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세포외 분비물질을 이용한 체세포배 생산성의 향상

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Extracellular compounds can enhance development of carrot somatic embryos

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ABSTRACT

The enhancing effect of excreted cell factors on the production of somatic embryos from suspension cultures of *Daucus carota* was studied as a function of factors including molecular size, harvesting time, injection period, and concentration of the extracellular compounds. The production of late-stage embryos was increased up to 1,500 embryos/ml compared with control cultures when high molecular size and extracellular factors, extracted from newly established embryo culture at early stationary phase, were added at the starting time. The stimulating effect on the production of somatic embryos can be attributed to the presence of high molecular size(>10 kDa) compounds.

INTRODUCTION

The first reports on somatic embryogenesis were published by Steward et al. (1) and Reinert (2) and many subsequent efforts have focused on the development of efficient propagation techniques to induce somatic embryos from a greater variety of mono- and di-cotyledonous plant species. Liquid phase cultivation of somatic embryos has been recognized as a valuable method of plant propagation for producing genetically superior, uniform and massive amounts of plants *in vitro* with a common objective to routinely produce an abundance of viable embryos. Typical approaches to improve the yield of somatic embryos include a manipulation of a wide variety of environmental conditions, including nutrients, hormone, and pH levels of the culture medium in addition to aeration, temperature, and light level adjustments. However, the lack of understanding on the biochemical mechanism of somatic embryogenesis has generally discouraged the efforts of many researchers to optimize such exogenous conditions. This study addresses the alternative strategy of investigating endogenous compounds as enhancing growth factors to control and maximize the production of somatic embryos in carrot cultures.

The promoting effect of conditioned medium

from embryogenic culture on the production of carrot somatic embryos at low cell densities was first observed by Hari (3) and by Warren and Fowler (4). However, very few studies into the role of the excreted cell factors during embryo development have been published. Huang et al. (5) observed a similar stimulating effect of conditioned media, with low inoculum, during somatic embryogenesis of *Brassica napus*. They hypothesized that responsible compounds may be synthesized and released into culture medium from embryos as opposed to other undifferentiated cell types in the culture. In work with carrot cultures, de Vries et al. (6) also reported that embryogenic potentials were stimulated by the addition of concentrated spent media isolated from embryogenic culture, even in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D), and indicated that these factors may be polypeptides because of a noted heat lability. A related, but counteracting, efxfect has also been observed by Sung and Okimoto (7) in that conditioned media obtained from cell culture media containing 2,4-D favors cell proliferation but inhibits embryo development. From such consistent reports of a conditioning effect on somatic embryogenesis, some excreted cell factors from cell culture medium are likely to be involved with the mechanism of embryo development. These factors may have the potential to function as growth enhancing compounds to increase the production of somatic embryos.

In order to examine such factors more closely, it is important to recognize and account for the fact that the production of somatic embryos is a multi-step process. Somatic embryos pass through the successive stages of so-called globular, heart, and torpedo embryos and finally a young plantlet which has recognizable root and shoot meristems. Recently developed automated analytical procedures using imaging technology and pattern recognition software for objectively quantifying the embryo concentration (8) has been utilized. These techniques are valuable for monitoring morphological changes and were utilized in this study in order to assess the effects of culture modifications on embryo development. The overall objective is to develop production strategies for increasing yields of somatic embryos in a plant cell culture. This study particularly explored the potential of excreted compounds as growth and development enhancing factors to accelerate the process of somatic embryogenesis and thereby increase the production of somatic embryos. We explored molecular size and optimum concentration of excreted factors that maximize embryo production. The necessary addition time for these factors and the best culture period for harvesting spent medium were also determined.

MATERIALS AND METHODS

Plant cell cultures and culture media. Plant callus was initiated from the hypocotyl segment of 12 day old juvenile carrot plants which were germinated from the seeds of Daucus carota L cv. Nantes and maintained on MS (9) media supplemented with 0.5 μM 2,4-D, 0.1 μM 6-γ, γ-dimethylallylaminopurine (DMAA) and 30 g/ℓ sucrose. The suspension cells were established by inoculating 5 g of callus to 50ml of MS medium supplemented with 0.5 μM 2,4-D in a 125ml Erlenmeyer flask. The pH was adjusted to 5.7. The carrot suspension cells were cultivated on a 7 day subculture cycle with continuous agitation using a gyrotary shaker at 165 rpm. For induction of embryogenesis, cells and cell aggregates were first screened for selection of sizes less than 500 μm, filtered, and washed three times with MS medium lacking 2,4-D. Subsequently, 0.5 gram fresh weight of cells were added to 50 ml 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 pressure dialysis using an Amicon YM 10 membrane (Amicon Corp., Danvers, MA) with a 10 kDa cut-off. The concentrate was mixed with 10% glycerol, and stored at -20℃. The concentrates and filtrates, referred as CEFM and FEFM respectively, were passed through 0.22㎛ filter membrane for sterilization.

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 fresh medium to an appropriate cell density in order to determine individual embryo numbers (globular, heart, torpedo and plantlet). The software (8,10), running with a Model 3000 Image Analyzer (Image Technology, Deer Park, N.Y.), enabled us to automatically count the individuals belonging to each embryo stage. Three samples were taken from each flask for analysis and all measurements were duplicated. The plantlet stage was defined as a post-torpedo embryo with a length greater than 1.3mm.

The filtrates were passed and collected through a 0.22㎛ membrane for the analysis of protein and carbohydrates concentrations. The amount of protein present in the culture medium was determined by a Bio-Rad assay. An HPLC system with a refractive index detector (Perkin-Elmer, Wilton, CT) was used for the measurement of carbohydrate concentration. An Aminex HPX-87H column (300mm×7.8mm, Bio-Rad) was operated with Milli Q water as the mobile phase at 0.6ml/min.

RESULTS AND DISCUSSION

Effect of molecular size of EFM fractions on embryo development

In previous experiments, EFM was shown to be

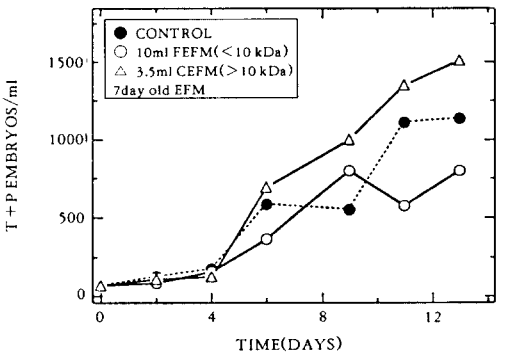

Fig. 1. Effect of different molecular size fractions from 7 day old EFM on the production of torpedo embryos and plantlets (T+P embryos).

generally effective for the stimulation of somatic embryo production and had potential as a growth enhancing factor (11). However, the optimum production of embryos is likely to depend on only a small fraction of the total number of compounds present in EFM. To determine the molecular sizes of the compounds that promote embryo production, 10-fold concentrated (CEFM) and filtrated (FEFM) EFM was prepared from 7 day old embryo culture. The addition of 3.5ml of CEFM (>10 kDa) and 10 ml of FEFM (<10 kDa) to 46.5 and 40ml of MS basal medium, respectively, was done at the same time the medium was inoculated with carrot cells. The major difference between CEFM and FEFM is that the former contains higher molecular size compounds (>10 kDa) and the latter contains lower molecular size compounds (<10 kDa) from the EFM group. A control culture consisted of 50ml of MS basal medium with the usual inoculum of cells and no additional EFM factors.

As shown in Figure. 1, the addition of 3.5ml CEFM increased the final production of torpedo embryos and plantlets (TP embryos) by about 30 % (to 1500 embryos/ml) and showed a faster production rate of TP embryos compared to the control culture. However, the addition of 10ml FEFM decreased the level of final TP embryo concentration by 30%. A larger volume of FEFM

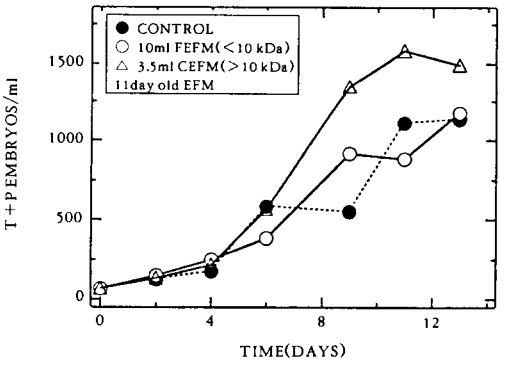

Fig. 2. Effect of different molecular size fractions from 11 day old EFM on the production of torpedo embryos and plantlets (T+P embryos).

did not improve the production of somatic embryos. This result indicates that extracellular compounds larger than 10,000 dalton, extracted from newly established embryo culture, are responsible for the stimulating effect on the production of late stage embryos (TP embryos). Lower molecular size compounds may be inhibitory to the formation of end stage embryos. Other factors such as additional metabolizable carbon sources present in EFM compounds are unlikely to be candidates since the sugar level increased less than 10 % after addition of EFM.

The same experiments were repeated with 11 day old CEFM and FEFM to further delineate any enhancing effects and to examine the embryo production response at low EFM carbohydrate levels. At 11 days, the level of carbohydrates in both CEFM and FEFM is negligible and corresponds to less than 1% of the total sugar level in control cultures. Other nutrients are also likely to be at extremely low levels after 11 days. CEFM and FEFM were prepared by the same procedures as before and the volumes of the two fractions were identical to those used previously (cf. Figure 1). Figure 2 represents the time course of the production of torpedo embryos and plantlets when 11 day old CEFM or FEFM was added during inoculation.

The embryo concentration in the presence of 3.5 ml CEFM shows a faster production rate and a higher production level, up to 30% (1500 embryos), in comparison to a control culture. The addition of 10ml FEFM, in contrast, resulted in the same final level of TP embryo production as that of control. When these results are compared to the experiments depicted in Figure 1, it is seen that the final production level of embryos after addition of 11 day old CEFM was the same as that seen when 7 day old CEFM was used. However, embryo production rates at intermediate times are accelerated in the presence of 11 day old CEFM. On the other hand, the presence of 11 day old FEFM did not influence the production of end stage embryos (TP embryos), whereas the addition of 7 day old FEFM slightly decreased the production of TP embryos. This suggests that the composition of excreted factors is changing with EFM harvesting time since different effects are observed. The embryo promoting effect after addition of EFM is likely to depend upon the composition of excreted compounds, particularly high molecular size compounds. In particular, extracellular compounds with molecular sizes greater than 10,000 dalton are likely to be responsible for the stimulating effect on the production of late stage embryos.

Effect of EFM harvesting time on embryo production profiles

Acceleration of end-stage embryo production by the addition of high molecular size EFM factors depends on the harvesting time of EFM from competent cultures. Production of early stage embryos must be enhanced to an equal or greater extent since all late-stage embryos can only be derived from progressively earlier developmental forms. To determine the complete embryo production profile as a function of EFM factors isolated at various harvesting times, three embryogenic cultures were run under similar experimental conditions except 4, 7, and 11 day old CEFM was added at a volume of 3.5ml. The composition over the entire range of embryo stages was monitored

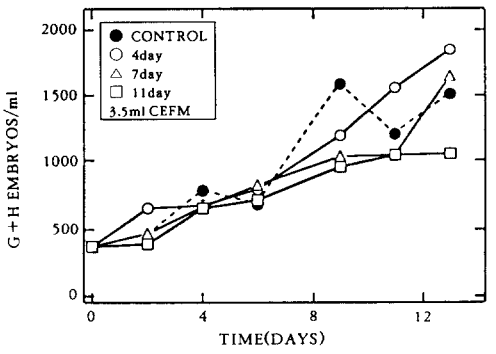

Fig. 3. Effect of harvesting time for manufacture of CEFM on the production of globular and heart embryos (G+H embryos).

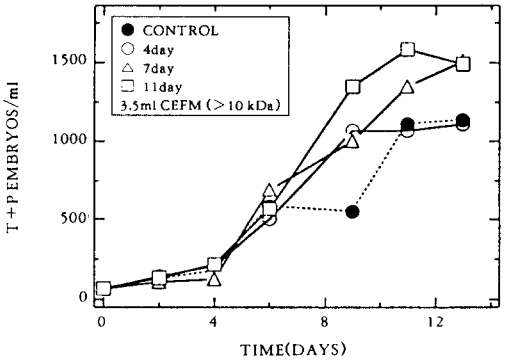

Fig. 4. Effect of harvesting time for manufacture of CEFM on the production of torpedo embryo and plantlet (T+P embryos).

and compared to appropriate controls.

As shown in Figure 3, no stimulating effect on the production of early-stage embryos (GH or globular and heart embryos) was observed after addition of both 7 and 11 day old CEFM compounds, but the addition of 4 day old CEFM slightly increased the production of GH embryos. Such an increase after addition of 4 day old CEFM may be attributed to availability of excess nutrients in CEFM which are not metabolized during a short 4 day culture period. Reduced production of GH embryos after addition of both 7 and 11 day old CEFM is most likely attributed to a faster conversion rate from early to late stage embryos due to the accelerating effect on development by high molecular weight CEFM compounds.

Figure 4 shows the concentrations of end stage (TP) embryos as a function of 4, 7, and 11 day old harvesting time for CEFM. For all conditions examined, CEFM accelerated the production rate compared with that of a control culture containing no enhancing factors. As mentioned previously, 11 day old CEFM shows the highest production rate and highest peak production level. This is followed by 7 day old CEFM and then 4 day old CEFM. The addition of 11 day old CEFM enhanced the production of TP embryos up to two and a half-fold at a particular time (9th day) in comparison with that of control culture and showed a 40% increase compared with that of 7 day old CEFM culture after the 9th day. On the other hand, the presence of 4 day old CEFM did not affect the production level and yielded the same level as control (1,000 embryos/ml). The 11 day old CEFM corresponds to the early stationary period in terms of dry weight growth. The addition of EFM harvested from late stationary phase (about the 16th day) did not increase embryo production levels above that of 11 day CEFM culture (data not shown here).

The comparison of Figures 3 and 4 also points out that the early GH and late TP stages are correlated: low levels of early stage embryos are observed when high levels of late-stage embryos are present. This can be explained by a general acceleration of embryo development which effectively drains the early-stage pool. Since plantlets are the final product, they accumulate. It is also possible that differential effects on different stages may be the result of compositional changes during EFM harvesting periods. This is unnecessarily complicated, however, since both a uniform and a differential effect can explain the available data at this point. Until the composition is more carefully measured, a uniform acceleration of embryo development can be assumed.

Since the factors responsible for accelerated development arise from EFM during the period at

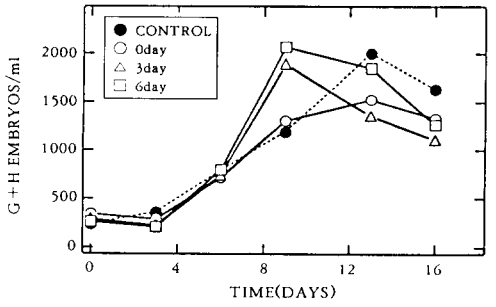

Fig. 5. Response of early embryo development to 5 ml of 14 day old EFM added at different growth phases.

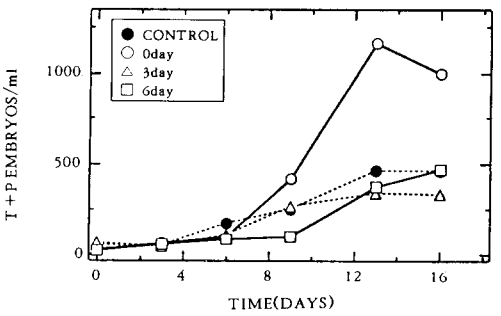

Fig. 6. Response of late embryo development to 5 ml of 14 day old EFM added at different growth phases.

which end-stage embryos are predominant, it is likely that they are secreted by the embryos and not nonembryogenic cells. A defining characteristic of these factors at this point is that they are high molecular weight compounds secreted by embryogenic cells that promote uniform acceleration of embryo development.

Addition time for EFM

Embryogenesis culture is composed of a mixture of single cells, cell aggregates, globular, heart, and torpedo embryos, and finally plantlets, and it may be that each stage requires a different environmental and physiological condition to continue development. EFM, prepared from newly established embryo culture during early stationary phase, stimulates embryo development and was previously seen to accelerate the transition between stages. This experiment examined the effect of feeding time of EFM to see whether it could differentially effect a specific embryo development step.

A volume of 5ml of 14 day old EFM was dosed at different growth phases corresponding to 0, 3, and 6 days. The addition of EFM at any growth phase did not influence the cell growth or lag period, in terms of dry weight changes, beyond a slightly higher growth rate in the case of EFM injection during inoculation (dry weight data not shown here). In Figure 5, it is seen that accumulation of early embryos (GH embryos) was reduced by addition at the inoculation step, but a later injection at both 3 and 6 days resulted in an increased level of early stage embryo production compared with the control culture. As shown in Figure 6, the maximum concentration of torpedo embryos and plantlets, when EFM was added at inoculation, was more than two-fold (1,000 embryos/ml) higher than that of control culture (500 embryos/ml) as well as cultures exposed to a later injection of EFM.

This result indicates that the addition of EFM at the starting time is the best strategy to maximize the production of end-stage embryos. Later injections do not improve the overall production of TP embryos. The addition of EFM factors at any time up to day 6 can accelerate the globular and heart embryo developmental stages. However, any delay in adding EFM factors beyond the inoculation time does not allow for corresponding increased production of late-stage embryos. This may be due to the fact that the nutrient supply is continually decreasing, regardless of the presence of any EFM factors. In Figure 5, it is seen that the addition of EFM factors at 3 or 6 days leads to accumulation of early embryos. However, these embryos are apparently aborted and not subsequently transformed to the next stage when compared to control levels as shown in Figure 6. Therefore, nutrient availability is important for complete development when EFM factors are added, particularly in batch culture experiments

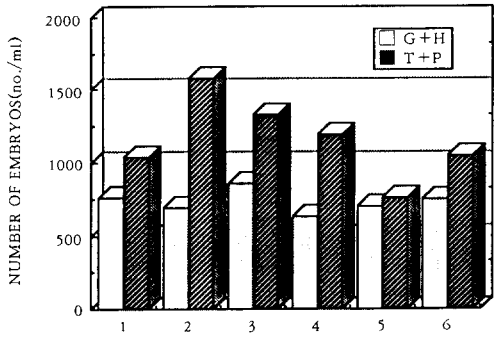

Fig. 7. Embryo production numbers (at day 17) as influenced by the variation in concentration of CEFM (>10 kDa) and EFM. No. 1 is a control without CEFM and 2-5 are, respectively, the addition of 2, 4, 6, and 8ml of CEFM. No. 6 is the addition of 6ml EFM.

such as those reported here.

Optimum concentration of EFM

To examine the appropriate volume of EFM to yield the best production of end-stage embryos, an extended range of concentrations from 2ml to 8ml of 10 times concentrated CEFM and 6ml of EFM were added during inoculation. The total amount of excreted protein in the CEFM and EFM was 38.1 and 21.9 μg/ml, respectively. CEFM and EFM were prepared from 2 weeks old embryo culture medium and CEFM was concentrated with an Amicon YM 10 membranes at 10 kDa cut-off.

Figure 7 represents the production of early and late stage embryos with different levels of CEFM and EFM, measured after 17 days culture. The final production level of GH embryos was not dramatically influenced by different levels of CEFM and EFM. However, the concentration of TP embryos was sharply increased with addition of 2ml of CEFM. As the volume of CEFM increased, the productivity fell off and inhibition effects were noted at high volumes of CEFM. This may be due simply to the dilution of the media with respect nutrient concentrations since the CEFM fractions contain little or no available nutrients. The addition of 6ml EFM showed the same level of TP embryos production compared with that of control. In this last case, the dilution and stimulating effects are balanced. The best yield in late stage embryo production was obtained from the addition of 2ml of CEFM (>10 kDa) containing 38.1 μg protein/ml. Overall, the optimum concentration of CEFM to maximize the production of torpedos and plantlets is dependent on the molecular size of excreted factors, the harvesting time for EFM, and the injection timing.

In conclusion, the stimulating effect on the production of end-stage embryos indicates that the enhancing effect comes from extracellular, high molecular size (>10 kDa) compounds that are probably protein molecules. Early stationary phase collection of EFM and early injection are the best conditions to maximize the late-stage embryo production. In addition, sufficient availability of standard nutrients is important to realize the promoting effects of the EFM factors. This excreted factor can be utilized as a general tool to regulate and stimulate embryo development and also to restore the embryogenic potential from an impaired embryogenic cell line. The combination of bioreactor technology with this strategy will be of direct interest to any commercialization of large-scale plant cloning processes that use somatic embryogenesis.

요 약

당근세포(*Daucus carota*)로 부터 체세포배 생산성 증대를 위하여 체세포 배 현탁 배양액으로부터 추출한 세포외 분비 물질을 이용하여 다음과 같은 효과를 측정하였다. : 분자량의 차이, 세포외 분비 물질 추출 시기, 주입 시기, 주입 농도에 따른 각종 체세포 배 생산성 증감 효과. 체세포배 생산량은 최대 1,500개/ml 까지 얻었으며, 10 kDa 이상 분자량이 포함된 세포외 분비 물질을 초기에 주입시켰을 때 생산성이 특히 증가함을 보여 주었다.

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NOMENCLATURE

EFM = embryo free medium
CEFM = concentrated EFM
FEFM = filtrated EFM
GH = globular and heart embryo
TP = torpedo embryo and plantlet

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