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INFLUENCE OF ELICITOR AVAILABILITY ON LIMONENE AND LINALOOL ACCUMULATION FROM *CITRUS GRANDIS* CELL CULTURES

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Suspended callus cultures of Citrus grandis were elicited with chitosan, a polycationic polimer and also a permeabilizing agent. The procedure, which is based on measurements of the conductivity of the culture medium after addition of chitosan ranging from 0.5 to 7.0 mg per g fresh weight callus, has been applied to modified Murashige and Skoog (MS) medium. Low concentration of chitosan (0.5 mg/g fr.wt.) stimulate limonene production and at the same time increase linalool content. Maximum limonene and linalool accumulations were observed from cultures elicited with 1.0 mg chitosan/g fr. wt. callus incubated for 2 hours. Chitosan successfully influenced limonene and linalool accumulation in a short period and not to permeabilization of the cells.

Keywords: *Citrus grandis, Chitosan, Elicitation, Limonene, Linalool.*

INTRODUCTION

Limonene and linalool are among the active terpenes and major constituent of citrus essential oils. Limonene, the principal component of orange peel oil, has been identified as a non toxic agent with potential for cancer chemotherapy (Hardcastle *et al.* 1999). The used of limonene and linalool in skincare treatment, aromatherapy treatment and as effective insecticidal were reported elsewhere (Braddock and Cadwallader 1995, Gabrielyan *et al.* 1992). Growing interest towards these compounds resulted on extensive study in producing maximal limonene and linalool via biotechnology techniques.

An attempt has been made to produce plant valuable compounds by adding precursors to several culture species. There are several other manners to influence bioconversion capacities of cells, e.g. elicitation (Kurtz *et al.* 1987), permeabilization (Felix 1982), irradiation (Galun *et al.* 1985) or other forms of stress, such as pH and osmotic shock. Among those techniques, elicitation has been shown to be very effective in

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influencing secondary metabolites production in cell cultures. Elicitation is adapted from plant defence mechanism that leads to activation of antimicrobial metabolite production. Chitosan, a polycationic polimer and also a permeabilizing agent, has been shown to have elicitor effects in a number of secondary product studies (Kohle *et al.* 1984).

Reported on the addition of soluble chitosan to suspension-cultured *Glycine max* cells shows an inhibition on the rate of increase in cell fresh weight, increased their glyceolin content and altered their cell wall composition (Kohle *et al.* 1984). A positive effect on the production of anthracena derivatives was exerted in particular by chitosan (1 mg/30 ml) elicitation of *Rheum palmatum* L. suspension and callus cultures (Kasparová and Siatka 2001). Similar results have been reported by Gagnon and Ibrahim (1997) for roots of white lupin seedlings when exposed to chitosan resulted in dramatic increase in the amount of genistein and 2-hydroxygenistein monoprenyls as well as major increase of all isoflavonoids in the exudates.

The impact of elicitors on *Citrus spp.* cultures on terpenoid production has not been extensively studied. Therefore, the possibility of increasing the accumulation of limonene and linalool by adding elicitor on interest, chitosan, is studied. The objective of the investigation reported here was to establish a strategy to improve limonene and linalool productivity in *C. grandis* cell culture.

METHODS

Plant Material, Media Preparation and Callus Initiation

Young *C. grandis* fruits about 4-5 cm in diameter were obtained from established plantation at Bukit Selambau, Kedah. Sterilization technique was established by immersing the whole fruit into 20% Clorox® for 2 hours prior peeling and cutting. Callus was initiated on Murashige and Skoog (1962)(MS) salts with 3x phosphate and supplemented with 30 g/l sucrose, 3 mg/l 2,4-D, 3 mg/l kinetin and 0.2 mg/l ABA based on Jenimar (2001) for optimum callus growth. Culture was incubated in the dark for 24 hours at room temperature (25 ± 2°C) for about a month before calli were ready to be used for elicitation study.

Elicitor Preparation

A 500 mg of chitosan (Fluka, Japan) was first dissolved in about 30 ml of glacial acetic acid and the solution was titrated with 1 N NaOH to give a final pH of 5.7. The stock solution was sterilized by autoclaving at 121°C, 15 psi for 20 minutes.

Determination of Optimum Concentration of Chitosan

About 30 g of calli from stock cultures were cleaned from attached media and chopped into small pieces. Those calli were inoculated into liquid media at an inoculum density of 10 g fresh weight per 100 ml culture media. The suspended callus were incubated in the dark 24 hours a day at room temperature (25 ± 2°C) for 2 days in incubator shaker (Microtech, USA) at speed of 120 rpm to allow adaptation before adding chitosan. The cultures were divided later on into 20 ml portions and the weight of suspended callus was determined by pouring out the media. Each portion of suspended callus was added into 20 ml fresh media in a 150 ml flask. Chitosan was added into fresh media at different concentrations ranging from 0.5 to 7.0 mg per g fresh weight callus. The treated cultures were incubated in the dark 24 hours a day at room temperature (25 ± 2°C) for 2 days in incubator shaker at speed of 120 rpm.

Determination of Optimum Incubation Period with Chitosan

Chitosan with optimum concentration study was added into the suspended cultures and incubated in same condition at different incubation period of 0.5, 1, 2, 3, 4 and 5 hours.

Conductivity Determination

Conductivity of media was determined immediately after removal of callus by filtration using conductivity meter (Hach, USA).

Limonene and Linalool Determination

Extraction of limonene and linalool were carried out for cultures regarding to appropriate chitosan concentration and incubation period. Treated calli of *C. grandis* (2.0-3.0 g) were extracted with methanol (70 ml) using soxhlet apparatus according to Morris *et al.* (1985). Quantification of limonene and

linalool were made by gas chromatography (Shimadzu GC-17A) equipped with FID detector. Column used was BP-20 (0.25 µm, 30 m x 0.25 mm i.d.) from SGE, Florida, USA. The experiments were performed in triplicates and data were subjected to analysis of variance (ANOVA) at P = 0.05 for mean comparison using SPSS Software ver. 10.0.

RESULTS AND DISCUSSION

Effects of Chitosan Concentration and Incubation Period on Media Conductivity

The effects of chitosan concentration on the media conductivity in suspended cultures of *C. grandis* was shown in (Fig. 1). Three stages in conductivity profile were observed within added chitosan concentrations ranging 0.5-7.0 mg/g fr. wt. The first stage is between 0.5 to 1.5 mg chitosan/g fr. wt. callus exhibited constant value in conductivity; the second stage is between 2.0 to 5.0 mg chitosan/g fr. wt. callus showed gradually increase in conductivity and the final stage is between 5.0 to 7.0 mg/g fr. wt. demonstrated again a constant value in conductivity. Addition of chitosan at a concentration of around 5.0 mg chitosan/g fr. wt. callus leading to an increase of media conductivity of around 11 mS. Further addition of chitosan at corresponding concentration did not result in any significant change of conductivity. Meanwhile results from Figure 2 shows the effects of elicitation as a function of incubation period on changes in media conductivity. From the observation, there was an increase in conductivity within 0.5 to 2 hours time interval. When elicited at longer period, conductivity value seems to be maintained at constant level.

From the result it was found that chitosan at concentrations 1.5 to 5.0 mg/g fr. Wt. had affect cell membrane permeability. This phenomenon was shown by increased in media conductivity within the concentration range. This is due to leakage of various ions and cell metabolites into the media effect from chitosan on plasma membrane permeability. Completed permeabilization of the *C. grandis* cells was observed at concentration of 5.0 mg chitosan since no significance change of conductivity was obtained with addition of higher chitosan concentration.

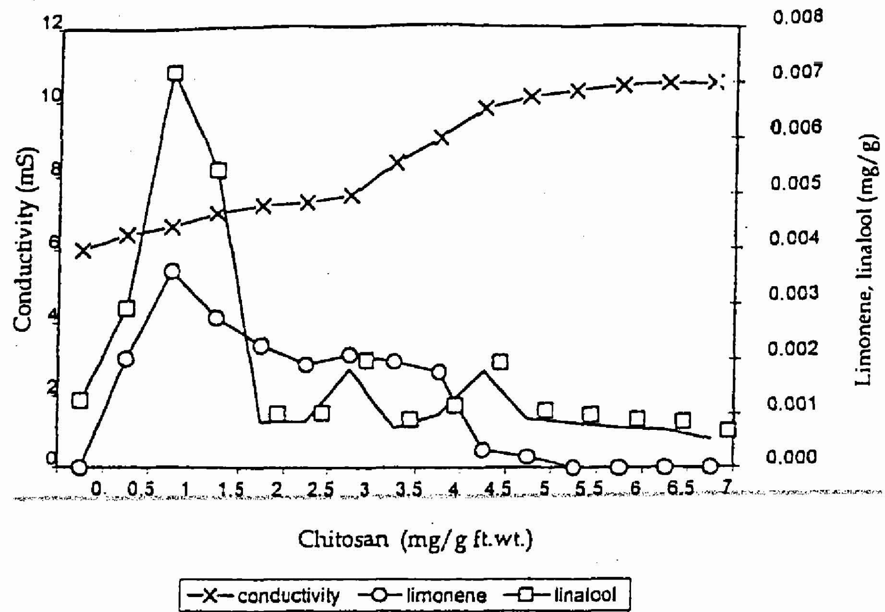

Fig. 1: Effects of chitosan concentration on conductivity, limonene and linalool accumulation in *C. grandis* cell cultures.

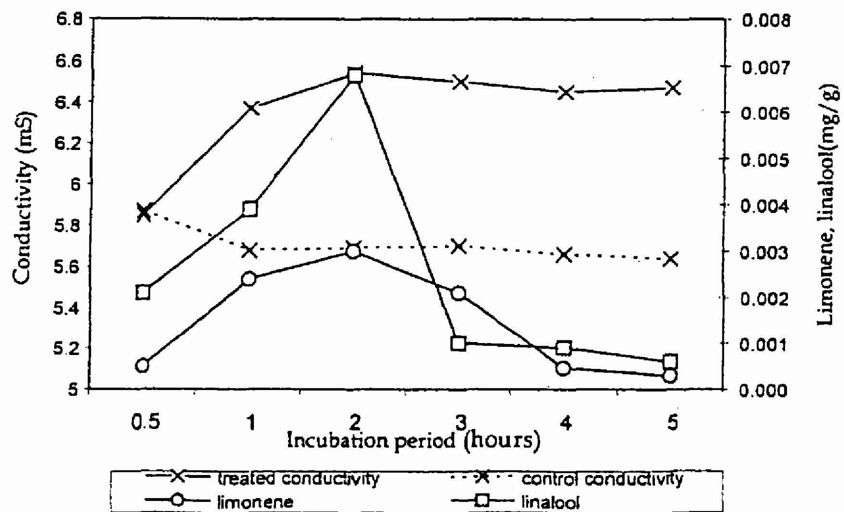

Fig. 2: Effects of 1.0 mg/g chitosan on conductivity limonene and linalool accumulation as a function of incubation period in *C. grandis* cell cultures.

Chitosan has the ability to increase plant tissue membrane permeability due to its polycationic nature, and also binds to galacturonate, a component of plant cell wall that permits leakage of electrolytes, protein and UV absorb-material (Young *et al.* 1982). In this regard it can be suggested that the addition of 1.0 mg chitosan/g fr. wt. callus leads to elicitation of cultured *C. grandis* cells but do not permeabilize the cells. An opposite results in conductivity was observed when culture elicited with 1.0 mg chitosan per g fresh weight as a function of incubation period ranging from 0.5 to 5 hours. In this case, elicited cultures are suppose to show an equal conductivity as compared to control cultures. However, low conductivity was observed for the control and the reduction in the conductivity of the media for control cultures reflects nitrate assimilation from media into the cells is in agreement to the suggestion of Hahlbrock *et al.* (1974).

Conductivity of the media can be related to cell viability for completed cell permeabilization. This can act as indicator of damaged in plant membrane systems. It was concluded that short-term incubation with chitosan could maintain cell viability for subsequent limonene production. Successful permeabilization requires efficient release of the desired product while retaining cell viability (Parr *et al.* 1986). Same problems has been experienced by Beaumont and Knorr (1987) when acid-soluble chitosan were used leading to subsequent release of intracellular products as well as a decrease in cell viability of *Catharanthus roseus* cells with increasing chitosan concentration (Köhle *et al.* 1984). The detrimental effect of acid-soluble chitosan and cell viability could be reduced by the utilization of the available water-soluble chitosan salts (Beaumont and Knorr 1987).

Effects of Chitosan Concentration and Incubation Period on Limonene Accumulation

The accumulation of limonene as a function of chitosan concentration is shown in (Fig. 1). The amount of limonene was determined 2 hours after addition of chitosan to the media. There was a sharp increase to the maximum limonene formation in the presence of chitosan at 1.0 mg chitosan/g fr. wt. callus. At higher concentrations (> 1.0 mg/g fr. wt.) the amount of limonene accumulated declines to the same level as that of untreated callus.

Limonene accumulation was analysed from elicited callus within a period of time after inoculation (Fig. 2). It was obvious that limonene production was influenced as soon as the elicitor was introduced in the media. This production was continuously increased within the period of 0.5 to 2 hours. Maximum accumulation occurred around 2 hours after elicitation. Further incubation with chitosan led to a reduction of extractable limonene content.

The effects of elicitation on limonene accumulation in cells of *C. grandis* using chitosan have been investigated in this study. Chitosan influenced limonene accumulation at a time earlier than to those in untreated cultures. Chitosan at low concentration had triggered apparently high limonene concentration in a relatively short period of time. Elicitation is a process to introduce stress condition to plant tissue and as consequences of plant hypersensitivity response to stress by producing phytoalexins. The sensitivity of cells to elicitor is important to determine success eliciting effects (Lu *et al.* 2001). It is clear that *C. grandis* cells were very sensitive to traces of chitosan which can result in increase limonene production. Maximum limonene accumulation at concentration of 0.0036 mg/g was achieved by induction with 1.0 mg chitosan/g fr. wt. callus.

In many instances, rate of limonene accumulation due to the activity of an elicitor is probably related to the stress it causes to the cells in culture. Consequently, increased in total limonene content was achieved appropriated to different concentration of elicitor used. At concentration higher than 1.0 mg/g fr. wt. callus, limonene accumulation was low resulted from the leakage of plasma membrane. The effects of chitosan in *C. grandis* cell cultures was similar to alkaloid formation on cultures of *Eschscholtzia califormica* (Brodelius *et al.* 1989).

Limonene obtained from this study was rather low compared to extracted from intact plant (Braddock and Cadwallader 1995). There are many factors could possibly contribute to low phytoalexins production in cultured cells. One of those is the occurrence of catabolism phenomenon in which the accumulated compounds are broken down into their intermediates (Whitehead and Threlfall 1992). The de novo synthesis and release of extracellular enzymes, e.g. peroxidases was identified to be responsible in phytoalexins catabolism as demonstrated in freshly subcultured cell suspension cultures of *Nicotiana tabacum* (Mäder and Walter 1986). The similar agreement was reported by Moreno *et al.* (1996) to explain the lack of induction of indole alkaloid accumulation after elicitation in *Catharanthus roseus* cell suspension cultures.

Other factor contribute on restricted limonene accumulation is the availability of certain endochitinases in plant tissues that can possess chitosanase activities (Mayer *et al.* 1996). Naturally, chitosanase hidrolyse chitosan, which is a component of fungal cells wall upon infection. Therefore, it is suggested that those enzymes had hydrolysed certain amount of added chitosan resulted on relatively low elicitation effect in *C. grandis* cultures.

Effects of Chitosan Concentration and Incubation Period on Linalool Accumulation

Upon induction with low concentration of chitosan, it was found that the concentration of detactable linalool was increased (Fig. 1). Linalool content reached maximum concentration when elicited with 1.0 mg chitosan/g fr. wt. callus and rapidly decrease to about the same level as that of untreated callus. During reduction, fluctuated level in linalool content was observed at several points before steadily declining at corresponding concentrations. Effects of various incubation period with chitosan on linalool accumulation is shown in Figure 2. An introduction of chitosan in the media had influenced linalool accumulation at a considerable level from 0.5 to 2 hours. The reduction in linalool content can be seen after 2 hours of elicitation.

Cultures depicted a similar pattern on limonene and linalool accumulation throughout 5 hours incubation period. Both compounds increased within the first 2 hours incubation period and reduced after the following hours. This profile was contradicted to that reported by Attaway *et al.* (1967) and Kekelidze *et al.* (1989) where an inverse relationship between limonene and linalool accumulation was obtained during the same time interval. These authors observed a remarkable trend where a marked decreases in linalool corresponding an increase in limonene in orange and tangerine peel oils. The reasons was not clear and unexplained but one possible reason is that such treatment could lead to substantial change in the cellular metabolism, where cells could immediately modify their metabolic reactions to the fungal attack (Moreno *et al.* 1996).

CONCLUSION

The objective of this work was to review the effect of elicitation on limonene and linalool accumulation in *C. grandis* suspended callus.

Permeabilization of membrane cells by using chitosan at higher than 1.0 mg/g fr. wt. callus was to take into consideration of its permeability effects. Changes in conductivity directly relate to changes in total amount of extractable limonene and linalool. Stress response of *C. grandis* cell cultures to elicitor was observed immediately to occur with the addition of chitosan, reflected by the accumulation of limonene and linalool at the early of incubation period. Cell cultures showed great sensitivity towards elicitation by producing limonene and linalool when supplemented with chitosan at concentration as low as 0.5 mg/g fr. wt. callus. The use of chitosan at higher concentration and at a long term of incubation resulted on decrease in limonene and linalool accumulation.

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