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Calcium increases the yield of somatic embryos in carrot embryogenic suspension cultures

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Abstract. An upward shift in the concentration of calcium present in the medium during somatic embryogenesis increased the number of embryos produced approximately two-fold. This was observed when embryogenic suspension cells grown in 2,4-D medium with the normal calcium concentration of 10^{-3} M were transferred to hormone-free medium containing 10^{-2} M calcium and when embryogenic suspension cells grown in 2,4-D medium containing 10^{-4} M calcium were transferred to hormone-free medium with 10^{-3} M calcium. At calcium concentrations between $6 \cdot 10^{-3}$ and 10^{-2} M globular stage somatic embryos were found in cultures supplemented with $2 \cdot 10^{-6}$ M of 2,4-D indicating that elevated calcium counteracts the inhibitory effect of 2,4-D on somatic embryogenesis. No qualitative changes were found in the pattern of extracellular polypeptides as a result of growth and embryogenesis in media with different calcium concentrations.

Key words: Calcium - *Daucus carota* - Somatic embryogenesis

Introduction

In plants, intracellular calcium is involved in a large number of physiological processes (reviewed by Ferguson and Drøbak 1988 and Hepler 1988), while many external stimuli have been shown to result in changes in intracellular calcium concentration and compartmentalization modulated by calmodulin (Roberts et al. 1986). Calcium is also a major ion in plant cell walls, being implicated in the maintainance of cell - cell adhesion by cross-linking of pectins present in the middle lamella (Jarvis 1984), as well as being involved in the control of activity of cell wall peroxidases (Penel 1986) and in the binding of polyamines to cell walls (Mariani et al. 1989).

Carrot somatic embryogenesis is used extensively as a model system for early plant development (Nomura and Komamine 1986). However, few reports exist on the possible role of calcium in this system (Nomura 1987, Timmers et al. 1989). In this present work we show that an upward shift in $CaCl_2$ in the growth medium results in a marked increase of the embryogenic potential of a carrot suspension culture. In addition, increased medium calcium is able to counteract the inhibitory effect of 2,4-D on somatic embryogenesis.

Materials and Methods

Plant material and culture conditions. Embryogenic cultures of *Daucus carota* cv. "Flakkese" SG 766 were obtained as described (De Vries et al. 1988b) and maintained on a rotary shaker at 100 r.p.m. under an 18 h light period at 25°C on a 14 d subculture cycle by inoculating 2 ml of packed cell volume in 50 ml of B5 medium (Gamborg et al. 1968) containing $2 \cdot 10^{-6}$ M 2,4-D, corresponding to an initial cell density of approximately 10^6 cells ml^{-1}. For initiation of embryogenesis, high density suspension cells from cultures 7 d after transfer were size fractionated between 125 and 50 µm nylon meshes. The enriched proembryogenic masses obtained this way were suspended at a density of $2 \cdot 10^4$ cells ml^{-1} (approximately 1,500 proembryogenic masses ml^{-1} in 2,4-D free medium yielding somatic embryos) or at a density of $2 \cdot 10^4$ cells ml^{-1} in B5 medium with 2,4-D yielding proliferating cultures. Culture viability was determined by fluorescein diacetate/lissamin green staining (Larkin 1976). After 12 d the number of somatic embryos was determined by direct counting, while unorganized proliferation was determined by cell counting after chromic acid dispersion (Sung 1976). All experiments were performed in triplicate.

Media containing concentrations of $CaCl_2$ below 10^{-3} M were adjusted with KCl to account for the reduction in chloride content. Suspension cultures grown in media with 10^{-4} M $CaCl_2$ were subcultured at least 3 times before use. Media were prepared using deionized water purified with a Millipore MilliQ system (R > 20 MΩ/cm). Medium $CaCl_2$ concentrations were not corrected for remaining trace amounts of $CaCl_2$.

[^{35}S]methionine labeling of extracellular proteins. Labelling, preparation and electrophoresis of extracellular proteins was as described previously (De Vries et al. 1988a).

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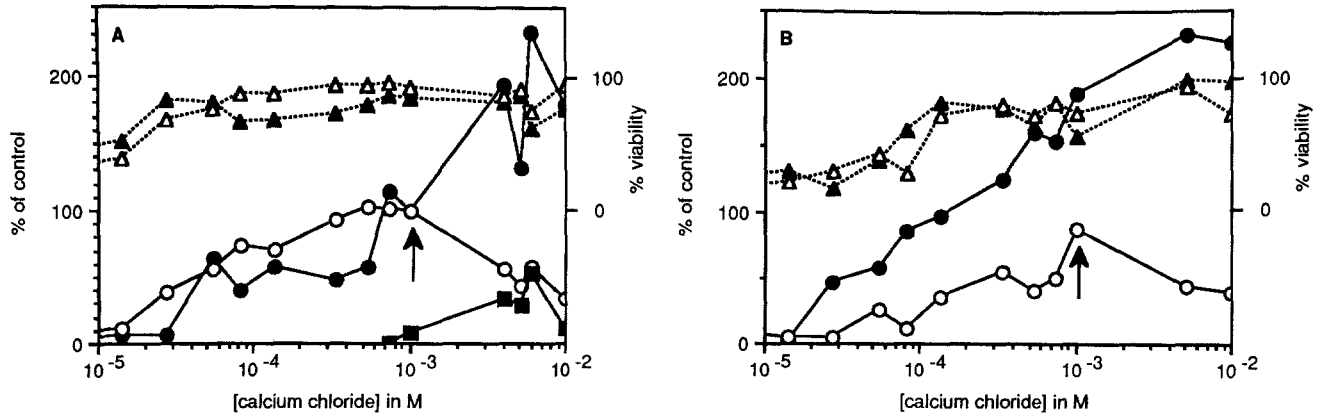

Fig.1. Effect of calcium chloride on somatic embryogenesis (●) and unorganized cell proliferation (O). Included in the figure is cell viability in embryo cultures (▲) and in proliferating cultures (Δ). A Culture pregrown in 10^{-3} M $CaCl_2$; somatic embryos formed in the presence of 2,4-D are denoted by (■). B Culture pregrown in 10^{-4} M $CaCl_2$. For the experiments of Figure 1A as well as 1B the number of proliferating cells and the number of embryos are presented as the percentage of parallel control cultures pregrown in normal B5 medium with 10^{-3} M $CaCl_2$ and subsequently incubated in normal B5 medium with 10^{-3} M $CaCl_2$, with and without 2,4-D for proliferating and embryo cultures respectively. After 12 days, control embryo cultures contained 1200-1400 somatic embryos ml^{-1} and proliferating cultures contained 7 - $8 \cdot 10^5$ cells ml^{-1}. The arrow in Fig. 1A and 1B indicates somatic embryos and proliferating cells obtained in normal B5 medium containing 10^{-3} M of $CaCl_2$ from cultures pregrown in 10^{-3} M $CaCl_2$ (A) and in 10^{-4} M $CaCl_2$ (B)

Results

In Figure 1A the effects are presented of increasing concentrations of calcium on somatic embryogenesis in the absence of 2,4-D, on unorganized cell proliferation in the presence of 2,4-D as well as on the viability of both embryo and proliferating cultures. The cells used in this experiment were obtained, after sieving and dilution as described in Materials and Methods, from an embryogenic suspension culture maintained in B5 medium with 10^{-3} M of calcium . The results show that between a calcium concentration of $1.5 \cdot 10^{-5}$ M and the normal concentration of 10^{-3} M both the amount of somatic embryos obtained (closed circles) and the cell density in proliferating cultures (open circles) increase with the concentration of calcium. This indicates that at these reduced concentrations of calcium there is no specific effect on embryogenesis but rather a general inhibitory effect on cell metabolism. Below $3 \cdot 10^{-5}$ M there is a decrease in cell viability (open and closed triangles for proliferating and embryo cultures respectively), presumably due to calcium depletion of the medium. At calcium concentrations over 10^{-3} M there is a clear increase in the amount of somatic embryos which is actually paralleled by a simultanous decrease in cell proliferation. No change was observed in the rate of development of somatic embryos as a function of the calcium concentration (data not shown).

In Figure 1B the effects of increasing concentrations of calcium on embryogenesis and cell proliferation obtained from embryogenic suspension cells pregrown at 10^{-4} M calcium are presented. No changes in growth rate and the number of proembryogenic masses present were observed between a suspension culture maintained in medium with 10^{-4} M calcium when compared to a control suspension culture in 10^{-3} M (data not shown). The results show that there is an increase both in the amount of embryos obtained (closed circles) and the cell density in proliferating cultures (open circles) up to 10^{-3} M of calcium. At calcium concentrations over 10^{-3} M there is a further slight increase in the amount of somatic embryos formed, that is not paralleled by a simultanous increase in cell density in proliferating cultures. The viability of the cultures was slightly lower when compared to the cultures used in the experiment portrayed in Figure 1B.

When the data from Figure 1A and 1B are compared it is clear that the same increase of embryogenesis can be achieved when proembryogenic masses are transferred to medium with a ten - fold higher concentration of calcium, regardless of the initial concentration. This indicates that the change in calcium concentration during the initial stages of embryo development is responsible for the increased amount of somatic embryos rather than the actual concentrations used. The effect is best illustrated by the two-fold increase in the amount of somatic embryos obtained at 10^{-3} M calcium in Figure 1B when compared to 1A. An unexpected effect was that at calcium concentrations between $6 \cdot 10^{-3}$ and 10^{-2} M (closed squares in Figure 1A), a small amount of, mainly globular stage, somatic embryos developed in cultures supplemented with $2 \cdot 10^{-6}$ M of 2,4-D, a concentration normally sufficient to prevent embryogenesis completely. The described effects of calcium were essentially similar in one other embryogenic carrot cultivar tested, while no effect was seen on a non-embryogenic carrot suspension culture (not shown).

To determine whether the observed increase in the number of somatic embryos after transfer of cells to

media with higher concentrations of calcium could be correlated with increased amounts of embryo-related extracellular proteins (De Vries et al. 1988a), [^{35}S]methionine labelled extracellular proteins from cultures grown at different calcium concentrations in the presence and absence of 2,4-D were analyzed by SDS-PAGE. The results (not shown) indicate that no qualitative nor quantitative changes in the pattern of extracellular proteins occurs, other than those due to the removal of 2,4-D. This suggests that the promoting effect of calcium during initiation of embryogenesis is not correlated with a simultanous alteration in the amount and number of embryo-related extracellular proteins.

Discussion

In carrot suspension cultures, somatic embryos develop directly from one or several cells on the surface of meristematic cell clusters termed proembryogenic masses. In proembryogenic masses, the inner cells appear to be more vacuolated (Halperin and Jensen 1967). Upon initiation of somatic embryogenesis by dilution and simultanous removal of 2,4-D one or several of the cells on the surface of a proembryogenic mass divide parallel rather then perpendicular to the surface of the proembryogenic mass. The outer cell resulting from this unequal division then develops into a globular embryo, while the inner cell remains adhered to the proembryogenic mass and develops into a suspensor-like structure.

In this work we have demonstrated that by shifting the concentration of calcium present in the growth medium during early somatic embryogenesis the number of somatic embryos obtained can be increased approximately two-fold. This finding may be either the result of a higher efficiency of initial formation of somatic embryos from the same proembryogenic mass, or of more proembryogenic masses producing somatic embryos; the overall effect however, is a shift from unorganized proliferation towards embryo development, as indicated by the observed increase in the amount of somatic embryos while parallel proliferating cultures remained constant or even decreased in cell density. So far this is the only method we have found that increases embryogenic potential, i.e. the number of somatic embryos produced by a fixed number of precursor cells; in newly initiated suspension cultures the embryogenic potential of the cultivar used in these studies was achieved more rapidly, but was not increased by the use of conditioned media (De Vries et al. 1988b). We did not observe any changes in the type and amount of extracellular proteins that we recently showed to be correlated with carrot somatic embryogenesis (De Vries et al. 1988a).

The finding that at elevated concentrations of calcium the synthetic auxin 2,4-D is not able to completely prevent somatic embryogenesis, suggests that calcium partially counteracts the inhibitory action of 2,4-D on somatic embryogenesis. Increased concentrations of $CaCl_2$ in the medium has also been shown to promote embryo development from single suspension cells of carrot (Nomura 1987). In that work it was observed that the distribution of free calcium in developing early carrot somatic embryos was highly polarized, while addition of 2,4-D resulted in a uniform redistribution of free calcium.

Regardless of the exact mechanism of the promoting effects of calcium on somatic embryogenesis, it is clear from the results presented here that although the standard composition of B5 medium is optimal for unorganized proliferation, it is suboptimal for somatic embryogenesis. Therefore, optimizing the calcium concentration in media used for initiation of somatic embryogenesis is clearly beneficial.

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