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세포외 단백질을 이용한 장기 배양 식물세포(*Daucus carota*)에서의 Embryo 생성에 관한 연구

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Effects of Extracellular Proteins on the Recovery of Embryogenic Potential in Long-term Cultures of *Daucus carota*

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ABSTRACT

A declining tendency in embryogenic capability was seen during 6 months culture period during which embryo production decreased from 1000 embryos/ml to 500 embryos/ml. The presence of extracellular factors extracted from newly established embryo cultures restored the embryogenic capability and even enhanced the embryo production up to 5 times (2500 embryos/ml) for old carrot suspension cultures compared with that of control cultures. The stimulating effect on the embryo production indicates that the enhancing effect comes from extracellular compounds that are probably protein molecules.

INTRODUCTION

Conventional vegetative micropropagation such as cutting methods have been generally used to avoid loss of beneficial traits that frequently occur during sexual fertilization, but these are considered as a labor intensive, manual operation. Elite genotypes may often need to be propagated vegetatively, but on a larger, and more convenient scale than traditional vegetative propagation methods allow. In addition, forest trees in which the conventional reproductive cycle is many years, and crops which are unable to be produced through traditional clonal propagation can be included. As an alternative, somatic embryogenesis has been acknowledged to be a valuable alternative propagation technique for making disease-free, genetically uniform, massive amount of plants cheaply and efficiently without losing beneficial traits. The utilization of liquid suspension cultures would be also beneficial for the automation and online control of such process.

However, the application of micropropagation technique relies upon the availability of long term cultures with a reliable embryogenic capacity. It has been reported that suspension cultures, which are originally capable of producing somatic em-

bryos, often lose their embryogenic potential through repeated subcultures. Fridborg and Eriksson(1) reported that the addition of activated charcoal helped to induce somatic embryos from carrot suspension cells which had lost embryogenic ability. They(2) indicated that the addition of activated charcoal might reduce the endogeneous auxin compounds, and other excreted phenolic compounds which could suppress embryo development. Smith and Street(3) also indicated that the genetic changes in growing cells through long term subcultures may contribute to the loss of embryogenic capability.

This declining phenomenon in somatic embryogenesis has deterred considerable efforts to commercialize the micropropagation technique as an alternative to conventional propagation methods. The objectives of this study were not only to restore the embryogenic capability, but also enhance the embryo production from long term cultures by the addition of different extracellular compounds, which were shown to be effective to stimulate somatic embryogenesis in our previous study(4).

MATERIALS AND METHODS

Plant cell cultures and culture media

Daucus carota L cv. Nantes seeds were used as a starting material to establish suspension cultures. The callus was initiated from the hypocotyl segment of 12day old juvenile carrot plant and maintained on Murashige and Skoog (MS) medium supplemented with 0.1 μM 6-γ, γ-dimethylallylaminopurine (DMAA), 0.5 μM 2, 4-dichlorophenoxyacetic acid (2, 4-D), and 30g/ℓ sugar. The suspension cells were established by inoculating 5g of callus to 50ml of MS medium adjusted at pH 5.7 with 0.5 μM 2, 4-D in a 125ml Erlenmeyer flask. The carrot suspension cells were cultivated at a rotary shaker at 165rpm and subcultured each weeek. Suspension cells were maintained for over 6months of culture period. For induction of embryogenesis, suspension cells were screened for selection of sizes less than 500 μm, filtered, and washed three times with MS medium in the absence of 2, 4-D. 0.5g fresh weight of cells was added to 50ml of the wash medium devoid of 2, 4-D and monitored for two weeks.

Preparation of excreted cell factors

Spent media, referred to as a embryo-free media (EFM), was collected from established embryo culture in the absence of 2, 4-D and then passed through Whatman No. 1 filter paper. EFM was further concentrated 10-fold by Amicon (Danvers, MA) pressure dialysis using Amicon YM 10 membranes at 10 kDa cut-off and mixed with 10% glycerol, and then stored at-20℃. The concentrates, referred to as a concentrated EFM (CEFM), were passed through 0.22 μm filter membrane for sterilization. Salt precipitated proteins, which were precipitated with a 70% of ammonium sulfate, were redissolved into 20mM Tris buffer solution (pH 7.5).

Analytical methods

For the fresh and dry cell weight determination, cells were collected using Whatman No. 1 filter paper. They were placed in preweighed weigh dishes and dried for one week at 60℃ for dry weight measurement. Known volumes of culture samples were diluted in a petri dish with a fresh medium to obtain a suitable cell density and determine the number of each embryo stage (globular, heart, torpedo and plantlet). Our software enabled us to count the number of each embryo stages through a Model 3000 Image Analyzer (Image Technology, Deer Park, N. Y.) (5, 6). Three samples taken from each flask were analyzed and all measurements were duplicated. Total number of embryos and the concentration for each stage were estimated and plantlet was defined as post-torpedo embryo with more than 1.3mm size in length.

RESULTS AND DISCUSSION

As shown in Figure 1, the results for the peak production of torpedo and plantlet (TP embryos)

from the embryo cultures in the presence of various concentrates were recorded during a 6months period. In control culture, the peak concentration of TP embryos gradually decreased from 1,000 embryos/ml to less than 500 embryos/ml, as the culture age increased from 2.5months to 5. 5months. The declining tendency of embryogenic capability in carrot suspension cultures was also reported by Smith and Street(3). They also suggested that more frequent subculturing causes a faster decline of embryogenic potential in somatic embryogenesis. Verma and Dougall(7) used the high levels of sucrose to restore the embryogenic potential in cell cultures of *Daucus carota*. Vuke and Mott(8) reported that the presence of other carbohydrates helped to induce the somatic embryogenesis for different cell lines. However, these approaches have some limitations to enhance and recover the embryo production and also are variant to each cell lines and even same species. Recently, we observed that the addition of excreted compounds extracted from already established embryo cultures enhanced the production of somatic embryos(4). Similar reports(9, 10) have been suggested that the presence of spent media prepared from embryo cultures in the absence of 2, 4-D was effective to initiate normal embryo development from carrot suspension cultures.

The utilization of extracellular compounds to recover the embryogenic capability for long term cultures of carrot cell lines is presented in Figure 1. The characteristics of different extracellular compounds which were tested in our study are shown in Table 1. From the comparison between runs 1 and 2, the production of TP embryos from control cultures showed almost the same value, but the addition of EFM compounds increased the concentrations of TP embryos up to 50%. In the case of run 2 with the addition of 2ml of CEFM, the production of TP embryos yielded almost the same level of run 1 with the addition of 7.5ml of EFM, even if less extracellular compound was added. This result indicated that the enhancement of somatic embryos by excreted compounds de-

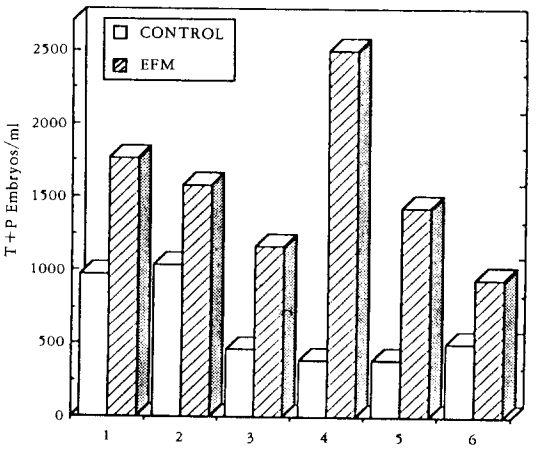

Fig. 1. Long term stability on the production of torpedo embryo and plantlet.

Table 1. Characteristics of various EFMs which were added during inoculation in figure 1 experiments

Run No.	Age of cell lines	Concentrates used	M. W. size cut-off	Volume of EFM	Culture period
1	2.5months	EFM	no	7.5ml	13days
2	3months	CEFM	>10kDa	2ml	17days
3	3.5months	EFM	no	5ml	14days
4	4months	Proteins	>10kDa	1.1㎍/ml of Proteins	14days
5	4.5months	CEFM	>50kDa	1ml	16days
6	5.5months	CEFM	>10kDa	1ml	13days

pends on the characteristics of extracellular compounds such as the sizes of molecular weight, harvesting time, and concentration of excreted factors.

In run 3, the decrease in the production of TP embryos was evident from the comparison of control cultures for run 1 and run 3 cell lines. The addition of 5ml of EFM stimulated the concentration of TP embryos up to two times higher. EFM restored the embryogenic capability from old cell lines and even enhanced the embryo production. The increasing effect by the presence of EFM was apparent. In run 4, the addition of salt precipitated proteins strikingly enhanced the embryo production up to 2,500 embryos/ml (5times

higher than that of control culture), which was the maximum production level among all tested embryo cultures. In run 5 and run 6 experiments, the increasing effects by the addition of EFM to aging cell lines were evident. The embryo production was increased up to 50% by the addition of high molecular weight(>50 KDa) compounds in comparison with that of low molecular weight(> 10 KDa) compounds. This result suggests that high molecular weight, excreted factors may be more effective than low molecular weight compounds to enhance the embryo production.

In conclusion, extracellular protein compounds among EFM compounds excreted from embryo cultures had the highest potentials to restore the embryogenic capability for aging suspension cell lines and also stimulate the embryo production. It is also clear that understanding the roles of secreted cell factors during somatic embryogensis will be helpful to regulate the biochemical process and also development of practical somatic embryogenesis schemes. These or similar extracellular protein factors may also prove to be valuable with other cell lines that undergo somatic embryogenesis. This approach will obviously benefit to increase the yield of somatic embryos and could be utilized as a general tool to regulate and stimulate embryo production.

요 약

Control culture에서 embryo 생성률이 6개월 동안 1000embryos/ml에서 500embryo/ml로 감소되었다. 이러한 현상을 극복하기 위한 방법으로 embryo 배양액에서 추출된 세포외 물질의 첨가는 embryo 생성률을 control culture와 비교하여 최대 (2500embryos/ml)까지 증가시켰다. 또한 세포외 단백질이 embryo 수율 향상에 기인하는 것으로 추측된다.

REFERENCES

1. G. Fridborg and T. Eriksson(1975), *Physiol. Plant*, **34**, 306-308.
2. G. Fridborg, M. Pedersen, L. Landstrom and T. Eriksson(1978), *Physiol. Plant* **43**, 104-106.
3. S. M. Smith and H. E. Street(1974), *Ann. Bot.*, **38**, 223-241.
4. Wook-Jin Chung(1992), *Ph. D. Thesis*, Rutgers University.
5. D. Cazzulino, H. Pedersen and C. K. Chin (1990), *Develop. Indust. Microbiol.*, **31**, 285-292.
6. D. Cazzulino, H. Pedersen and C. K. Chin, eds.(1991), *Bioreactors and Image Analysis for Scale-up and Plant Propagation.* In : Scaleup and Automation in Plant Tissue Culture, **8**, pp. 147-177, New York : Academic Press.
7. D. C. Verma and D. K. Dougall(1977), *Plant Physiol.*, **59**, 81-85.
8. T. M. Vuke and R. L. Mott(1987), *Plant Cell Rep.*, **6**, 153-156.
9. V. Hari(1979), *Z. Pflanzenphysiol.*, **96**, 227-231.
10. G. G. Warren and M. W. Fowler(1981), *New Phytol.*, **87**, 481-486.