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A PROCEDURE FOR THE DETERMINATION OF OPTIMAL CHITOSAN CONCENTRATIONS FOR ELICITATION OF CULTURED PLANT CELLS

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Key Word Index—*Nicotiana tabacum*, Solanaceae; *Eschscholtzia californica*, Papaveraceae, chitosan; elicitation; permeabilization, suspension cultures.

Abstract—An experimental method for determination of the optimum chitosan concentration for elicitation of plant cell suspension cultures is presented. The procedure, which is based on measurements of the conductivity of the culture medium after addition of chitosan, has been applied to suspension cultures of *Nicotiana tabacum* and *Eschscholtzia californica*. Increased conductivity of the medium (due to permeabilization of the cells) results in decreased secondary product formation and cell growth. Maximum product formation is observed for cells elicited with the highest chitosan concentration which does not affect membrane permeability

INTRODUCTION

Chitosan (polyglucosamine) is widely distributed in nature. This polycationic polymer appears to be involved as a regulatory molecule in a number of different biological systems Of particular interest is its role in plant–pathogen interactions. Upon infection of a plant by a fungus the host often produces chitinases which may act on the fungal cell wall to liberate chitosan. This chitosan may inhibit the growth of the fungus through inhibition of RNA synthesis or induce gene expression in the host plant [1]. The induction of phytoalexin production [2], lignification [3] and synthesis of proteinase inhibitors [4] are some examples of chitosan-induced plant defence reactions.

Plant cell cultures have been used to study the effects of chitosan on plant cell metabolism. The formation of the phytoalexins glyceollin and acridone alkaloid epoxides in suspension cultures of *Glycine max* [5] and *Ruta graveolens* [6], respectively, has been reported. The wider use of chitosan as an elicitor for the induction of secondary products in plant cell cultures is complicated by the fact that chitosan is also a permeabilizing agent [7, 8] Treatment of suspension cultured cells with relatively high chitosan concentrations lead to cell death, most likely due to the permeabilization of various membranes [9].

Here we describe a method to determine the optimum concentration of chitosan leading to elicitation of cultivated plant cells but not to permeabilization of the cells. Cell suspension cultures of *Nicotiana tabacum* and *Eschscholtzia californica* have been used for these model studies

RESULTS AND DISCUSSION

Extensive studies on the effects of microbial elicitors on the formation of secondary products in plant cell suspension cultures have been carried out during recent years [10 and references therein]. In our laboratory we have used a glucan isolated from yeast extract (subsequently called yeast elicitor) to increase the yield of secondary products (alkaloids) in various cell suspension cultures [11,12]. However, this elicitor has not been effective in inducing secondary metabolism in all cultures tested. Chitosan is believed to be a more generally applicable biotic elicitor [13] As outlined above it is effective in inducing various reactions in plant cell suspension cultures. During our own studies we have noticed that the amount of chitosan added to a suspension culture is critical for the response of the cells. At high chitosan concentrations the cells are permeabilized leading to decreased viability and product formation

Consequently, it is important to determine a chitosan concentration resulting in elicitation but not in permeabilization of the cells for maximum product formation. For this purpose we have developed a simple and rapid method based on measurements of the conductivity of the medium after addition of chitosan If the intracellular conductivity is very close to the conductivity of the medium the procedure described here cannot be employed without modification (e.g. dilution of the medium to reduce the extracellular conductivity) However, cultivated plant cells are generally most sensitive to elicitor treatment in late exponential or in stationary growth stage [11,12]. The conductivity of the medium is at this point relatively low (< 3 mS) as shown in Fig. 1 for the two cell lines used in this study

The effects of chitosan concentration on the medium conductivity in cell cultures of *N. tabacum* are shown in Fig. 2. An increase in conductivity is observed within a certain chitosan concentration interval (i e. 1–3 mg chitosan per g fresh weight of cells). We ascribe this increase in conductivity to the permeabilization of the plasma membrane of the cells leading to leakage of various ions and cell metabolites into the medium. Complete permeabilization of the tobacco cells used in this experiment is observed at a concentration of around 3 mg chitosan per g fresh weight of cells leading to an increase of

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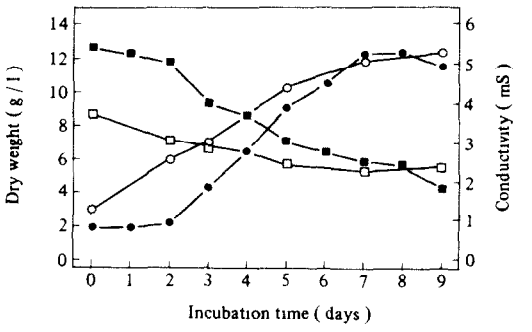

Fig 1 Dry weight of cells and conductivity of growth medium as function of incubation time after subcultivation (○ ●) Dry weight, (□ ■) conductivity Open symbols *Eschscholtzia californica*, solid symbols *Nicotiana tabacum*

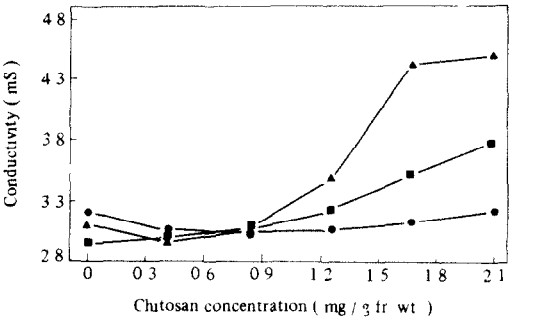

Fig 3 Effects of chitosan on cultures of *N tabacum* The cells were treated five days after subcultivation The conductivity of culture media was determined 30 min after elicitation The cell concentrations were 0 12 (●), 0 24 (■) and 0 48 (▲) g fresh weight of cells per ml medium

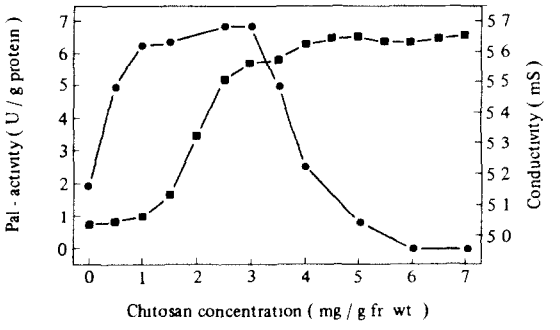

Fig 2 Effects of chitosan on cultures of *N tabacum* The cells were treated two days after subcultivation The cell density was 0 10 g fresh weight per ml Conductivity (■) was measured 30 min after addition of chitosan to the medium PAL (●) was determined in extracts of cells collected 150 min after elicitation

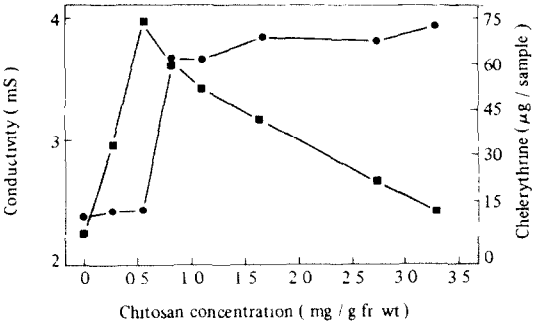

Fig 4 Effects of chitosan on cultures of *E californica* The cells were treated five days after subcultivation The cell density was 0 18 g fresh weight per ml medium Conductivity (●) of the culture medium and total (medium + cells) chelerythrine content (■) were determined 0 5 and 8 hr after elicitation, respectively

medium conductivity of around 0.5 mS. Addition of chitosan at corresponding concentrations to medium after the removal of cells does not result in any significant change of conductivity. The plasma membrane appears to be unaffected by chitosan at concentrations below 1–1.5 mg per g cells

Phenylalanine ammonia lyase (PAL) is involved in secondary metabolism (initial enzyme of the general phenylpropane pathway) of plants [14] and it has been used in this study as a marker enzyme of induced secondary metabolism. Chlorogenic acid and scopolin are examples of products from the phenylpropane pathway that have been isolated from these tobacco cultures [Gugler and Brodelius, unpublished]. As can be seen from Fig 2 the enzyme is already maximally induced at a concentration of 1 mg chitosan per g fresh weight of cells At this chitosan concentration no effects on membrane permeability can be observed. However, at higher chitosan concentrations, leading to complete permeabilization of the cells (>3 mg/g cells), a decreased PAL activity is observed.

It appears important to define the amount of chitosan added on basis of the biomass within the culture and not on basis of the culture volume Permeabilization of cells occurs at a defined ratio of chitosan to biomass (*ca* 1 mg chitosan per g fresh weight of cells for the tobacco culture) independent of cell concentration in the culture as indicated in Fig. 3

The rate of permeabilization of suspension cells by chitosan is similar to those reported for the permeabilization of different suspension cultured plant cells by various chemicals (data not shown) [9] The relatively rapid changes in membrane permeability allow the determination of optimum chitosan concentrations for elicitation according to the method presented here within less than one hour

Treatment of suspension cultures of *Eschscholtzia californica* with the yeast elicitor leads to a decreased conductivity of the medium [12] indicating an uptake of ions from the medium Furthermore, the yeast elicitor is efficient in inducing the formation of various benzophenanthridine alkaloids in this culture [12] Sanguinarine and chelerythrine are formed within a few hours after elicitor addition, while the end product macarpine is produced at a later stage [12] The effects of chitosan on the synthesis of these benzophenanthridine alkaloids has been investigated The formation of chelerythrine as function of chitosan concentration is shown in Fig. 4 The amount of alkaloid was determined 6 hr after addition of chitosan to the medium Maximum alkaloid formation is observed at a chitosan concentration resulting in no permeabilization of the cells (i e around 0 5 mg/g cells). At

higher chitosan concentrations (> 1 mg/g cells) the cells are permeabilized, as reflected by the increased conductivity of the culture medium, and the amount of alkaloid produced is decreased. At relatively high chitosan concentrations no increase in product formation is seen.

The effect of chitosan on the growth of *Eschscholtzia* cells has been investigated. Fresh medium was added to treated cells 6 hr after addition of chitosan (Fig. 5). At moderate chitosan concentrations (< 1 mg/g fresh weight) a somewhat lower final dry weight (4.5 to 5 g/l) is obtained as compared to untreated cells (7 g/l) while at high chitosan concentrations a considerable decrease in biomass (to *ca* 3 g/l) is observed. This represents a very limited growth since the dry weight of the inoculated cells was 2 1 g/l. The growth pattern of *E. californica* cells treated with the yeast elicitor is somewhat different from that observed for chitosan treated cells [12]. At moderate elicitor concentrations an increase in final dry weight was observed, while at higher elicitor concentrations only a moderate reduction in final dry weight (around 5 g/l) was obtained.

The production of macarpine is shown in Fig. 5, the maximum alkaloid formation is seen at a chitosan concentration which does not affect the plasma membrane (i.e. 0.5 mg/g cells). It may be pointed out that the maximum yield of macarpine was 6 mg/g dry weight in cultures treated with the yeast elicitor [12], while chitosan-treated cells yielded 7 mg/g dry weight of cells. When used at an appropriate concentration, chitosan is as efficient as the yeast elicitor in inducing alkaloid formation in suspension cultures of *E. californica*. Essentially no induction of alkaloid formation is observed for permeabilized cells (≥ 1 mg chitosan per g cells).

In contrast to the results reported here, chitosan has been reported not to be effective in eliciting benzophenanthridine alkaloid formation in another cell suspension cultures of *E. californica* [15]. No experimental details were reported and therefore it is difficult to explain this difference. However, a possible explanation may be that a permeabilizing concentration of chitosan was used in the latter case. It may be pointed out that the two *Eschscholtzia* cultures respond similarly to elicitation by the yeast elicitor [12,15].

Chitosan is considered to be a generally applicable elicitor [13]. However, a number of reports can be found in the literature stating that chitosan has not been effective in inducing secondary metabolism in plant cell cultures [15–17]. A possible reason for these negative results may be that the elicitation experiments were carried out under permeabilizing conditions The procedure presented here has been tested on a number of other cell cultures (the results will be reported elsewhere) and in our experience it appears to be generally applicable for the determination of elicitation conditions. These experimental conditions may be established within less than one hour with the method presented here.

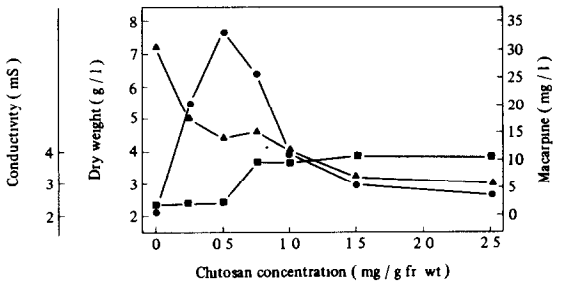

Fig. 5 Effects of chitosan on cultures of *E californica*. Cells were harvested five days after subcultivation by filtration and cells (2 0 g fresh weight) were suspended in the collected medium (20 ml) Conductivity (■) of the culture medium was measured 30 min after addition of chitosan. Fresh medium (30 ml) was added to the treated cells after 6 hr The cultures were harvested after six days and dry weight (▲) and total macarpine content (●) were determined

EXPERIMENTAL

Chemicals Chitosan was from Chugai Boyeki (Japan). Gamborg's B5 and Murashige and Skoog media were from Flow Laboratories (Scotland).

Culture conditions Stock cultures of plant cells were incubated on gyratory shakers (120 rpm) in the dark at 26° in the following media: *N tabacum* (Wisconsin 38) MS-medium [18] supplemented with 0.22 mg/l 2,4-D and 3% w/v sucrose Cells were transferred to fresh medium every 7 days at an inoculum density of 7.5 g fresh weight per 100 ml culture medium *E californica*: B5 medium [19] supplemented with 1 mg/l 2,4-D, 0.1 mg/l kinetin and 2% w/v sucrose. Cells were transferred to fresh medium every 7 days at an inoculum density of 7.5 g fresh weight per 100 ml culture medium

Preparation of chitosan solution A stock solution of chitosan was prepared by adding 500 mg of the polymer to 25 ml of H_2O As the chitosan dissolved the solution was titrated with HCl to give a final pH of 6 0 The total volume was then adjusted to 50 ml with H_2O This stock solution was sterilized by autoclaving at 121° for 20 min

Treatment of cell cultures with chitosan Elicitor concentration dependence stock cultures were divided into 10 or 20 ml portions in small flasks The amount of cells (fresh weight) was determined. Chitosan was added at different concentrations to a set of flasks The treated cells and medium were separated by filtration after 30 min for analysis *Cell concentration dependence.* three cell concentrations were obtained by diluting (1 : 1 with medium) and concng (to half the volume by removing medium) stock cultures. Chitosan was added at different concns to sets of flasks After 30 min the cells were removed by filtration and the conductivity of the medium was measured *Time course* chitosan was added at appropriate concentrations to stock cultures The treated stock cultures were divided into 10 ml portions in small flasks One flask was taken at the appropriate time for analysis

Analytical procedures. Fresh weight cells were collected by filtration under reduced pressure onto a nylon net (50 μm). *Dry weight.* samples of known fresh weight were dried at 50° on preweighed Al foil until constant weight. *Conductivity* determined immediately after removal of cells by filtration with a conductivity meter (Orion Research, U S A) *PAL activity*: PAL activity was determined in cell free extracts as previously described [11] *Alkaloid extraction* and *quantitation* alkaloid extraction and analysis were carried out as described elsewhere [12]

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