyceollin (11) in resistance expression (11, 16). Mycelial walls of the fungus have been shown to be potent elicitors of glyceollin accumulation and their possible role in the induction of phytoalexin accumulation in infected plants has been suggested (1–3, 10, 11). Yoshikawa *et al.* (14, 18) recently demonstrated that fungus-associated elicitors such as mycelial wall preparations induced glyceollin accumulation in soybean tissues in a manner similar to that occurring in infected tissues, thus providing evidence for the *in vivo* involvement of elicitors. The isolated mycelial wall preparations are, however, column-like structures (see Fig. 1) that are insoluble in water. Although harsh treatments such as autoclaving (2, 3, 5, 13) or alkaline treatment (10) can solubilize active elicitor moieties from mycelial walls, such conditions are unlikely to exist in biological environments. This raises the question of how insoluble elicitor molecules on or in mycelial walls are recognized by plant cells during natural infection processes. Unless wall-associated elicitors are proven to be surface-borne or detected in some other way by plant cells during pathogenesis, their physiological role remains questionable. We report here that a soluble elicitor(s) is released from mycelial walls of *P. megasperma* var. *sojae* immediately after contact with soybean tissues, and suggest that such a released elicitor may be responsible for the initiation of phytoalexin accumulation in fungus-infected soybean plants.

MATERIALS AND METHODS

Isolation of Mycelial Walls. Race 1 of *P. megasperma* var. *sojae* was grown in an asparagine-glucose medium (9) supplemented with autoclaved soybean hypocotyl extract (16) at 25 C for 7 days. Mycelia were collected by suction filtration and washed with deionized H_2O. Mycelial walls were isolated by the method previously described (14) with slight modification to avoid use of organic solvents which may alter native wall structures or molecules. About 5 g batches of mycelia were finely pulverized in a mortar under liquid N_2, and subsequent steps were performed at 0 C. The resulting powder was suspended in 20 ml of 20 mM Tris-HCl (pH 7.2) and briefly sonicated (1 min, 5 times) to disperse small particles present inside mycelial fragments into the buffer medium. The suspension was centrifuged at 100*g* for 5 min. The pellet was resuspended and washed by centrifugation with the same buffer three times. The wall suspension was again sonicated as described above, and the walls were washed with deionized H_2O by centrifugation 7 times. The washed walls were lyophilized and before use, finely suspended in deionized H_2O with a Waring Blendor. The isolated wall preparations appeared to be free from cytoplasmic contaminants under the microscope (Fig. 1), and the supernatant fraction of the wall suspension did not contain elicitor activity when assayed as described below.

Release of Soluble Elicitor from Mycelial Walls by Soybean Cotyledons and Cell-free Extracts. The soybean cultivar Harosoy 63 was grown as described previously (15). Cotyledons from 8-day-old seedlings were wounded by cutting away the lower surface (about 1-mm thick). A 50-μl aliquot of aqueous mycelial wall suspension (500 μg/ml) was placed onto the wounded areas of 30 cotyledons, and these were incubated on moist filter papers in Petri dishes at 25 C for the indicated time periods. At the end of incubation, the droplets of the wall suspension on cotyledons were recovered and added to 2 volumes of absolute ethanol to terminate incubation. The suspension was evaporated to dryness at 40 C *in vacuo*, and the resulting residue suspended in 6 ml of 10 mM Tris-HCl (pH 7.2). The suspension was centrifuged for 20 min at 30,000*g* to precipitate mycelial walls. The resulting supernatant fraction was filtered through a membrane filter (Millipore, 0.45 μm) to ensure removal of insoluble cell walls. The soluble filtrates thus obtained were referred to as the "released elicitor" fractions.

Cell-free extracts from soybean cotyledons or hypocotyls were prepared as follows. Ten g of either tissue were chopped into small pieces and homogenized with 30 ml of 20 mM Tris-HCl (pH 7.2) containing 2 mM $MgCl_2$ and 3 mM DTT in a Waring Blendor for 3 min at full speed. The homogenate was squeezed through 2

[1] This research was supported in part by Grant 256040 from Ministry of Education of Japan to M. Y.

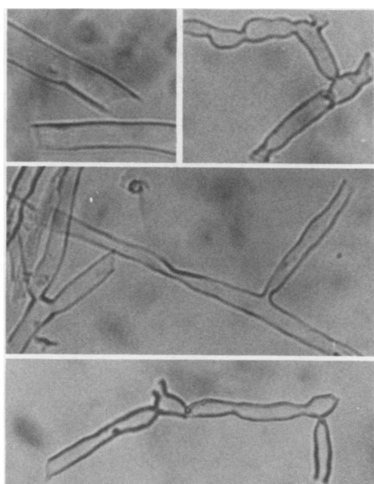

FIG. 1. Microscopic appearance of a mycelial wall preparation isolated from *P. megasperma* var. *sojae*.

layers of gauze and centrifuged at 20,000*g* for 20 min. The resulting supernatant fraction was brought to 70% saturation by addition of solid $(NH_4)_2SO_4$, stirred for 1 h, and centrifuged as above. The pellet was dissolved in 10 ml of 10 mM Tris-HCl (pH 7.2). The solution was dialyzed against two changes of the same buffer overnight and then centrifuged as above. The resulting supernatant was used as a cell-free extract. The above procedures were performed at 0 C. The extract (0.8 ml) was incubated wtih 0.2 ml of mycelial wall suspension (2 mg/ml) at 35 C for the indicated time periods. The incubation was terminated by addition of 1 ml of 0.1 N $HClO_4$ at 0 C since it was found that ethanol addition was not sufficient to terminate this *in vitro* reaction. The incubation mixture was centrifuged at 30,000*g* for 20 min at 0 C to precipitate proteins and residual mycelial walls. The resulting supernatant fraction was neutralized with 0.1 N KOH. After standing at 0 C for 30 min, the $KClO_4$ crystals that formed were removed by centrifugation at 30,000*g* for 10 min. The supernatant fraction was passed through the membrane filter as above and the filtrate used as the released elicitor fraction. These procedures terminating the reactions with ethanol or $HClO_4$ were employed mainly in kinetic experiments in which rapid termination of the reactions was necessary. In other experiments, the reactions were terminated without addition of the agents, at 1 h after incubation unless otherwise specified, by pelleting residual mycelial walls by centrifugation followed by filtration through the membrane filter as described above to avoid possible structural alteration of the released elicitor by the agents. Control experiments showed that the addition of ethanol or $HClO_4$ under the described condition did not destroy the elicitor activity of the released fraction or release the soluble elicitor from mycelial walls.

Assay for Elicitor Activity. Detached cotyledons from 8-day-old soybean seedlings were wounded as described above. An 80-μl aliquot of the released elicitor fractions was placed onto the wounded areas of 10 cotyledons, and these were incubated on moist filter papers in Petri dishes at 25 C for 24 h. Rifampicin and ampicillin at 10 and 500 μg/ml, respectively, were added to the released elicitor fractions to suppress bacterial growth during incubation. These antibiotics alone neither induced glyceollin accumulation in wounded cotyledons nor suppressed glyceollin accumulation in elicitor-treated cotyledons (14). Glyceollin concentrations in the wound droplets were determined as described previously (14).

RESULTS

Release of Soluble Elicitor by Cotyledon Tissue. Suspensions of insoluble mycelial walls isolated from *P. megasperma* var. *sojae* were potent elicitors of glyceollin accumulation in soybean cotyledons (Fig. 2). Inasmuch as the isolated walls were empty columns (Fig. 1), how were the insoluble elicitor molecules on or in the walls recognized by plant cells? It was observed that drops with wall fragments rapidly acquired a soluble glyceollin elicitor after contact with soybean cotyledons (Fig. 3). Release of the soluble elicitor by the tissue was also more efficient than by autoclaving, a commonly employed method used to release mycelial wall elicitors (2, 3, 5, 13). The release of the soluble elicitor was a relatively rapid reaction since significant elicitor activity was observed in the soluble fraction after only 2 min, and maximum activity was obtained by 30 min (Fig. 4). The released elicitor did not appear to originate from the plant tissue or from autonomous degradation of mycelial walls inasmuch as neither exudates from water-treated cotyledons nor the soluble fraction from wall suspensions that were not incubated with cotyledon tissue showed elicitor activity (Figs. 3 and 4). These results suggested that the elicitor molecule recognized by plant cells may be released from insoluble forms following contact of the fungal cell walls with

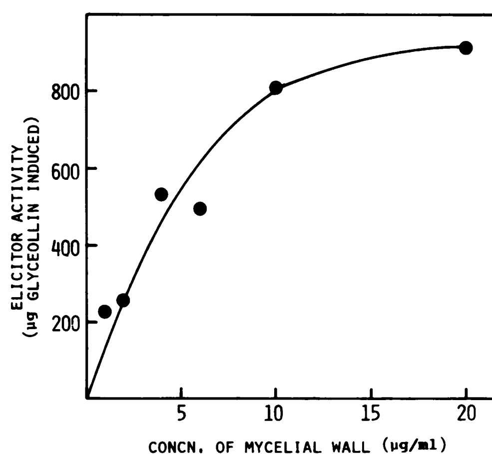

FIG. 2. Elicitor activity of insoluble mycelial walls isolated from *P. megasperma* var. *sojae* when assayed on soybean cotyledons.

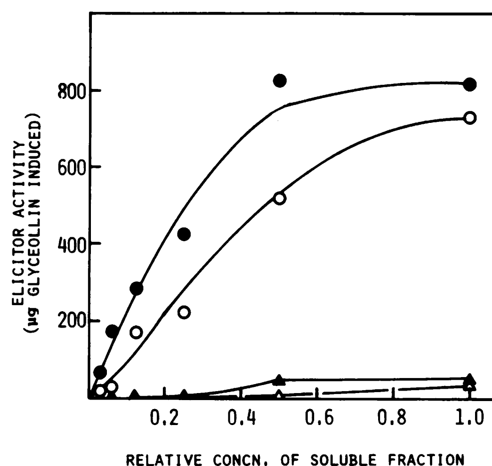

FIG. 3. Elicitor activity of the soluble fractions obtained after incubation of mycelial walls of *P. megasperma* var. *sojae* with soybean cotyledon tissue (●) or after autoclaving the mycelial walls (○). Mycelial wall suspension (500 μg/ml) was incubated with the cotyledon tissue for 1 h or autoclaved at 121 C for 2 h and the soluble fractions were prepared as described. The corresponding soluble fractions from the mycelial wall suspension that was neither incubated with the tissue nor autoclaved (▲) or from water-treated cotyledon exudate (△) served as controls. The final volume of each soluble fraction was adjusted to 6 ml and the relative concentration of the solution was designated by 1.

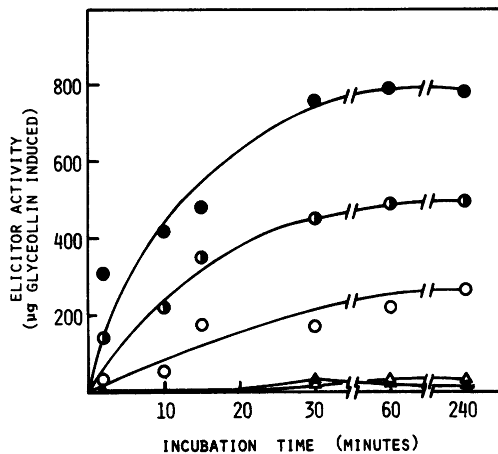

FIG. 4. Time course of the release of a soluble elicitor after incubation of mycelial walls of *P. megasperma* var. *sojae* with soybean cotyledon tissue. The soluble fractions were obtained at various times of incubation and diluted to give one-third (●), one-ninth (◑), and one-twenty-seventh (○) of the relative concentrations shown in Figure 3. The soluble fractions corresponding to the relative concentration of 1 from the mycelial wall suspension that was not incubated with the tissue (▲) or from water-treated cotyledon exudate (△) served as controls.

plant tissue.

Elicitor activity in the released fraction was not destroyed by proteolytic enzymes, nucleases, and cellulase, or by autoclaving (Table I) but was diminished by periodination suggesting that the activity resides with a carbohydrate(s) other than β-1,4-glucan (Table I). The released elicitor was partially soluble in 50% ethanol but not in 70% or higher concentrations of ethanol (data not shown). The released elicitor did not adsorb to a cation exchange resin (Dowex 50, H^+ form) but bound to an anion exchange resin (Dowex 1, OH^- form), indicating that the elicitor molecule is negatively charged (Table I). Sephadex G-50 column chromatography of the released elicitor fraction showed that at least two size classes possessed elicitor activity (Fig. 5). The approximate mol wt were 10,000 or greater for the larger elicitor and 2,000 for the smaller one, based on the fractionation of standard dextrans on the column.

Release of Soluble Elicitor by Cell-free Extracts. Incubation of mycelial walls with cell-free extracts from soybean cotyledons or hypocotyls also resulted in the release of a soluble elicitor (Fig. 6). Kinetics of the release with cell-free extracts were similar to those with living cotyledon tissue, and significant elicitor activity was observed in the soluble fraction after 2 min incubation. The cell-free extracts from cotyledons appeared to contain higher levels of the releasing activity than those from hypocotyls, based on tissue fresh weights. The Sephadex G-50 column chromatographic profile and sensitivity to periodination and various lytic enzymes of the elicitor released by cell-free extracts were also similar to that released by cotyledon tissue (data not shown), suggesting that the released elicitors were similar or identical. The releasing activity in cell-free extracts was greatly diminished by incubation of the extracts at temperatures above 70 C (Fig. 7). About 60% of the releasing activity was precipitated between 30 and 50% saturation of $(NH_4)_2SO_4$, and almost all the activity was precipitated at 70% saturation (data not shown). These results indicated that the factor in the soybean tissues responsible for releasing soluble elicitors from fungal mycelial walls is a protein, possibly an enzyme which attacks native fungal walls.

DISCUSSION

We found that a soluble glyceollin elicitor was released from insoluble fungal mycelial walls upon contact of the walls with

Table I. *Effects of Various Treatments on the Activity of the Elicitor Released from Mycelial Walls of P. megasperma var. sojae by Soybean Cotyledon Tissue*

Elicitor treatment	Elicitor activity Relative concn. of elicitor fraction[a]	
	1/3	1/9
	µg glyceollin induced	
Control (untreated)	816	564
Trypsin[b]	886	616
Protease[b]	884	544
RNase + DNase[b]	872	692
Cellulase[b]	806	460
Autoclaved (121 C, 20 min)	896	612
Periodination[c]	116	44
Periodination control[d]	824	380
Effluent from cation exchange column (Dowex 50, H^+ form)	772	404
Effluent from anion exchange column (Dowex 1, OH^- form)	0	12

[a] The released elicitor fraction was obtained after incubation of wall suspension with cotyledon tissue for 1 h. After various treatments, the fraction was diluted to give the indicated concentrations (see Fig. 3 legend) and assayed for elicitor activity.

[b] The released elicitor fraction was treated with each enzyme at 100 µg/ml in 20 mM Tris-HCl (pH 7.2) for 2 h at 35 C except nuclease treatment to which $MgCl_2$ was added to 2 mM. Each enzyme solution alone at the employed concentrations did not induce glyceollin accumulation in the cotyledon assay.

[c] The released elicitor fraction (0.5 ml) was treated with 2 mM sodium metaperiodate for 4 h at 25 C and the reagent consumed by 0.15 ml ethylene glycol.

[d] Similar to footnote c but sodium metaperiodate was first consumed by ethylene glycol and then incubated with the released elicitor fraction.

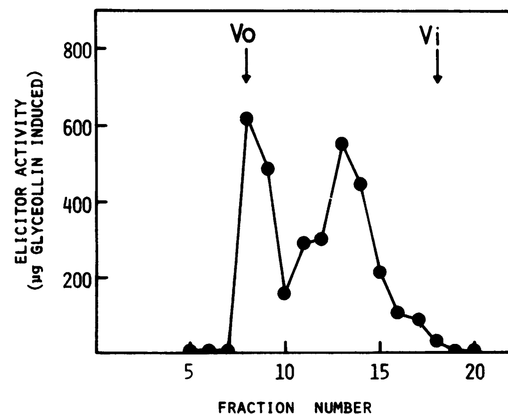

FIG. 5. Sephadex G-50 column chromatography of the soluble fraction obtained after incubation of mycelial walls of *P. megasperma* var. *sojae* with soybean cotyledon tissue. The soluble fraction (5 ml) was applied to a column (1.5 × 60 cm) of Sephadex G-50 and eluted with 10 mM Tris-HCl (pH 7.2). The column void (V_o) and inclusion (V_i) volumes were determined by blue dextran and glucose, respectively.

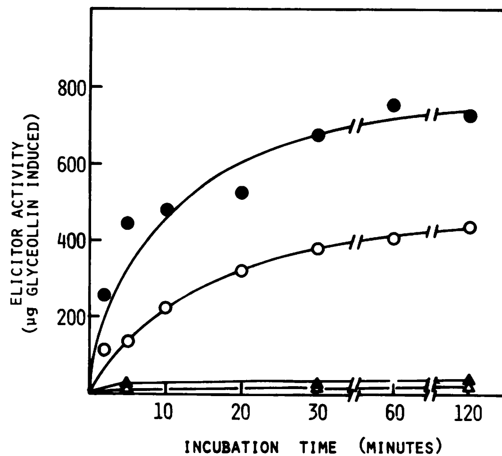

FIG. 6. Release of a soluble elicitor after incubation of mycelial walls of *P. megasperma* var. *sojae* with cell-free extracts prepared from soybean cotyledons (●) or hypocotyls (○). Concentrations of the extracts from both the tissues were adjusted to 1 ml/g fresh weight. The corresponding soluble fractions from the mycelial wall suspension that was incubated with an autoclaved extract from the cotyledons (▲) or an extract that was not incubated with the wall suspension (△) served as controls.

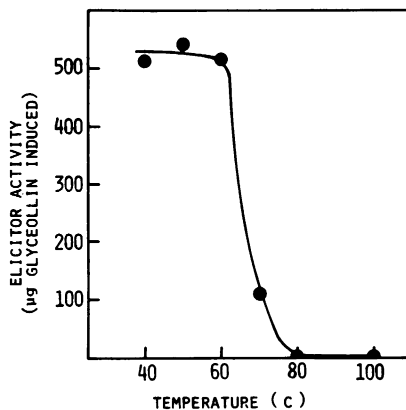

FIG. 7. Temperature stability of the elicitor-releasing activity in cell-free extracts from soybean cotyledons. The extracts were treated with the indicated temperatures for 10 min before incubation with mycelial walls of *P. megasperma* var. *sojae*.

soybean tissues. Probably, the released soluble elicitor is responsible for the initiation of phytoalexin accumulation in soybean tissues treated with mycelial walls. Although unproven, it is also possible that similar release of elicitors occurs in fungus-infected soybean tissues. If correct, this would offer new insight into the early events of recognition occurring between incompatible fungus pathogens and host plant cells.

Despite frequent demonstration that cell wall preparations from various fungi possess potent elicitor activity, there has been doubt whether they, in fact, have a physiological function in host-parasite interactions (11). This is in part due to the fact that most soluble elicitors from fungal walls have been obtained by harsh treatments such as autoclaving (2, 3, 5, 13), which do not occur in biological environments. The present study, however, showed that an active elicitor was released almost immediately after the contact of fungal cell walls with soybean tissues, a condition very similar to the natural infection process. This may accordingly indicate that the soluble elicitors detected here are important in the initial stages of host-parasite interaction.

Ayers *et al.* (2, 3) extracted soluble elicitors from mycelial walls of *P. megasperma* var. *sojae* by autoclaving and fractionated them into four fractions containing either branched β-1,3-glucans or mannan-containing glycoproteins. It is uncertain whether all of these elicitor fractions are recognized by plant cells during the infection process. Although we have not extensively characterized the elicitor(s) released by soybean tissue or cell-free extracts, the available data (Table I) suggest that the elicitor activity is associated with a carbohydrate moiety other than β-1,4-glucan. Further characterization of the released elicitor should disclose which component of the mycelial walls is involved in the initiation of phytoalexin accumulation.

It is unlikely that the soluble elicitor observed in the present study is released from plant tissues (7, 8) rather than mycelial walls, because neither cotyledon tissue exudates (Figs. 3 and 4) nor cell-free extracts (Fig. 6) contained elicitor activity. The soybean tissues were instead found to contain a heat-labile enzyme that attacks mycelial walls to release soluble elicitors. A similar observation was reported recently in pea-*Fusarium solani* interaction (6) while this paper was being submitted. Purification and characterization of the presumed elicitor-releasing enzyme is being conducted.

Acknowledgments—The authors are grateful to N. T. Keen for critically reviewing the manuscript. They also thank J. D. Paxton for kindly supplying soybean seeds.

LITERATURE CITED

1. ALBERSHEIM P, BS VALENT 1978 Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. J Cell Biol 78: 627–643
2. AYERS AR, J EBEL, B VALENT, P ALBERSHEIM 1976 Host-pathogen interactions. X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. Plant Physiol 57: 760–765
3. AYERS AR, B VALENT, J EBEL, P ALBERSHEIM 1976 Host-pathogen interactions. XI. Composition and structure of wall-released elicitor fractions. Plant Physiol 57: 766–774
4. DEVERALL BJ 1972 Phytoalexins and disease resistance. Proc R Soc Lond 181B: 223–246
5. GARAS NA, M DOKE, J KUĆ 1979 Suppression of the hypersensitive reaction in potato tubers by mycelial components from *Phytophthora infestans*. Physiol Plant Pathol 15: 117–126
6. HADWIGER, LA, JM BECKMAN 1980 Chitosan as a component of pea-*Fusarium solani* interactions. Plant Physiol 66: 205–211
7. HARGREAVES JA, JA BAILEY 1978 Phytoalexin production by hypocotyls of *Phaseolus vulgaris* in response to constitutive metabolites released by damaged bean cells. Physiol Plant Pathol 13: 89–100
8. HARGREAVES JA, C SELBY 1978 Phytoalexin formation in cell suspensions of *Phaseolus vulgaris* in reponse to an extract of bean hypocotyls. Phytochemistry 17: 1099–1102
9. KEEN NT 1975 Specific elicitors of plant phytoalexin production: determinants of race specificity in pathogens? Science 187: 74–75
10. KEEN NT 1978 Surface glycoproteins of *Phytophthora megasperma* var. *sojae* function as race specific glyceollin elicitors in soybean. Phytopathol News 12: 221
11. KEEN NT, B BRUEGGER 1977 Phytoalexins and chemicals that elicit their production in plants. Am Chem Soc Symp Ser 62: 1–26
12. KUĆ J 1972 Phytoalexins. Annu Rev Phytopathol 10: 358–374
13. THEODOROU MK, IM SMITH 1979 The response of French bean varieties to components isolated from races of *Collectotrichum lindemuthianum*. Physiol Plant Pathol 15: 297–309
14. YOSHIKAWA M 1978 Diverse modes of action of biotic and abiotic phytoalexin elicitors. Nature 275: 546–547
15. YOSHIKAWA M, H MASAGO, NT KEEN 1977 Activated synthesis of poly(A)-containing messenger RNA in soybean hypocotyls inoculated with *Phytophthora megasperma* var. *sojae*. Physiol Plant Pathol 10: 125–138
16. YOSHIKAWA M, K YAMAUCHI, H MASAGO 1978 Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol Plant Pathol 12: 73–82
17. YOSHIKAWA M, K YAMAUCHI, H MASAGO 1978 *De novo* messenger RNA and protein synthesis are required for phytoalexin-mediated disease resistance in soybean hypocotyls. Plant Physiol 61: 314–317
18. YOSHIKAWA M, K YAMAUCHI, H MASAGO 1979 Biosynthesis and biodegradation of glyceollin by soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol Plant Pathol 14: 157–169