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Study of Morphogenesis and Embryogenesis in *Daucus carota* L. in Different Sucrose Concentrations

***Abstract*: Many studies have been conducted on *Daucus carota* L. regeneration by somatic embryogenesis. Parameters like auxin concentration and sucrose have been altered to observe the net effect of the same on callusing and embryogenesis. We could obtain globular embryos in callus tissue grown in moderately high auxin-containing media for six months and subcultured in a solid MS basal medium. Suspension cultures without auxin but with varying sucrose concentrations when initiated with this callus also witnessed embryo formation at different rates.**

Keywords: MS basal medium, *Daucus carota*, 2,4-D, Callus culture, Morphogenesis, suspension culture, family.

Daucus carota (Family –Apiaceae) has long been the subject of study for *in vitro* regeneration of shoots through indirect somatic embryogenesis.[1,2,3,4] These studies have shown that the initiation of indirect somatic embryogenesis competence takes time in the presence of auxin.[5,6] The embryogenic cultures of carrots are initiated and multiplied in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). Removal or reduction of auxin is necessary to prevent inhibition of post-globular stages of embryo development.[7,8] On such a proliferating medium (with 2,4-D) callus differentiates into localized groups of meristematic cells called 'pro embryonic masses (PEMs) or "State I" cell clusters.[9] Thus, a high auxin concentration leads to asymmetric divisions with no embryo formation.[10] Generally, the embryogenic cultures of carrots are initiated and multiplied in a medium containing 2,4-D in the range of 0.5-1mg L^{-1} but embryo development happens when the callus is transferred to a medium with a very low level of auxin (0.01-0.1mg L^{-1}) or no auxin at all.[11,12]

There are also instances where embryogenesis is obtained without the aid of growth regulators but with high sugar concentration[13,14,15,16,17], or low pH of 5.6.[18] Among the available sugars, sucrose has been the most tested carbon source and osmoticum for somatic embryogenesis, usually at a concentration of 2-5% in plant species.

This experiment was carried out with the aim of determining whether a change in certain parameters, e.g. auxin (2,4-D) application, sucrose concentration in a liquid medium, and discontinuous shaking of suspension culture were capable of producing plantlets.

Materials and Methods: A fresh underground stem of *Daucus carota* was taken and washed thoroughly under running tap water to remove all surface debris and cut into 3-4 cm thick pieces. The underground stem pieces were then dipped into 1% Extran (e.Merck, Germany) for 10 mins and then washed, followed by surface sterilization by immersing in 0.1% (w/w) Mercuric chloride ($HgCl_2$) for 5mins. The root pieces were washed three times with sterile distilled water to remove the traces of $HgCl_2$. A series of transverse slices of 1 cm in thickness were cut from the underground stem with a sterile scalpel under a laminar airflow chamber. A block of $4mm^3$ across the cambium was cut from each piece so that each small piece contained a part of phloem, cambium, and xylem and inoculated in MS basal medium[19] with 3 mg L-1 2,4-D. The Uniform size and thickness of the explants were maintained. After inoculation, the culture tubes were transferred to a culture room maintained at 22 +/-2 °C with a relative humidity of 80-85% and a photoperiod of 16 hrs of light for a future experiment.

A portion, of the six-month-old callus, was further cut into small pieces of about $5mm^3$, and two different sets of subculturing were done. One part was subcultured in solid MS basal with 3 mg L-1 2,4-D (Set A) as control and another portion was cultured in solid MS basal medium without 2,4-D (Set B) for further dedifferentiation.

A morphological study was also done with the remaining callus tissue. The loose aggregate of cells was taken on a slide, squashed, and observed under the light microscope. A cytological study was done by staining some isolated cells in a 2% aceto-orcein N HCL (9:1) solution for an hour. After 1 h the cells were taken on a slide, squashed, and observed under the microscope.

After four weeks the dedifferentiated cells from Set B were further divided into 2 parts. Cell suspension culture was initiated with one part by transferring 0.2g of callus tissue to four 250ml Erlenmeyer flasks, containing 100 ml of the liquid MS basal media and different concentrations of sucrose each (30, 40, 50, and 60g L^{-1}, respectively). The liquid cultures were kept on the shaker for 4-5 h daily, instead of a continuous 24h. The other portion of the callus from Set B was allowed to grow in a solid MS basal medium without 2,4-D (same as Set B).

The rate and pattern of morphogenesis and embryogenesis were studied in both solid and liquid cultures.

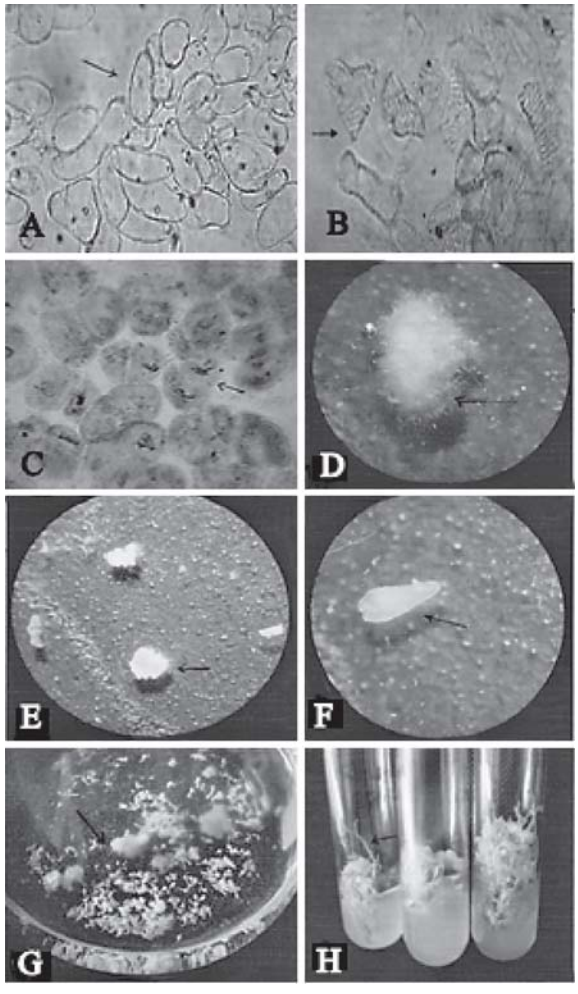


Fig. 1. Morphological and cytological study of cells in callus tissue, the different stages of embryogenesis and organogenesis in *Daucus carota*. (A) Oval to elongated cells in callus tissue. Bar= 1.5cm. (B) Callus tissue showing xylogenesis with tracheary elements, Bar= 1.5cm. (C) Dense cytoplasm without vacuole. Bar= 1.5cm. (D) Loosely aggregated cells in the suspension culture (40X). (F) Torpedo-shaped embryo (40X). (G) A large number of embryos in the suspension culture and shoot initiation. (H) Root and shoot development from callus grown in solid MS basal media without 2,4-D.

Results and Discussions: Callusing was observed within four days from the cambial tissues, inoculated in MS media with 3 mg L-1 2,4-D.

Morphological study of the callus of *D. carota* showed that there were round, oval and elongated cells (Fig. 1A). Callus tissue of *D. carota* also showed xylogenesis with tracheary elements[20] having a continuous spiral deposition of secondary wall materials (Fig.1B). Cytological study of callus tissue showed densely stained chromatin and dense cytoplasm and absence of vacuole (Fig.1C).

The rate of growth of the callus tissue subcultured in Set B was almost equal to that subcultured in Set A (Fig. 2), but it showed morphogenesis which was not found in Set A.

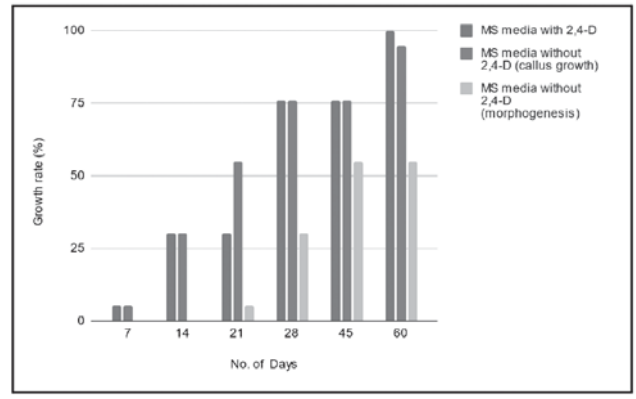


Fig. 2: Graph showing the rate of callus growth and morphogenesis of *D. carota* in solid MS medium.

Loosely aggregated cells were observed in the suspension cultures (Fig. 1D). Globular callus (Fig.1E) was observed after 20 days in the callus tissue of carrot inoculated in media without 2,4-D.

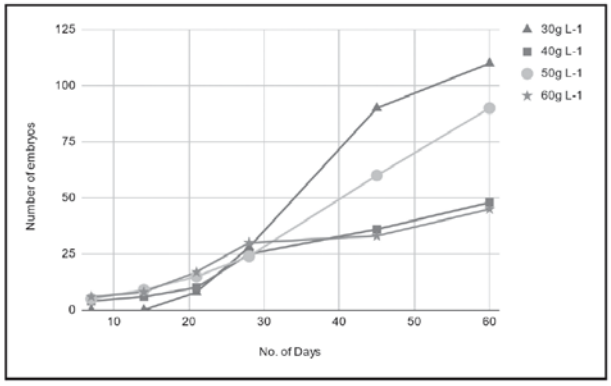
In the first set of suspension cultures (30g L-1 sucrose), the formation of an organ-like structure occurred first within 7 days, followed by the formation of embryos (Fig.1F) which were observed only after the end of the third week. After about 6 weeks there were numerous embryos and at the end of 2 months, a few matured embryos' showed initiation of shoot primordial (Fig. 1G).

In the second set (40g L-1 sucrose), initiation of embryo formation started within 7 days along with the process of organogenesis. With time, the number of embryos exceeded the number of organ-like structures. Few embryos were found in clumps, others developed separately.

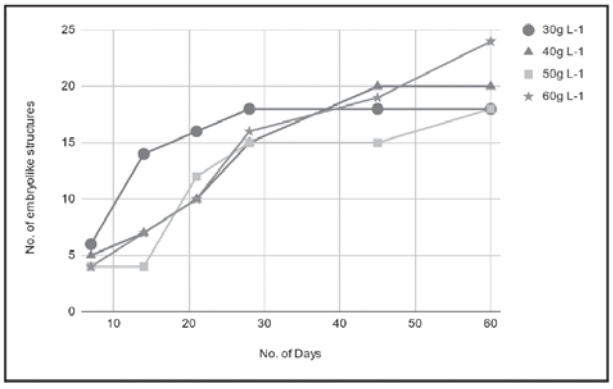
In the third set (50g L-1 sucrose), initiation of embryo formation started within 7 days along with the process of

morphogenesis. Embryo formation was mainly in clumps and very few embryos were found in isolation.

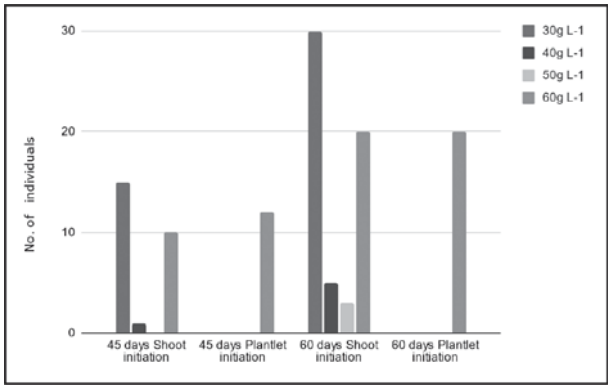
Finally, in the fourth set (60g L^{-1} sucrose), embryo formation started within three days of culture. The number of organ formations was more than in the above sets. Embryos formed mainly in clumps. After a month numerous embryos showed initiation of shoot primordial followed by root.



(a)



(b)



(c)

Fig. 3: Comparative studies of rates of embryogenesis, morphogenesis, shoot initiation, and plantlets formation in suspension cultures in four different sucrose concentrations. (A) Rate of embryogenesis. (B) Rate of morphogenesis. (C) Rate of shoot initiation and plantlets initiation in 45 days and 60 days.

From the results, we saw that callus could be initiated under a high amount of auxin (3 mg L^{-1} 2,4-D). Thereafter, embryogenic induction could occur without auxin. This deviation from the general observation could have happened because of the age of the callus. The callus was grown in auxin for 6 months before subculturing into an auxin-free media. Usually, callus were subcultured within a few weeks to initiate embryogenesis. However, here we found this prolonged exposure had not only initiated callusing but had also caused embryogenic induction. Hence, when the callus was a subculture in an auxin-free media, globular embryos were formed.

Usually, a high amount of sucrose was used to initiate embryogenesis under auxin-free media. Here also embryogenesis occurred under the high range of sucrose (30g, 40g, 50g, and 60g L^{-1}). The impact of sucrose concentration on embryogenesis was also different. Low amounts of sucrose showed delayed embryogenesis as compared to higher levels of sucrose used but ultimately set 1 (30g L^{-1}) had the maximum number of embryos than the other sets (Fig. 3A). Higher sucrose showed clumped embryo formation. The amount of morphogenesis in the fourth set (60g L-1) was more than in the first, second, and third set (Fig. 3B). Shoot development in the suspension culture started after two months (Fig. 3C). Even the callus developing in MS media without 2,4-D showed numerous shoot development followed by root formation (Fig.1H). So, it can be concluded that callus genotype or age can have an impact on the general pattern of somatic embryo formation.

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