Note
Duration of Treatment of Carrot Hypocotyl Explants with 2,4-Dichlorophenoxyacetic Acid for Direct Somatic Embryogenesis

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Received September 25, 1995

The relationship between the concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) and the duration of treatment with 2,4-D on direct somatic embryogenesis from carrot hypocotyl explants was examined. Elevated concentrations of 2,4-D (up to 100 mg/liter) reduced the duration of pretreatment required for successful embryogenesis.

Key words: carrot; hypocotyl; 2,4-dichlorophenoxyacetic acid; embryogenesis

Carrot somatic embryogenesis is usually done in a two-stage process. The first stage involves initiation of the growth of unorganized cells, such as callus cells or cells in suspension cultures, and carrot cells from various sources are cultured in a standard culture medium with 1 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-D). The second stage involves morphological development of these cells into somatic embryos occurred upon removal of 2,4-D from the culture medium. However, we developed a novel embryo-forming system in which carrot somatic embryogenesis occurs in the absence of 2,4-D directly in hypocotyl explants that have been incubated in a liquid Murashige-Skoog (MS) medium containing 2,4-D for 12-24 h. In this system, somatic embryos develop via the formation of a cluster derived from organized cell division of epidermal cells without an intervening callus or suspension-culture phase. The concentration of 2,4-D used in this system was originally chosen as 1 mg/liter, as is generally used for induction of callus and suspension cultures. In view of the potential role of 2,4-D in somatic embryogenesis, this study was done to examine the relationship between the concentration of 2,4-D and the required duration of treatment with 2,4-D of hypocotyl explants for somatic embryogenesis. We show here that an increased concentration of 2,4-D allows a considerable reduction in the duration of treatment of carrot hypocotyl explants for somatic embryogenesis.

Carrot (Daucus carota L. cv. Koshingosum) seeds were surface-sterilized in 70% ethanol under a vacuum and then with 5% sodium hypochlorite for 10 min. They were washed extensively in sterile water and then allowed to germinate for about 2 weeks at 26 °C in darkness on basal MS medium that contained 3% (w/v) sucrose and had been solidified with 0.2% Geltrite. Hypocotyl explants without apical meristems were removed from the seedlings and cut into 1-cm pieces. These hypocotyl explants (36 pieces) were placed on solidified MS medium with 2,4-D (100 mg/liter) and sucrose (3%, w/v). After treatment with 2,4-D for 5 min to 12 h, the hypocotyl explants were washed thoroughly with hormone-free MS liquid medium that contained 3% sucrose, transferred to hormone-free solidified MS medium and then cultured for 30 d.

Figure 1 shows the relationship between duration of pretreatment of carrot hypocotyl explants with 2,4-D and the concentration of 2,4-D required for somatic embryogenesis on solidified MS medium. The explants were cultured on hormone-free solidified MS medium for 30 d after they were treated with 2,4-D at the indicated concentration for the indicated period. The rate of somatic embryogenesis is represented by the number of hypocotyl explants that formed globular embryos on their epidermal tissues. Pretreatment of explants with 2,4-D concentration of 1 mg/liter used in the previous system required more than 6 h for formation of somatic embryos (Fig. 1). An increase in the concentration of 2,4-D allowed a considerable reduction in the duration of treatment of explants. Treatment within 60 min of explants with 2,4-D at concentrations from 10 to 100 mg/liter allowed formation of embryos. Embryos were formed from 10% and 70% of the whole pieces of explants by 5 min- and 6-h-treatment with 10 mg/liter of 2,4-D, respectively. More than 50 mg/liter of 2,4-D caused embryogenesis at high frequency with treatment for 5 min. However, the frequency of embryogenesis were decreased considerably by treatment for more than 60 min in the case of these concentrations of 2,4-D.

Figure 2 shows the course of formation of globular embryos on explants on MS medium without hormones after treatment with 2,4-D at 10 mg/liter for 5 min to 24 h. For comparison, treatment of explants with 1 mg/liter of 2,4-D, as used in our

**Fig. 1.** The Relationship between the Concentration of 2,4-D and the Duration of Treatment of Hypocotyl Explants with 2,4-D Required for Formation of Somatic Globular Embryos

Explants (36 pieces) that have been treated with 2,4-D at concentration from 1 to 100 mg/liter for 5 to 720 min were transferred to MS solid medium without 2,4-D and then cultured for 30 d. The frequency represents the percentage of explants on which globular embryos appeared.

**Abbreviations:** 2,5-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; NAA, naphthaleneacetic acid; 2,4,6-T, 2,4,6-trichlorophenoxyacetic acid; PCIB, p-chlorophenoxyisobutyric acid.
original embryo-forming system, was also examined. Globular embryos began to form on day 16 of culture on medium without hormones after treatment with 10 mg/liter of 2,4-D for 30–60 min and torpedo-shaped embryos and plantlets began to form on day 21 and 25, respectively. The timing of formation of globular and torpedo-shaped embryos and plantlets after treatment with 1 mg/liter of 2,4-D for 24 h was in fair agreement with that observed after treatment with 10 mg/liter of 2,4-D for 60 min. When we treated samples with 2,4-D at 10 mg/liter, a duration of treatment of 60 min was most effective for embryogenesis. This result differed from that shown in Fig. 1. The best duration of treatment varied a little in each experiment, there was always a marked tendency for an increase in the concentration of 2,4-D during treatment of explants to allow reduction in the required treatment time for subsequent embryogenesis. As the duration of pretreatment was prolonged to 24 h, embryogenesis was greatly delayed. The data from treatment with 10 mg/liter of 2,4-D in Figs. 1 and 2 which were obtained from independent experiments showed that embryogenesis sometimes occurred after treatment for 5 min, but the most effective treatment times for embryogenesis were 60 min and 6 h. Thus, the results in experiments that were repeated three times fluctuated. Moreover, embryogenesis was greatly inhibited or prolonged by treatment with 2,4-D at higher than 10 mg/liter.

Embryos that were formed after treatment with less than 20 mg/liter of 2,4-D developed steadily into normal plantlets (data not shown). However, embryos that were induced by a brief treatment with 100 mg/liter of 2,4-D did not develop beyond the torpedo stage and no normal plantlets were generated (data not shown). Explants that had been treated with 50 mg/liter of 2,4-D for 10 min yielded both torpedo-shaped embryos and plantlets (data not shown).

In our novel embryo-forming system, we attempted to find whether other auxins such as IAA and NAA and anti-auxins such as TIBA, 2,4,6-T, and PCIB as well as 2,4-D were capable of inducing embryogenesis. The explants were treated with these auxins (1 mg/liter) and anti-auxins (1 mg/liter) for 24 h and then cultured in the solid medium without auxins and anti-auxins for 30 days. However, no embryogenesis occurred on the explants that were treated with IAA and NAA, and TIBA, 2,4,6-T, and PCIB, although formation of adventitious roots was induced on explants (data not shown).

Kamada et al.\(^3\) reported that somatic embryogenesis can be induced by such stresses as a high osmotic pressure due to sucrose, lengthy (two-week) exposure to heavy metal ions, and sodium hypochlorite. When apical meristems of carrot seedlings are put under these conditions, somatic embryogenesis occurs at a high frequency on the surfaces of the leaves that have developed during culture, without formation of visible callus. There was a possibility that in our somatic embryo-forming system, a high concentration of 2,4-D for a brief time may play an key role in the induction of embryogenesis as stress.

In this system, epidermal cells of hypocotyl explants seem to acquire the capacity to develop into somatic embryos during treatment with high concentration of 2,4-D for a very short time. Further work is needed to discover the early function of 2,4-D in the induction of embryogenesis.

References