

EMBRYOGENESIS INDUCTION WITH IAA AND IAA CONJUGATES IN CARROTS

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Abstract:

In order for cells to form embryos they must initially be exposed to auxins before being transferred into an auxin-free medium. Synthetic auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), generate embryos with genetic variations and other negative side effects. Natural auxins, like indole-3-acetic acid (IAA), can provide an alternative way to induce somatic embryogenesis. Our research has shown that it is possible to use amide-conjugated IAA in place of 2,4-D to induce embryo formation. It may be possible to use free IAA to accomplish the same feat. It has also been determined that ethanol-based stock solutions of IAA alter a culture's ability to form embryos, but water-based solutions of IAA do not. Water-based stock solutions of IAA are generated using the potassium salt of IAA, which does not substantially change the pH of culture medium. Cellular metabolism of IAA has been determined by examining the concentrations of IAA and its conjugates in cells and culture media that have been treated with IAA over a period of 24 hours. Based on preliminary data using ethanol-based IAA solutions, it would be necessary to replenish IAA daily to ensure that it acts as an effective auxin.

Introduction:

The ability to form embryos is conferred upon carrot cells by exposure to high concentrations of auxins (1,3). After this incubation, cells must be placed in an auxin-free environment before embryos can begin to develop (Figure 1). Researchers frequently use synthetic auxins, like 2,4-dichlorophenoxyacetic acid (2,4-D), to induce embryogenesis. Such synthetic auxins, however, do not yield uniform results. Specifically, 2,4-D may introduce genetic variation into embryos, which is not always desirable. Additionally, some plant species do not respond to 2,4-D at all.

Indole-3-acetic acid (IAA) is a naturally occurring auxin which is less likely to share the faults of its synthetic compatriots. Using IAA or conjugates of IAA instead of 2,4-D would allow somatic embryogenesis, without genetic variation, of rare or endangered plant life. Such somatic embryogenesis might also be used as a comparative model against zygotic embryos (5).

Our research is designed to test the feasibility of using IAA and IAA conjugates as auxins that may be used in conjunction with, or in place of, synthetic auxins such as 2,4-D.

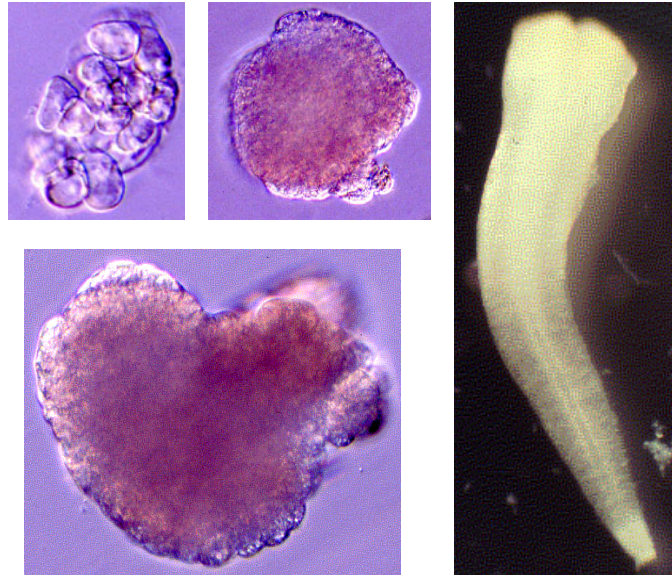


Figure 1. Stages in embryo development: A) undifferentiated carrot cells, B) globular, C) heart-stage, and D) torpedo-stage embryos.

Methods:

Culture Conditions:

Cultures were grown in medium containing MS salts and vitamins (Murashige and Skoog, 1962; commercial preparation) supplemented by 30g/L sucrose (pH 5.7). Cells were subcultured weekly.

Analyzing Cellular Metabolism of IAA:

Cells were grown in medium with IAA. Periodically, cells were harvested and examined for their free IAA, ester-linked IAA, and amide-linked IAA concentrations. Free IAA fractions were examined without treatment. Free + ester-linked IAA fractions were examined after a 1N hydrolysis with NaOH, and free + ester-linked + amide linked IAA fractions were examined after a 7N hydrolysis with NaOH (2).

Testing Conjugate Embryogenic Potential:

Cultures of embryogenic cell lines were filtered through mesh screens of varying sizes. Cell clusters between 43 and 109 μm in diameter were grown in medium without auxins for 1 week to remove residual 2,4-D. Cells were then grown in medium with conjugates for 4 weeks. Afterwards, cells were transferred into media without auxins and observed for embryo formation.

Testing IAA Embryogenic Potential:

For 4 weeks, IAA was added to cells in growth medium at regular intervals. At the end of the 4 week period, cells were transferred into media without auxins and observed for embryo formation.

Results and Discussion:

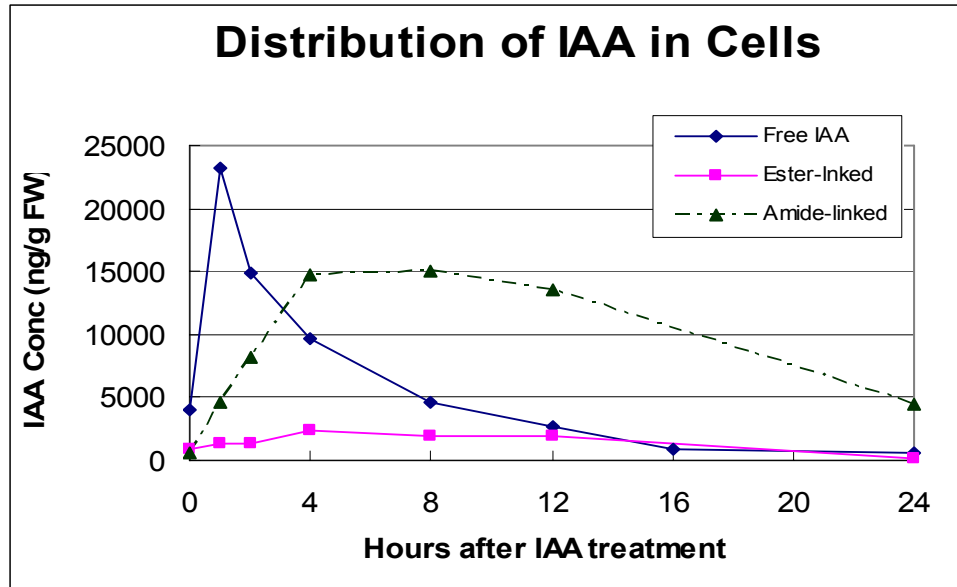


Figure 2: The metabolism of IAA and IAA conjugates in carrot cells over a period of 24 hours. At time 0, the medium contained 17 μM IAA. Peak at time 1 represents 4% of initial IAA concentration, or 2.9 μg .

It is evident that IAA does not remain in its free state for an extended period of time. Instead, it is converted primarily to amide linked IAA conjugates for the purpose of storage. Under natural conditions, IAA is degraded by light and enzymes, thus necessitating a plant's use of conjugates to maintain a sufficient concentration of auxins (2). Based on the above rate of degradation, it would be necessary to replenish the IAA concentration approximately every 24 hours if IAA is to act as an effective auxin.

We decided to test IAA as a natural auxin by adding it to medium either every day or every third day (Table 1). By adding IAA every third day we hoped to determine if IAA was an effective auxin in extremely low concentrations.

Treatment	Cell Lines Tested	Treatment Period			Post-Treatment
		2 Weeks	3 weeks	4 weeks	2 weeks
No auxin	DA, DB, DD, DE, DF, DG, DH, DI, DJ, DL, DM, DN, DO, DP, DQ, DR, DS, DT	Globular	heart & torpedo	torpedo	Torpedo
IAA added daily	DN	None	None	none	Globular
IAA added every 3 rd day	DA, DB, DD, DE, DF, DG, DH, DI, DJ, DL, DM, DN, DO, DP, DQ, DR, DS, DT	Globular	globular	globular	Globular
IAA-glycine	DN, DO	None	globular & heart	globular & heart	Globular & Heart
IAA-leucine	DA, DB	None	None	none	Globular
IAA-histidine	DA, DB	None	None	none	Globular
IAA-alanine	DF, DN	None	None	none	None
IAA-phenylalanine	DF, DO	None	None	none	None

Table 1. Embryogenesis during and after four weeks treatment with free IAA or IAA-conjugates. Stock solutions (20 mg/ml 95% ethanol) of IAA-conjugates were filter sterilized and added to culture medium to reach a final concentration of 57 μ M. Sterile IAA solution (20 mg/ml 95% ethanol) was added to reach a final concentration of 17 μ M.

The data in Table 1 demonstrates that amide conjugates IAA-leucine and IAA-histidine show promise as natural substitutes for 2,4-D. Our data also suggests that adding IAA to culture medium every third day is not sufficient to prevent embryogenesis, although it may slow embryo growth. It is interesting to note that after cells were transferred to an IAA free environment, the embryos that had already formed did not develop any further.

Up through this point in the experiment, IAA and IAA conjugates were applied to medium dissolved in ethanol. It is possible that ethanol, even at the concentrations in which we use it (8.7 μ M) may retard embryo formation. Previous research has shown that concentrations of 10 mM ethanol can be toxic to carrot cells. This research has also determined that the noxious effects of ethanol are derived from the acetaldehyde that is oxidized from ethanol, rather than the ethanol itself (4). Given that our experiment used ethanol in concentrations that were over 1000 times more dilute than that which was shown to be toxic, we did not expect undesirable results. This

expectation was not entirely bourn out—we did observe retarded embryo growth. We did not, however, encounter the levels of toxicity outlined in the aforementioned research.

To verify the hypothesis that ethanol was responsible for the observed retarded embryo formation, we grew various cell lines in 2,4-D medium, then transferred them to auxin-free medium and treated half the cells with ethanol (Table 2).

Cell Line	Age of Cell Line	EtOH? (Y/N)	Development after 1 week	Development after 2 weeks
DH	2 yrs	Y	No embryos	Globular to heart
		N	> 50% globular	Globular to torpedo
ED	1 yr	Y	Pre-globular	Pre-globular
		N	Pre-globular	>50% Globular
FA	2 months	Y	Pre-globular	Globular and Torpedo (3mm long)
		N	Heart to Torpedo	Plantlets (up to 1 cm tall)

Table 2: The inhibitory effects of ethanol on embryo formation. For the ethanol treatments, we added ethanol to 25ml of medium to reach a concentration of 8.7 μ M.

By the second week of observation, those cells that did not have ethanol added to them displayed more embryogenic potential than those that did. This furthers the idea that dilute ethanol slows embryogenesis and may therefore have influenced our earlier results.

It should be noted, however, that although our previous data was obtained using IAA in ethanol, the cell lines used were youthful enough to allow them some measure of protection from the effects of ethanol. Our most recent data suggests that although ethanol may retard embryo formation, it does not prevent embryos from growing beyond a certain stage (Table 2). Thus, the lack of further development of cells that had IAA added every third day may be attributed to the presence of IAA—to a certain extent, IAA acts as an effective auxin at extremely low concentrations. It is the frequency with which IAA is added to medium which controls its effectiveness (Table 1).

We are currently re-testing the cell metabolism of IAA using the potassium salt of IAA dissolved in distilled water. Adding this water-based stock solution to growth medium alters the pH by only $.0308 \pm .01$ pH units: an acceptable difference given that autoclaving growth medium can modify the pH by $.507 \pm .1$ pH units.

We believe that the overall rate of metabolism of IAA is not ethanol-dependant.

It is probable, however, that the initial spike in the free IAA concentration seen in Figure 2 was due to the presence of ethanol, given that ethanol generates a wounding response in plants.

Due to the relative youth of the cell lines used and the low concentration of ethanol, it is not likely that our results are overly skewed by the presence of ethanol. To verify this assumption, we are currently using a water-based IAA stock solution to confirm that IAA-leucine, IAA-histidine, and free IAA can be used as natural substitutes for 2,4-D.

References:

1. Ammirato, V (1985). *Patterns of development in culture*. In: Henke RR, Hughes KW, Constantin MP, and Hollaender A, eds., *Tissue Culture in Forestry and Agriculture*, pp. 9-29. Plenum Press, New York.
2. Cohen JD and Bandurski RS (1978). *The bound auxins: protection of indole-3-acetic acid from peroxidase-catalyzed oxidation*. *Planta* 139:203-208.
3. Kamada H and Harada H (1979). *Studies on the organogenesis in carrot tissue cultures. I. Effects of growth regulators on somatic embryogenesis and root formation*. *Z. Pflanzenphysiol.* 91:255-266.
4. Perata Pierdomenico and Alpi Amedeo (1990). *Ethanol-Induced Injuries to Carrot Cells*. *Plant Physiology: The Role of Acetaldehyde*. *Plant Physiology* 95:748-752.
5. Zimmerman JL (1993). *Somatic embryogenesis: a model for early development in higher plants*. *Plant Cell* 5:1411-1423.

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