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박하세포의 현탁배양에 대한 Fungal Elicitor, Pluronic F-68과 Methylcellulose의 영향

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Effect of Fungal Elicitor, Pluronic F-68 and Methylcellulose on Suspension Culture of *Mentha piperita* Cells

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ABSTRACT

The effect of fungal elicitor, Pluronic F-68 and methylcellulose on suspension culture of *M. piperita* cells was investigated in shake flasks. About a two-fold increase in oil production was observed in response to the treatment of the fungal elicitor prepared from *Rhodotorula rubra*. Low concentration of Pluronic F-68 or methylcellulose enhanced peppermint cell growth at 100 rpm of agitation.

INTRODUCTION

In cultures of *Mentha piperita*, it has been reported that some cell lines can synthesize essential oils (1). Some efforts have been made to produce essential oil in callus or cell suspension culture (2, 3) and the effects of many culture conditions such as carbon source, exposure time to light, initial seeding density, pH, agitation and hormone concentration on cell growth and essential oil production have been investigated (4, 5, 6); however, some problems still remain unresolved in improving the peppermint oil production.

It is widely accepted that microbial invasion of higher plants leads to the synthesis of secondary metabolite. This microbial attack can be mediated by virus, bacterial or fungal contact. Although many molecules act as elicitors or inducers of secondary metabolism, fungal elicitors are the most important for improving secondary metabolite production (7, 8, 9).

Pluronic F-68 and methylcellulose have been extensively used in insect cell culture to protect against mechanical damage by sparging (10, 11). While there is growing interest in the effects of Pluronic F-68 and methylcellulose on insect cells, there has been few reports of corresponding studies with plant cells (12).

Therefore, in this study the effect of fungal elicitor, Pluronic F-68 and methylcellulose on suspension culture of *Mentha piperita* cells was examined using shake flasks.

MATERIALS AND METHODS

Cell Line and Seed Culture Preparation

Peppermint cell line was derived from the leaves of *Mentha piperita*. The basic medium was Lin-Staba (LS) medium supplemented with 0.2 mg 2, 4-dichlorophenoxyacetic acid and 20 g sucrose per liter. The cells were subcultured every 12 days into 200ml of baffled Erlenmeyer flasks containing 50ml of the liquid medium and incubated at 27℃ in the white fluorescent light for 16 hrs per day on a gyratory shaker at 100 rpm. The initial pH of the medium was adjusted 5.7 before autoclaving.

Suspension Culture Operation

200ml baffled shake flasks with a working volume of 50ml were used for the experiments. The temperature was controlled at 27℃ and agitation speed was 100rpm.

Fungal Elicitor Preparation

Alternaria mali, *Rhodotorura rubra*, *Aspergillus niger*, *Chatomium globosum*, *Fusarium moniliforme* and *Rhizopus arrihizus* were obtained from Korean Collection of Type Culture(KCTC) and Korea Culture Center of Microorganism (KCCM). All of fungi were transferred to 50ml of YEB medium and incubated at 26℃, 100 rpm. After 6 days, the mycelia were homogenized in its medium by the homogenizer(Yamato, Japan) at 20000 rpm for 10 min. and subsequently autoclaved at 121℃, 15 psi for 30 min. and filtered using Whatman paper No. 1. The filtrates were stored at -5℃, used as elicitor without further purification for the experiments and the concentration of carbohydrate component of elicitors in all experiments was about 3mg carbohydrate per liter culture medium.

Biomass, Sugar and Essential Oil Analyses

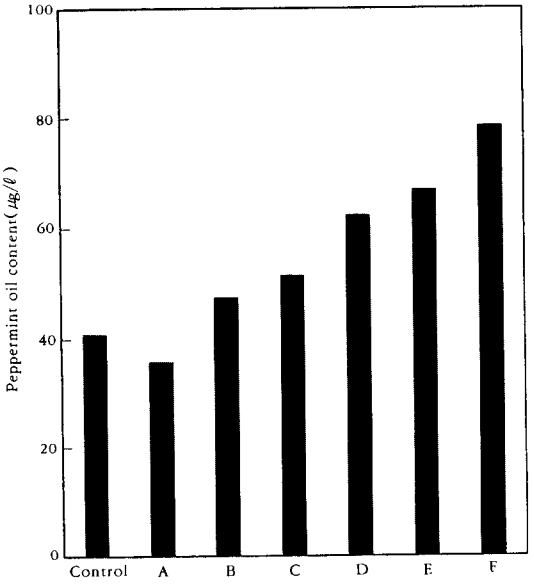
The cell suspension was centrifuged in a 15ml graduated tube at 3000 rpm for 20 min. and the percentage of cell volume after centrifugation was considered as the packed cell volume (PCV). The concentration of carbohydrate in the elicitors was determined by orcinol-sulphuric acid method (13). Essential oil analysis is as follows: cells were separated from culture broth after filtration. Cells were washed with dd H_2O, added with 50ml dd H_2O, and homogenized by the homogenizer at 20000 rpm for 10 min. Cells or culture broth was extracted for 2 hrs using pentane-dichloromethane 2:1(v/v) in order to analyze intracellular oil or extracellular oil content. Peppermint oil content was measured by a gas chromatograph (GC) equipped with a flame ionized detector. The GC conditions are as follows: injection volume, 1 μl; fused silica capillary column coated with SE-30, 15 m x 0.54 mm I.D.; oven temperature 80℃(2 min), 80℃-200℃(at the increasing rate of 5℃/min), 200℃-240℃(at 20℃/min), 240℃(3 min). Oil components were identified as limonene, menthone, menthol, isomenthol, pulegone and menthylacetate with an authentic standard.

RESULTS AND DISCUSSION

Effect of Fungal Elicitor on Peppermint Oil Production

A series of experiments were conducted to determine whether peppermint oil production varied with different fungal elicitors from *Alternaria mali*, *Rhodotorura rubra*, *Aspergillus niger*, *Chatomium globosum*, *Fusarium moniliforme* and *Rhizopus arrihizus*. 14-day-old peppermint cells were treated with prepared elicitors for 48 hrs and peppermint oil contents were determined. As shown in Fig. 1, maximum accumulation of peppermint oil was observed when the elicitor from *R. rubra* was used. The formation of peppermint oil in elicitor-treated culture was increased 1.7 fold than that of control. The amount of accumulation for the four other elicitors was still better than that for the control.

In a preliminary experiment, *M. piperita* cells were treated with fungal elicitors from *R. rubra*



A: *R. arrihizus* B: *F. moniliforme*
C: *C. globosum* D: *A. mali*
E: *A. niger* F: *R. rubra*

Fig. 1. Effect of various fungal elicitors on pepperminit oil production.

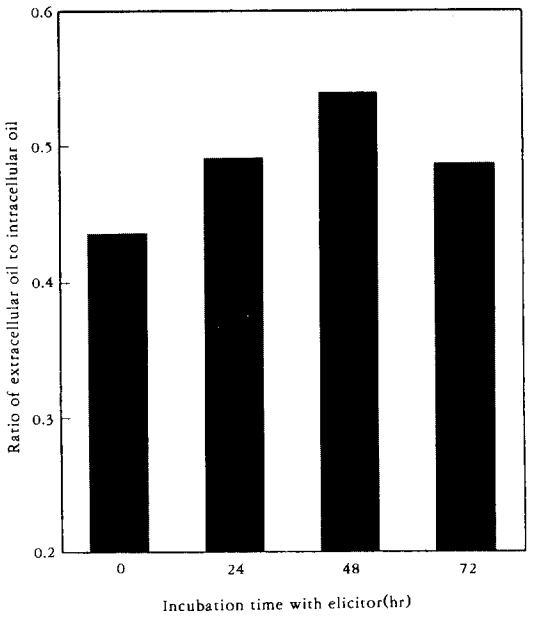


Fig. 2. Effect of fungal elicitor from *R. rubra* on excretion of peppermint oil.

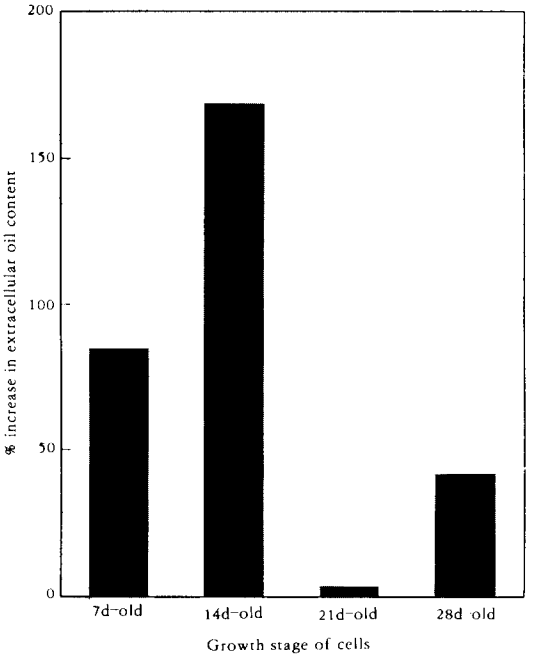


Fig. 3. Effect of growth stage of peppermint cells with fungal elicitor from *R. rubra* on peppermint oil production.

at different incubation time of 24, 48 and 72 hrs. Maximum accumulation of oil in *M. piperita* cells occurred at 48 hrs of incubation, but prolonged exposure of more than 48 hrs to elicitors resulted in a decline in oil accumulation (data not shown). Ration of extracellular oil content to intracellular oil content was also increased in the elicitor-treated culture, as show in Fig. 2. It is probable that fungal elicitor treatment influences on oil excretion from *M. piperita* cells.

Aiso, *M. piperita* cells were treated with fungal elicitor from *R. rubra* for 48 hrs to see if the growth stage of cells affects peppermint oil production. When 7-day-old (early exponential phase), 14-day-old (late exponential phase), 21-day-old (stationary phase) and 28-day-old cells (death phase) were tested with fungal elicitors, 14-day-old cells were best (Fig. 3). This result indicates that the growth stage of cells is an important parameter in the treatment of fungal elicitors to cells.

It is not completely understood how elicitors

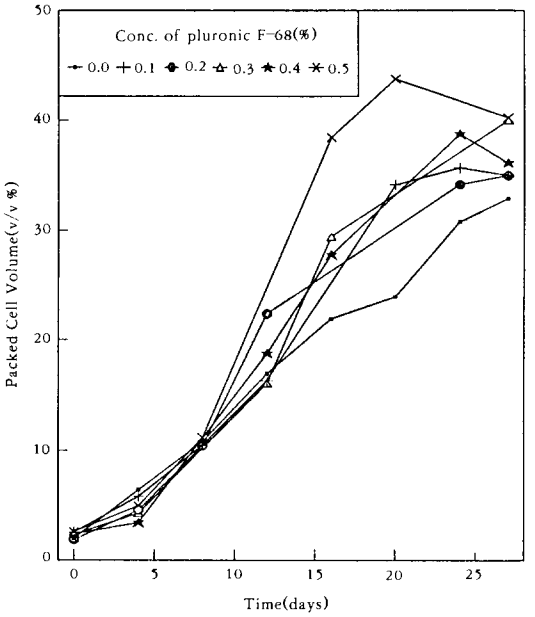


Fig.4. Effect of Pluronic F-68 on peppermint cell growth.

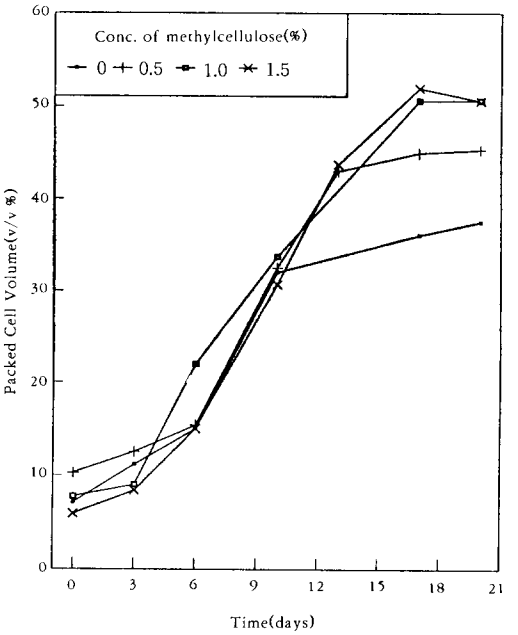


Fig.5. Effect of methylcellulose on peppermint cell growth.

enhance secondary metabolite biosynthesis in cultured plant cells. However, our findings is that. *M.* piperita cells accumulate peppermint oil greatly in shake flasks in response to fungal elicitor.

Effect of Pluronic F-68 and Methylcellulose on Peppermint Cell Growth

Pluronic F-68 or methylcellulose was shown to protect insect or plant cells(10, 11, 12). To investigate the protective effect of Pluronic F-68 and methylcellulose on *M. piperita* cells, experiments were carried out at 100 rpm agitation with cells using shake flasks. As shown in Fig. 4 and 5, increase of the concentration of Pluromic F-68 from 0% to 0.5% improves *M. piperita* cell growth by 22.4%. Also increase in concentration of methylcellulose from 0% to 1.5% enhances the cell growth by 34.9%. These results show that either Pluronic F-68 or methylcellulose at a low concentration improves peppermint cell growth. Therefore, the applications of Pluronic F-68 and methylcellulose in insect cell culture may well be applied to plant cell culture.

It has been hypothesized that the mechanism of protection of these compounds in insect cell culture involves interactions with the cell membrane(10, 11). Further investigation is needed to better characterize our observations. However, our studies prove that the surfactant such as Pluronic F-68 or methylcellulose may be a valuable supplement in plant cell culture for protection of cells against mechanical damage.

요 약

Shake flask를 사용하여 *M. piperita* 세포의 현탁 배양에서의 fungal elicitor, pluronic F-68, methylcellulose의 영향에 대하여 연구하였다. 그 결과 *Rhodotorula rubra*라는 균주에서 추출한 fungal elicitor를 처리하여 약 2배 정도 박하정유 생산의 증가를 관찰하였고 100 rpm의 교반속도에서 낮은 농도의 Pluronic F-68, methylcellulose 첨가에 의해 박하세포의 성장이 증진되었다.

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REFERENCES

1. S. A. Kireeva, V. N. Mel'nikov, S. A. Reznikova and N. I. Meshcheryakova(1978), *Soviet Plant Physiology*, **25**, 438.
2. M. L. Lin and E. J. Staba(1961), *Lloydia*, **24**, 139.
3. H. Becker(1979), *Biochem. Physio. Pflanzen*, **161**, 425.
4. J. H. Kim(1989), *M. S. Thesis*. Dept. of Food Sci. & Tech., Seoul Nat'l. Univ., Korea.
5. E. B. Song(1990), *M. S. Thesis*. Dept. of Food Sci. & Tech., Seoul Nat'l. Univ., Korea.
6. J. H. Oh, C. H. Kim, H. J. Lee, Y. A. Chae and I. S. Chung(1992), *Biochemical Eng. for 2001*, (S. Furusakis, I. Endo and R. Matsuno, eds.), P. 292, Springer-Verlag, Japan.
7. F. Dicosmo and M. Misawa(1985), *Trends in Biotechnology*, **3**(12), 318.
8. D. J. Kim and H. N. Chang(1990), *Biotechnology Letters*, **12**(6), 443.
9. U. Mukundan and M. Hjortso(1990), *Biotechnology Letters*, **12**(8), 609.
10. D. W. Murhammer and C. F. Gooche(1988), *Biotechnology*, **6**, 1411.
11. S. Goldblum, Y. K. Bae, W. F. Hink and J. Chalmers(1990), *Biotechnology Progress*, **6**, 383.
12. A. T. King, M. R. Davey, B. J. Mulligan and M. C. Lowe(1990), *Biotechnology Letters*, **21**(1), 29.
13. C. Francois, R. D. Marshall and A. Neuberger (1962), *Biochem. J.*, **83**, 335.