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INCREASED SECONDARY PRODUCT FORMATION IN PLANT CELL SUSPENSION CULTURES AFTER TREATMENT WITH A YEAST CARBOHYDRATE PREPARATION (ELICITOR)

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Key Word Index—Yeast elicitor; suspension cultures; *Glycine max*; *Thalictrum rugosum*; glyceollin isomers; berberine.

Abstract—A carbohydrate fraction isolated from yeast extract by ethanolic precipitation was used as an elicitor to induce secondary product formation in plant cell suspension cultures. The elicitor preparation is effective in inducing glyceollin isomer synthesis (up to 200 μg glyceollin per g dry wt) in cells of *Glycine max* and enhancing berberine biosynthesis (up to four-fold) in cells of *Thalictrum rugosum*. The response of the cell cultures to the elicitor treatment is dependent on the amount of carbohydrate per unit of biomass and on the physiological state of the cells. Cells are optimally induced in late exponential or early stationary growth phases.

INTRODUCTION

Industrial utilization of plant cell cultures for the production of biochemicals has been hampered by low yields of the target substances. At present only one such commercial process has been reported [1]. Shikonin is produced in a two stage process with *Lithospermum erythrorhizon* cells in suspension culture.

In order to improve the yield of product various techniques have been developed for the selection of high-producing plant cell cultures [2 and references therein]. The basis for this selection is somaclonal variation within a cell population. Clones from single cells are selected and subcultured. However, such high-producing cultures often show a decline in productivity upon serial propagation and, therefore, scaling-up may be difficult. Reselections may be required at regular intervals.

Another approach to improve the yield of product in plant cell cultures is the alteration of cell metabolism by external factors (e.g. stress). Cultured plant cells are in principle totipotent and, therefore, any product present in the parent plant should also be synthesized in culture under the right incubation conditions. General methods for the induction of enzymes of secondary metabolism would be extremly valuable. It is well known that certain enzymes of secondary metabolism are induced in higher plants after infection by pathogenic microorganisms [3]. The host–pathogen interaction is most often species-specific and the induced product (phytoalexin) is toxic to the invading organism.

The host–pathogen interactions leading to enzyme induction in plants may also occur in cell cultures [4, 5]. Extracts of mycels or cell walls from the pathogenic microorganism are effective in inducing phytoalexin synthesis in such systems. However, many of the pathogenic organisms used as sources for elicitors are relatively difficult to cultivate on a large scale. Therefore, elicitor preparations which are readily obtained in substantial amounts would be beneficial. Such preparations should if possible also be effective towards different plant cell systems. Recently, we have initiated studies on the induction of enzymes of secondary metabolism in plant cell suspension cultures with an elicitor preparation from yeast extract. We now report on the effects of this glucan preparation on *Glycine max* and *Thalictrum rugosum* suspension cultures which are producing glyceollin isomers and berberine, respectively.

RESULTS AND DISCUSSION

Elicitors isolated from the fungus *Phytophthora megasperma* f.sp. *glycinea* induce phytoalexin synthesis in whole plants and suspension cultures of *G. max* [4–6]. The mechanism behind this induction has been extensively investigated and the enzymology involved has been well characterized. Phenylalanine ammonia lyase (PAL) has been shown to be the key enzyme induced. We have chosen this well characterized elicitor–phytoalexin system for initial studies on an elicitor isolated from yeast extract (*Saccharomyces cerevisiae*). The effects of the elicitor on PAL activity and glyceollin formation in suspension cultures of *G. max* H63 have been investigated. A similar elicitor preparation from yeast extract has been shown to be effective in inducing the phytoalexin synthesis in intact plants [7].

The induced PAL-activity in *G. max* cells is dependent on both elicitor (carbohydrate) and cell concentration (Figs 1 and 2). This is in line with the hypothesis that the elicitor irreversibly binds to receptors on the plant cells. The amount of elicitor added to a culture should preferably be based on the amount of cells (biomass) and not on culture volume. We have chosen to define our elicitor concentrations as μg per mg dry wt or per g fr. wt of cells. The amount of glyceollin isomers synthesized is also

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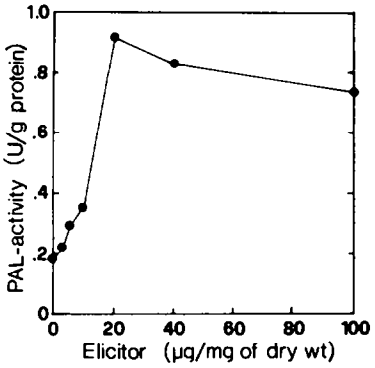

Fig. 1. PAL activity of *G. max* suspension cells as function of elicitor concentration measured 7 hr after addition of elicitor.

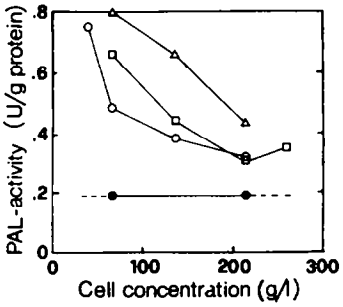

Fig. 2. PAL activity of *G. max* suspension cells as function of cell concentration at different elicitor concentrations (incubation time 7 hr). (—●—) No elicitor; (—○—) 50 μg/ml; (—□—) 100 μg/ml; (—△—) 200 μg/ml.

dependent on the amount of elicitor used for the induction (Fig. 3). Maximal PAL activity and glyceollin synthesis is obtained at a carbohydrate concentration of *ca* 20 μg per mg dry wt. Furthermore, maximal PAL activity and glyceollin isomer concentration within the cells are reached 7–8 and 10–12 hr after induction, respectively (Fig. 3). These induction times are independent of elicitor concentration. Furthermore, PAL activity declines to the same level as that of untreated cells 15–20 hr after addition of elicitor (Fig. 3) These observations are in good agreement with those reported for PAL in soybean suspension cells treated with elicitor from *Phytophthora megasperma* [5].

The glyceollin isomer concentration within the cells also decreases with time (Fig. 3). The major part of it can be refound in the medium but some of the glyceollin appears to be metabolized by the cells. Studies on whole plants have shown that the phytoalexin is quickly synthesized and secreted as a response to infection [3].

A second addition of elicitor 24 hr after the first treatment results in a second induction of PAL (Fig. 4). The somewhat lower maximum PAL activity observed after the second treatment may be due to the increase in biomass observed. The amount of elicitor added was the same for both additions (20 μg per mg initial dry wt of cells). It is interesting to note that the same cell population respond to a repetetive elicitor treatment.

The glucan isolated from yeast extract efficiently induces the synthesis of phytoalexins within suspension cultures of *G. max* (H63) in a similar manner to elicitors

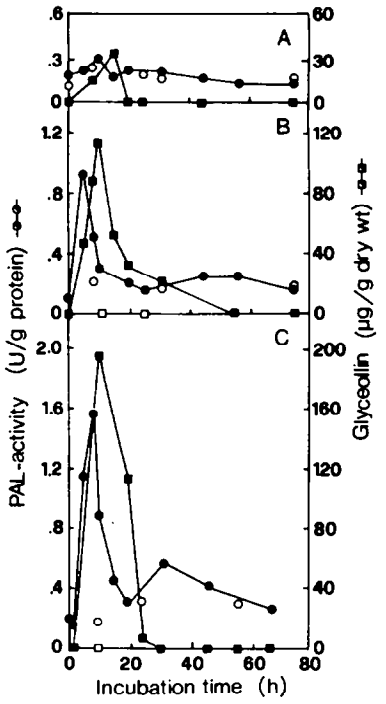

Fig. 3. PAL activity and glyceollin content of *G. max* suspension cells as function of incubation time at different elicitor concentrations. Open symbols = non-induced cells; solid symbols = induced cells. The elicitor concentrations used were in μg/g dry wt (A) 2, (B) 10 and (C) 20. For clarity no line has been drawn between the points representing non-induced cells.

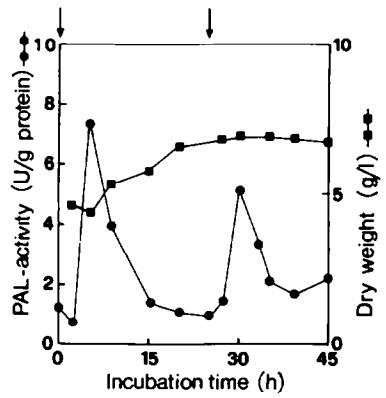

Fig. 4. Repeated induction of PAL in suspension cultured *G. max* cells with yeast elicitor (20 μg/mg initial dry wt). The arrows indicate times for addition of elicitor.

isolated from the natural pathogen *P. megasperma*. Treatment of another suspension culture of *G. max* (Fiskeby) with the yeast elicitor also results in induction of PAL and synthesis of phytoalexins (data not shown). The glucan structure required for elicitor activity has been shown to be a main chain of (1,6)-linked β-D-glucopyranosyl residues with one (1,3)-β-linked glucopyranosyl unit on every second glucose moiety [8]. The smallest active molecule is the glucoheptose. The yeast glucan obviously contains carbohydrates of this structure.

Further experiments have been carried out in order to investigate if the yeast elicitor can induce enzyme synthesis in other cultures. We have chosen to study the effect of the elicitor on the biosynthesis of the benzylisoquinoline alkaloid berberine in suspension cultures of *Thalictrum rugosum*. This biosynthesis does not involve the PAL. Berberine is synthesized from two molecules of tyrosine [9]. Another fundamental difference is that glyceollin is only found in soybean cells after induction while berberine is also present in non-induced *T. rugosum* cells. In addition to this the product (berberine) is of commercial interest.

A typical growth curve of *T. rugosum* in a batch suspension culture (shaker flask) is shown in Fig. 5. The synthesis of berberine appears to be growth associated and as soon as growth ceases (all sucrose consumed) the alkaloid synthesis stops (the concentration being 0.5–0.6 % dry wt). However, if a culture in stationary phase is treated with the yeast elicitor an induced berberine synthesis is observed.

The berberine concentration as a function of incubation time after treatment with various concentrations of elicitor is given in Fig. 6A. Non-treated cells show a constant berberine content (0.5 % dry wt) throughout the incubation period while treated cells show up to a fourfold increase in alkaloid concentration. The initial rate of

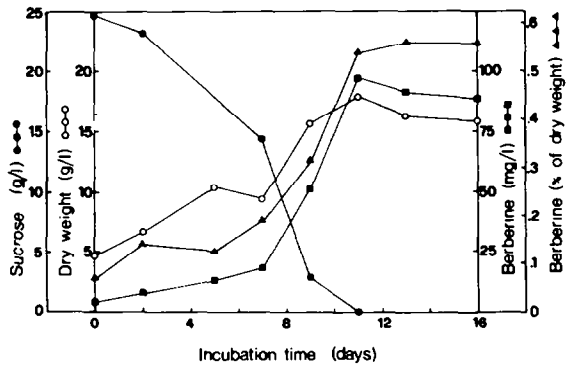

Fig. 5. Typical growth curve of *T. rugosum* in batch suspension culture.

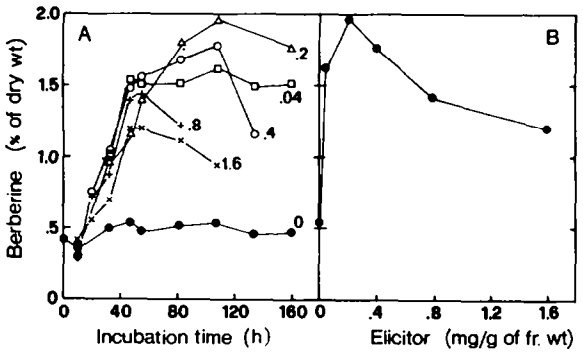

Fig. 6. A. Berberine content in *T. rugosum* suspension cells as function of incubation time after treatment with various concentrations of yeast elicitor. The elicitor concentrations (in mg/g fr. wt of cells) are indicated on each curve. B. Berberine concentration in *T. rugosum* suspension cells as function of elicitor concentration. The maximum berberine concentration for each curve in Fig. 6A has been plotted.

synthesis after induction appears to be independent of elicitor concentration. However, the maximum berberine concentration is highly dependent on elicitor concentration and the optimal concentration is *ca* 200 μg per g fr. wt of cells (Fig. 6B). At higher carbohydrate concentrations lower amounts of berberine are obtained which is most likely due to cell death. A culture treated with a relatively high concentration of elicitor quickly shows signs of necrosis (browning of cells). It is noteworthy that the synthesis of an apparently growth associated product can be induced in cells in stationary phase by treatment with elicitor. Treatment of the cells with the yeast carbohydrate preparation results in induction of one or more enzymes of the biosynthetic pathway of berberine. Further studies on which enzyme(s) is(are) induced are in progress in our laboratory.

Berberine has been classified as a prohibitin (a preinfectional metabolite with antimicrobial activity) [10]. Berberine in roots of *Mahonia trifoliata*, *Cooperia pedunculata* and *Sanguinar iacanadensis* protects these plants from invasion by the fungus *Phymatotrichum omnivorum* [10]. It is likely that berberine in other plant species has a similar function. It is interesting to note that the synthesis of such a prohibitin can be enhanced (at least in culture) by the action of a yeast carbohydrate fraction. It would be of interest to establish if the same glucan structure as described above for *G. max* is responsible for the induction of enhanced berberine biosynthesis in *T. rugosum*.

Cells of *T. rugosum* in different growth stages were treated with elicitor as described in the Experimental section. The results are summarized in Fig. 7. The final dry wt is somewhat lower for elicitor treated cultures (13 g/l.) as compared to an untreated culture (16 g/l.) (Fig. 7A). It is interesting to note that cultures treated with elicitor in stationary phase (treatments at day 10 or 12) decrease in dry wt to *ca* the same level as those cultures treated in exponential growth phase. Sucrose consumption is essentially the same for all cultures (Fig. 7B). Thus, elicitor-treated cells take up energy in the form of sucrose at the same rate as untreated cells even though less biomass is produced. It may be concluded that this energy is used at least partly for berberine synthesis (Fig. 7C). Exceptions are the cultures treated at day 2 or 4 which are not showing any significant increase in alkaloid synthesis.

Respiration of *T. rugosum* cells is also altered after elicitor treatment (Fig. 8). Increased cell respiration is observed indicating an overall enhanced cell metabolism as a response to the elicitor treatment. A relatively good agreement is seen between increased respiration and increased berberine synthesis (cf. Fig. 7C). An exception is cells treated on day 4 which show increased respiration but essentially no enhanced berberine synthesis. However, it may be concluded that the induced alkaloid synthesis is linked to an overall increase in cell metabolism.

From the above experiments (Figs 7 and 8) it is apparent that cells in the early exponential growth phase respond differently from those treated in the late exponential or stationary growth phase. Possible explanations for the differences in berberine synthesis may be that no precursors (e.g. tyrosine) are available in cells in the early growth stage due to high protein synthesis or that the enzyme(s) responsible for the enhanced berberine synthesis is(are) not induced in such cells. Additional studies are in progress to establish the reasons for the observed differences. Furthermore, treatment of cells that have been in stationary phase for some time (day 14) results in a

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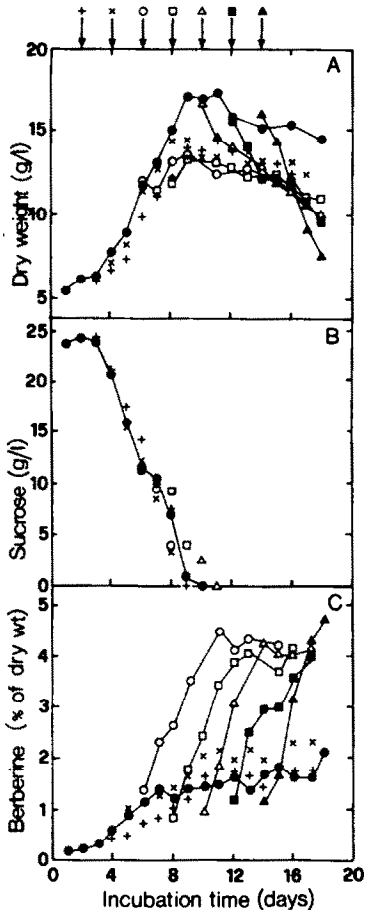

Fig. 7. Effects of yeast elicitor on dry wt (A), sucrose consumption (B) and berberine concentration (C) in suspension cultures of *T. rugosum* as function of incubation time. The arrows (labelled with symbols corresponding to respective curve) indicate time of addition of elicitor (200 μg/g fr. wt). Untreated cells (—●—); cells treated after 2 days (— + —); 4 days (— × —); 6 days (—○—); 8 days (—□—); 10 days (—△—); 12 days (—■—); 14 days (—▲—). For clarity no lines have been drawn between the points representing induction after 2 and 4 days in (A) and (C). Only one line representing the control has been drawn in (B).

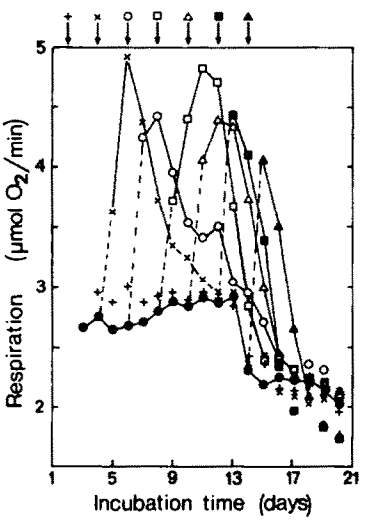

Fig. 8. Effects of yeast elicitor on respiration in suspension cultures of *T. rugosum* as function of incubation time. The arrows (labelled with symbols corresponding to respective curve) indicate time of addition of elicitor (200 μg/g fr. wt). Respiration is given as μmol O_2 consumed/min/g dry wt. Untreated cells (—●—); cells treated after 2 days (— + —); 4 days (— × —); 6 days (—○—); 8 days (—□—); 10 days (—△—); 12 days (—■—); 14 days (—▲—).

rapid decrease in dry wt with relatively limited increase in berberine productivity. Obviously, an 'aged' culture is more sensitive to the elicitor treatment than younger cultures. Some energy is likely to be required to overcome the 'shock' that such a treatment may be to the cells.

The highest berberine content (4–4.5% dry wt) is obtained in cultures treated in late exponential growth phase (day 6 or 8) or early stationary growth phase (day 10) (Fig. 7C). The productivity is also highest in these cultures (*ca* 0.5 g berberine/l. medium). The productivity of an untreated culture is *ca* 0.2 g/l. Thus, the induced cells show a considerable increase in berberine content and productivity.

From a biotechnological point of view the productivity increase after treatment with elicitor is of great interest. In the present study it was possible to obtain twice as much product without extending the cultivation time. However, the final berberine concentrations obtained in these studies are still too low for commercial exploitation. It should be pointed out that no selection for productivity has been attempted in this study. Berberine concentrations of 12.1 and 13.2% of dry wt have been reported for suspension cultures of *T. minus* and *Coptis japonica*, respectively, after selection of high-producing clones [11, 12]. The corresponding productivities were 0.65 and 1.39 g/l. medium. It would be of importance if the productivity of these relatively high-producing cell cultures could be increased by an appropriate treatment with yeast elicitor.

In conclusion, the carbohydrate fraction isolated from yeast extract appears to be effective in inducing phytoalexin production as well as enhancing prohibitin production in plant cell suspension cultures. Further studies on the effects of the carbohydrate preparation on other plant cell cultures are in progress in our laboratory. The optimal conditions for the treatment should be established for each system studied. The amount of carbohydrate added per unit of biomass and the growth stage of the cells appear to be of particular importance. A practical advantage of the yeast carbohydrate over other elicitor preparations may lay in the fact that it has been isolated from a microorganism approved for human consumption.

EXPERIMENTAL

Chemicals. Invertase (150 U/mg), phenylalanine and berberine were supplied by Fluka (Switzerland) and PVP and BSA by Sigma (U.S.A.). Glyceollin isomers (reference substance) were isolated from soybeans infected by mycels from *P. megasperma* according to the procedure previously described [6]. All other chemicals were of analytical grade and were obtained from commercial sources.

Cultivation of cells. Stock suspension cultures were cultivated on a gyratory shaker (120 rpm) in the dark at 26° as follows.

G. max H63 (kindly supplied by H. Griesebach, Freiburg, F.R.G.): B5-medium [13] supplemented with 4.5 μM 2,4-D. Cells (1 g fr. wt) were transferred to fresh medium (20 ml) every 7 days. *T. rugosum* (kindly supplied by J. Berlin, Stöckheim, F.R.G.): MS-medium [14] supplemented with 2 μM 2,4-D. Cells (1 g fr. wt) were transferred to fresh medium (20 ml) every 14 days.

Isolation of elicitor. Elicitor was isolated from yeast extract by EtOH precipitation as described in ref. [7]. The concn of carbohydrate was 5.2 mg/ml in the dialysed extract which was used without further purification for the induction expts.

Induction with elicitor. Elicitor was added at appropriate concns to stock cultures on day 6 and day 13 for *G. max* and *T. rugosum*, respectively. The treated stock cultures were divided into 10 or 20 ml portions in small flasks and subcultivated. One flask was taken at the appropriate time for each set of analyses. Cells of *T. rugosum* were treated with elicitor at different times during growth in the following way. On day 0 three 1 l. flasks each containing 300 ml medium were each inoculated with 15 g fr. wt of cells. On day 2 the three cultures were pooled and divided into nine 100 ml cultures in 500 ml flasks. On days 2, 4, 6, 8, 10, 12 and 14 one of these flasks was treated with elicitor (200 μg/g fr. wt). After addition of the elicitor the culture was divided into ten 10 ml cultures by pipetting in 50 ml flasks. One flask was taken for analysis of dry wt, respiration, sucrose and berberine concns at appropriate times.

Analytical procedures. Dry wt. Samples were collected on preweighed dry filter papers and dried at 60° until constant wt. *Protein concentration.* Determined in cell free extracts according to the Biuret method [15]. BSA was used as standard. *Sucrose.* To medium (100 μl) invertase (20 μl; 20 mg/ml) was added and the mixture incubated for 30 min at 37°. H_2O (880 μl) was added and the glucose concn determined with a glucose analyser (Yellow Springs Instrument Model 23A). *Respiration.* Cells were collected by filtration on a nylon net (50 μm) and subsequently a portion (200 mg fr. wt) was suspended in growth medium (5 ml). The mixture was satd with air and respiration measured with an O_2 electrode (Biometer, B. Braun Melsungen AG). *Phenylalanine ammonia lyase (PAL).* Cells of *G. max* (2 g fr. wt) were homogenized in a Waring blendor for 30 sec in 0.1 M Na borate buffer (6 ml), pH 8.8, containing PVP (80 mg/ml). The extract was stirred on ice for 10 min and then centrifuged at 20000 *g* for 20 min. PAL activity was determined as described in ref. [16]. One unit of activity is defined as the amount of enzyme required for the formation of 1 μmol of product in 1 min under the assay conditons.

Glyceollin isomers. Cells of *G. max* (2 g fr. wt) were homogenized in a Waring blendor for 2 min in EtOH (10 ml). After filtration EtOH was removed and the residue dissolved in H_2O (1 ml) and extracted with EtOAc (3 × 1.5 ml). The combined EtOAc extracts were evapd and the residue dissolved in EtOAc (200 μl). A portion (20 μl) was analysed by densitometric TLC (silica gel; toluene–$CHCl_3$–Me_2CO (8:5:7) at 285 nm (Camag TLC Scanner II). In this system glyceollin I, glyceollin II and glyceollin III appear as one spot, $R_f = 0.46$.

Berberine. Cells of *T. rugosum* (0.5 g fr. wt) were extracted with MeOH (2 × 5 ml) on a magnetic stirrer. A portion (20 μl) was applied to a silica gel TLC plate which was developed in MeOH–HOAc (50:1). The amount of berberine was determined by fluorometric densitometry (exitation at 370 nm) of the spot at $R_f = 0.32$.

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