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ENHANCED SOMATIC EMBRYO PRODUCTION BY CONDITIONED MEDIA IN CELL SUSPENSION CULTURES OF *DAUCUS CAROTA*

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Summary

The addition of conditioned media extracted from 8 day old embryo culture accelerated growth and production of torpedo embryos and plantlets in cell suspension cultures of *Daucus carota*. The production of late-stage embryos was increased a maximum three-fold (up to 1500 embryos/ml) compared with that of control culture, when spent medium was added during inoculation.

Introduction

It is known that plant cells in liquid suspension culture will not normally grow at low cell densities due to dilution of certain essential substances in the culture medium (Warren and Fowler, 1978; Hari, 1979; Sung and Okimoto, 1981). Compounds that have been found to be released to the culture medium include polypeptides, polysaccharides, amino acids, growth substances, vitamins and other small organic and inorganic molecules. Consequently, an optimum cell density is often emphasized in protocols for subculturing and growing cell suspensions of higher plants. To complement cell cultures showing poor growth in situations of unavoidable low inoculation density, the addition of conditioned media, in which cell cultures were previously actively growing, often compensates for diluted factors and enhances the culture growth. For example, Stuart and Street (1969) found that the minimum inoculation density could be reduced up to ten times by the addition of conditioned media. This phenomenon is now widely referred to as the nursing effect. This strategy is also effective in plant cultures undergoing developmental changes, such as somatic embryogenesis. In similar examples, spent media extracted from embryo cultures grown in the absence of 2,4-dichlorophenoxyacetic acid (2,4-D) hormone was found to be effective in promoting normal embryo development from carrot suspension cultures (Warren and Fowler, 1978; Hari, 1979) and in cultures of *Brassica napus* (Huang *et al.*, 1990). The optimum conditions are typically not delineated, however, due to the lack of a clear quantitative measure of embryo development. It has also been difficult to determine if enhancement occurs at a particular stage of development or throughout the sequence of steps leading to plant regeneration.

In this study, we investigate the effects of spent media extracted from actively growing carrot embryo cultures on the number density of somatic embryos at different stages of embryo development. The embryo concentration can be closely followed using image analysis techniques and a detailed, quantitative description of enhancing effects can be developed. The optimum volume of conditioned media and the appropriate feeding time in order to obtain the best yield of end-stage somatic embryos from suspension culture can be determined.

Materials and Methods

Plant cell cultures and culture media

Daucus carota L cv. Nantes seeds were germinated in the greenhouse to produce young carrot plants. Callus was initiated from the hypocotyl segment of a 12 day old juvenile carrot plant and maintained on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.1 μM 6-γ,γ-dimethylallylaminopurine (DMAA), 0.5 μM 2,4-D, and 30 g/l sugar. The suspension cells were established by inoculating 5 g of callus in 50 ml of MS medium adjusted to pH 5.7 and containing 0.5 μM 2,4-D in a 125 ml Erlenmeyer flask. The carrot suspension cells were cultivated on a rotary shaker at 165 rpm and subcultured weekly. For induction of embryogenesis, suspension cells were screened for selection of sizes less than 500 μm, filtered to remove bulk media, and washed three times with MS medium in the absence of 2,4-D. One gram fresh weight of cells was added to 50 ml of the wash medium devoid of 2,4-D and monitored for two weeks.

Preparation of conditioned media

Spent media, referred to as an embryo-free media (EFM), was collected from established embryo culture grown in the absence of 2,4-D. Cultures were first passed through Whatman No. 1 filter paper and the filtrate was used after filter-sterilization with a 0.22 μm filter membrane.

Analytical methods

For the fresh and dry cell weight determination, cells were collected using Whatman No.1 filter paper and placed in preweighed aluminum dishes. The fresh weight was recorded and cells were dried for one week at 60 °C for dry weight measurement. To determine the number of embryos at each developmental stage (classified as globular, heart, torpedo and plantlet), known volumes of culture samples were diluted in a petri dish with fresh medium and examined under a microscope equipped with a video camera. Image analysis software (Cazzulino *et al.*, 1990, 1991) was used to count the number of classified embryos through the torpedo stage that were present at a particular time. The plantlet stage was defined as a post-torpedo embryo larger than 1.3 mm in length and this classification was done interactively with the user to develop a reliable and accurate count. The equipment was built around a Model 3000 Image Analyzer (Image Technology, Deer Park, N.Y.). Three samples were taken from each flask and separately analyzed. All measurements were duplicated.

Results and Discussion

EFM was prepared from 8 day old, late exponential growth phase embryo culture medium. To ascertain the enhancing effect of EFM on the embryo development, 10 and 20 ml of EFM were added to 40 and 30 ml of MS basal medium, respectively, during inoculation. Control cultures contained no added EFM at a final volume of 50 ml. The total sugar concentration in the EFM was only 10% of the total sugar in the control cultures. In separate experiments, however, it was found that sugar levels within the range implied by this dilution (Chung, 1992) do not significantly alter embryo development. The data were individually measured for each of the four stages, but then pooled into conglomerate early-stage embryos, considered to be globular and heart forms, and late-stage embryos, considered to be torpedo and plantlet forms. This emphasizes gross differences in early and late-stage events that are due to the presence of EFM. More detailed interpretation of the data does not alter the conclusions reached based on this simplified presentation.

Time course changes of dry cell weight and pH were not affected by the level of added EFM compared with control cultures (Chung, 1992). As seen in Figure 1, addition of both 10 ml and 20 ml of EFM leads to a faster growth and higher production of torpedo embryo and plantlets compared with that of control culture. The production of late stage embryos (torpedo and plantlet) with the addition of 10 ml EFM reaches a maximum three-fold higher value (1500 embryos/ml) in comparison with that of control culture on the 9th day of culture. Higher production of torpedos and plantlets with the

addition of EFM compared with that of control culture could have resulted from availability of any of the remaining nutrients in the EFM as well as the stimulating effects of any growth enhancing factors that are the extracellular, metabolic products of an embryogenic culture. As the volume of EFM increased from 10 ml to 20 ml, the production of TP (torpedo and plantlet) embryos was not enhanced further. This suggests that the former hypothesis is less likely to explain the enhancement effect since any rate limiting nutrient compound, that always present in the culture, is not likely after 8 days to lead to a saturation response in its effectiveness. Nevertheless, further experiments were carried out in which sugar levels were independently varied in different culture media. No significant enhancing effects on embryogenesis were observed.

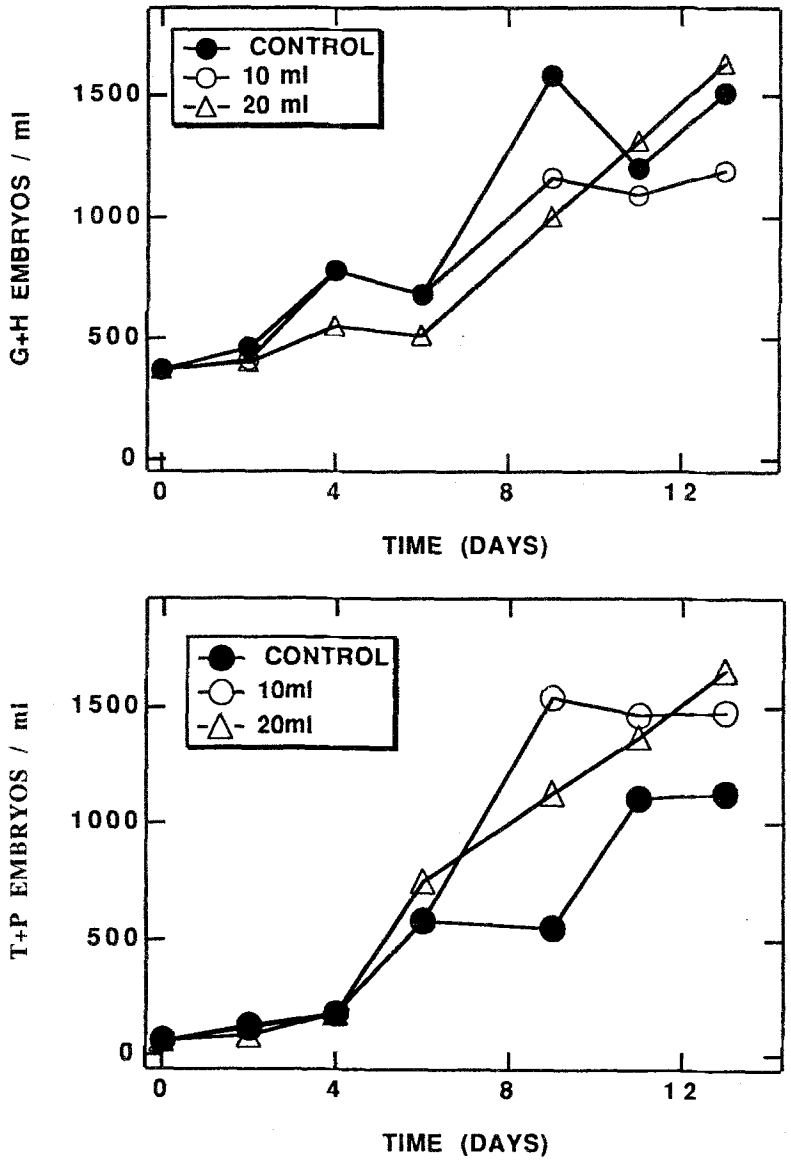

Figure 1. Effect of 8 day old EFM on the production of somatic embryos in carrot cell suspension cultures. Top: early stage; Bottom: late stage.

The production of early-stage embryos (globular and heart or GH embryos) was only slightly effected and even decreased after the addition of EFM. The apparent slow production of early embryos can actually be attributed to a faster conversion rate of early

to later embryos rather than an inhibiting effect by EFM. This is a consequence of the fact that all TP embryos must arise from the GH stages and therefore the production of early stage embryos must be at least as high as the number of late-stage embryos seen. These results suggest that extracellular compounds prepared from embryo culture might have a regulatory factor that functions to control the developmental processes of somatic embryogenesis.

Other experiments were carried out to examine the appropriate time for addition of EFM that would optimize the level of end-stage embryos. It was found that the highest levels were seen when EFM was added at the time of inoculation, corresponding to the data in Figure 1. No enhancement was seen if the conditioned media was added after the fifth day of culture. This indicates that the early stages of development are accelerated by the presence EFM, which at later times will, of course, lead to higher end-stage plantlets. This is also seen in Figure 1 by the higher levels of GH embryos over the first four days when EFM is present in the media. After four days, an increased rate of transitions to torpedo and plant stages takes place.

In conclusion, conditioned medium (EFM) extracted from established embryo culture in the absence of 2,4-D accelerates the production of all stages of embryo development. As a consequence, the production of later stages of torpedo and plantlet can be elevated to much higher levels compared with that of control culture. This is of direct interest in the development of large-scale plant cloning processes that use somatic embryogenesis as a means of rapidly multiplying plants. The plantlets, for example, can be directly transferred to soil. We have successfully demonstrated this in the greenhouse and phenotypical carrot plants are seen. Alternatively, torpedo stage embryos could be harvested and used in encapsulated form as so-called artificial seeds. These seeds would then be used in agricultural practice much as normal seeds.

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