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In vitro Responses of Embryoids of *Eschscholzia californica*

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Abstract. The *in vitro* embryoids of *Eschscholzia californica* do not form normal plantlets in cultures. However, 6-benzylaminopurine and gibberellic acid, when added to the medium, partially alleviate this inhibition. Experiments involving decotylization, and culture of embryoids at different stages, indicated the cardinal role of cotyledons in plumular growth.

The embryoids differentiate from the placental proliferations of *Eschscholzia californica* (KAVATHEKAR and GANAPATHY 1973). These do not develop into plantlets in the same culture medium in which they differentiate, or after transfer to a fresh medium of the same composition. This is in sharp contrast to *Daucus carota* (STEWARD 1970), and *Ranunculus sceleratus* (KONAR and NATARAJA 1969) where the embryoids develop into plantlets which even flower.

The present communication describes the behaviour of embryoids on basal Nitsch's medium, with or without casein hydrolysate, and experimental manipulations to obtain plantlets.

Material and Methods

The methodology for obtaining embryoids has already been described by KAVATHEKAR and GANAPATHY (1973). The cultures were maintained in a 12-h day photoperiod. The temperature was 22 ± 3 °C from September to April, and 28 ± 3 °C from May to August.

Subcultures

Embryoids which differentiated on the original explants were cultured in groups of more than two, along with a tiny portion of the parent callus, on the basal medium of NITSCH and NITSCH 1969 (hereafter referred to as NB) supplemented with casein hydrolysate, CH (500 ppm). These were allowed to grow for 18 weeks (wk), and then subcultured on the desired media with one embryoid a tube. One culture maintained on NB for more than 80 wk, and another on NB + CH for more than 110 wk, was subcultured nearly every 4wk.

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Decotylization

Mature embryoids were decotylized by sharp blades, under sterile culture conditions. Decotylated embryoids were then subjected to various treatments.

Stages of Embryoids Used

Embryoids of the following four stages were cultured:

Stage I = Late-globular (*ca.* 550 μm long);
Stage II = Cotyledon-initiation (*ca.* 750 μm long);
Stage III = Mature (*ca.* 2000 μm long); and
Stage IV = Hypocotyl elongation (*ca.* 1—2 cm long).
These were implanted singly on NB, and NB + CH.

Anatomy

For anatomical preparations routine procedures of fixation, dehydration, and embedding were employed (see JENSEN 1962). For localization of insoluble polysaccharides the sections were stained with periodic acid Schiff's reagent.

Results

I. Behaviour of Embryoids

The response of individual embryoids, subcultured on NB + CH (500 ppm), has been treated under three categories:

Behaviour of radicular pole

The radicular pole developed a translucent but compact callus, and the cells of peripheral layers had greater affinity to safranin-fast green. From these layers, secondary embryoids* differentiated. Within 2 wk globular embryoids were observed and, in another 2 wk, the embryoids matured. The differentiation of embryoids was asynchronous, and was accompanied by differential distribution of PAS-positive insoluble polysaccharides. The deep-seated cells of callus seldom differentiated embryoids. The embryoids that developed in contact with the medium were robust and had disproportionately massive and often fused cotyledons, whereas those that differentiated away from the medium showed a proportionate development. Their cotyledons were also discrete. The embryoids of both types rarely produced normal plantlets. Aberrant plantlets did develop and could be classified into four types: (i) plantlets in which the primary root was normal, but the hypocotyl and cotyledons were still embryonic; (ii) plantlets with normal primary root and hypocotyl, but the cotyledons were embryonic; (iii) plantlets with normal primary root and cotyledons, but the hypocotyl was embryonic; and (iv) plantlets with normal cotyledons and green foliage, but with abnormal hypocotyl and callusing of radicular pole.

Abbreviations used: NB — basal Nitsch's medium; CH — casein hydrolysate; wk — week.

* Hereafter the embryoid used as inoculum will be referred to as the primary embryoid, and the daughter embryoids arising from it as secondary embryoids.

Behaviour of hypocotyl

The hypocotyl of the explant showed slight enlargement, or it often callused. Further growth of the hypocotyl was retarded, due to proliferation at the radicular pole and the cotyledons. It could hardly be recognized during later stages.

Behaviour of cotyledons

In the majority of cultures the cotyledons neither elongated nor turned green. Instead, either they proliferated and showed a characteristic development resulting in the formation of embryonic mounds (KAVATHEKAR and GANAPATHY 1973) or callused. The callus was friable, and the distribution of starch within the callus varied depending on the position of cells, whether the cells were embryogenic or non-embryogenic, and whether specialized cells like tracheids were present or absent. The cells of the callus were capable of differentiating into embryoids. The origin, development, and structure of the embryoids resembled those from radicular pole callus.

II. Induction of Plantlet-Formation

The differentiation of secondary embryoids, from embryoids isolated and grown on NB + CH, was very common. Hence, attempts were made to transfer the embryoids to NB only (omitting casein hydrolysate) to determine if the differentiation of the secondary embryoid could be prevented, and normal plantlet formation consummated. To overcome the carry-over effect of CH, if any, the embryoids were repeatedly transferred to fresh NB medium. However, neither the capacity to differentiate secondary embryoids was lost, nor the inability to develop into plantlets overcome, even after 15 passages, each of 4—5 wk.

Effect of growth regulators

Some of the commonly used growth regulators were supplemented to NB, individually, at three concentrations (10^{-7}, 10^{-6} and 10^{-5}M), to induce 'germination' of mature embryoids. The growth regulators used were:

TABLE 1
Effect of decotylization on seedling formation in embryoids

Medium	Stage at inoculation	% cul			
		Tap root	CRE	Roots from CRE	Embryoids from CRE
NB	Cotyledons intact	0	83.3	0	75
NB	Cotyledons removed	0	83.3	0	83.3
NB + CH (500 ppm)	Cotyledons intact	0	100	0	100
NB + CH (500 ppm)	Cotyledons removed	8.3	83.3	0	83.3

* = The response of cotyledon(s) refers to cut surface.
Number of embryoids per treatment: 24, one embryoid per tube.

indole acetic acd (IAA); indole butyric acid (IBA); indole propionic acid (IPA); 2,4-dichlorophenoxyacetic acid (2,4-D); gibberellic acid (GA_3); 6-furfurylaminopurine (KN); and 6 benzylaminopurine (6-BAP).

In all the above treatments the response of mature embryoids was similar to that described on NB + CH. While there was no significant qualitative difference, there were marked quantitative differences.

Except with 2,4-D, with the other auxins there was prolific differentiation of secondary embryoids. With 2,4-D only callus developed. At 10^{-5}M the identity of explant was lost due to extensive proliferation. The callus was highly friable with intumescences; the core was, however, compact. When the callus was transferred to 2,4-D-free medium, 4—5 wk after culture, growth of the callus was inhibited but rhizogenesis occurred. If the callus was transferred to 2,4-D-free but CH-supplemented medium, extensive embryoid differentiation occurred within 2 wk but the growth of callus and rooting were poor. On continued maintenance on 2,4-D, a significant feature was the progressive differentiation of tracheids and inhibited embryoid differentiation.

Even on GA_3-supplemented medium the embryoids produced cotyledonary mounds, and the radicular pole callused. Occasionally, the hypocotyl also elongated.

The medium supplemented with cytokinins (KN or 6-BAP), led to partial greening of cotyledons and cotyledonary mounds. Secondary embryoid formation was poor. With BAP the plantlets showed only an active plumular pole. The cotyledons became green and flat, and 10—15 stubby leaves with reduced but dissected lamina were formed. Buds developed in the axils of cotyledons, as well as the leaves.

Thus, none of the seven growth regulators could bring about germination of mature embryoids into plantlets.

Decotylization

In the various treatments described so far, the embryoids behaved in a characteristic way with the cotyledons invariably overarching the shoot apical meristem (KAVATHEKAR and GANAPATHY 1973). This observation

tures

Hyp. callus	Hyp. embryonic	Callus from coty.	Mound from coty.	Embryoids from coty.	Leafy formation
41.6	58.3	50	50	83.3	0
16.6	75.0	66.6*	8.3*	25.0*	0
91.6	8.3	8.3	91.6	100	8.3
58.3	41.6	83.3*	16.6*	41.6*	50

Age of culture: 30 days (weekly observations were made).
CRE: Callus at radicular end.

permitted three assumptions: (i) the overarching of the shoot apical meristem by the highly active cotyledons suppressed the morphogenesis of plumule and, hence, plantlets did not form; (ii) the cotyledons act as a sink and, therefore, the chemical milieu fails to influence the shoot apical meristem; and (iii) both the physical (*i.e.*, i) and chemical (*i.e.*, ii) factors are jointly involved in plantlet formation. Logically, therefore, a decotylization experiment was conducted on 96 mature embryoids (*ca.* 2.0—2.5 mm) per replicate; embryoids with free cotyledons were chosen. Those with fused or cup-shaped cotyledons were deliberately avoided because, in them, the cotyledons could not be excised precisely. In each treatment half the number of embryoids were maintained as controls (Table 1); the remaining were decotylized.

Whether the embryoids were decotylized or not, in all the treatments the radicular pole callused followed by profuse differentiation of secondary embryoids. This indicates that presumably the cotyledons do not have any significant influence on the callusing and secondary embryoid formation at the radicular pole. In the decotylized embryoids the hypocotyl also callused, but to a lesser degree. Even the cut surfaces (due to excision of cotyledons) callused profusely. Three weeks after culture, the plumular pole of the embryoids did not show any response. On NB + CH 50 per cent decotylized embryoids produced normal leaves, and some had as many as 15 leaves in 30-day-old cultures. The leaves were present in a rosette, characteristic of the species. Leaf production being one of the parameters of development of embryoids into plantlets, this result is significant.

In the control cultures, the morphogenesis of plumular pole was rarely expressed. From this experiment the following conclusions can be drawn: (i) decotylization *per se* does not result in plantlet formation and, thus, the inability of embryoid to develop into plantlet is not due to the physical factor alone, *viz.*, the overarching of the shoot apical meristem by the cotyledonary mounds; (ii) neither the chemical factor alone, *viz.*, the addition/omission of CH to NB could induce the development of embryoid into plantlet; (iii) embryoids developed into plantlets with leaves only when they were decotylized and grown on NB + CH. Here, too, it was only in 50 per cent of the cultures.

Relation between stage of embryoid and plantlet development

In addition to the three conclusions referred to above, the decotylization experiment suggested that, in mature embryoids, the cotyledons might be exercising a chemical inhibition, rather than a physical one, on the morphogenesis of plumule. Therefore, the role of cotyledons was examined from two viewpoints: (i) is the presumed chemical inhibition of cotyledons related to the stage of embryoid development, or (ii) do the cotyledons contain inhibitory/promotery factors which do not permit the cotyledons to express themselves under experimental conditions? To follow this further (Table 2), the embryoids of four stages were cultured on NB, and NB + CH (500 ppm).

i) Response of embryoid stage I: The embryoids of stage I callused all over the surface, and secondary embryoids differentiated from the callus on both NB and NB + CH. Rooting also occurred, though much less, on NB + CH. The cotyledons did not initiate, and the embryoid failed to continue with normal, progressive development into stage II.

TABLE 2
Responses of different stages of embryoids

Stage of culture	Medium	% culture												
		Tap root	Laterals from tap root	Callused radicular polo (CRP)	Embryoids from CRP	Adventitious roots from CRP	Elongation of hypocotyl	Embryonic hypocotyl	Callus from hypocotyl	Embryoids from hypocotyl callus	Callus from cotyledon	Leafy cotyledons	Embryoids from cotyledons	Leafy condition
Embryoid globular stage	NB	0	0	100	91.6	91.6	0	0	0	0	0	—	—	8.3
	NB + CH (500 ppm)	0	0	100	83.3	33.3	8.3	0	0	0	0	8.3	0	0
Initiation of cotyledon	NB	0	0	100	100	8.3	0	0	0	0	0	0	83.3	33.3
	NB + CH (500 ppm)	0	0	91.6	91.6	0	0	0	0	0	0	58.3	41.6	50
Mature cotyledon	NB	4.1	4.1	95.9	54.1	16.6	12.5	79.1	8.3	0	8.3	0	79.1	12.5
	NB + CH (500 ppm)	0	0	100	79.1	20.8	33.3	41.6	25.0	0	16.6	4.1	79.1	4.1
Elongated hypocotyl	NB	50	41.6	45.8	33.3	41.6	100	0	8.3	8.3	12.5	54.1	29.1	25.0
	NB + CH (500 ppm)	54.1	54.1	45.8	41.6	37.5	100	0	8.3	8.3	8.3	75	4.1	37.5

ii) Embryoid stage II: The radicular pole of embryoid callused and differentiated into secondary embryoids, but no roots were formed. The greening of cotyledons occurred only on NB + CH. Leaf formation was observed on both the media, optimum on NB + CH. Thus it appears that with the initiation of cotyledons the embryoids acquire a capacity to express normal morphogenesis at the plumular pole.

iii) Embryoid stage III: The response was similar to that of stage I.

iv) Embryoid stage IV: Primary root formation occurred in nearly 50 per cent cultures in both treatments. In the remaining 50 per cent cultures the radicular pole callused. Often, the primary root also developed lateral roots. Irrespective of primary root formation, the hypocotyl elongated and became green; callusing was seldom noticed. In 25 to 50 per cent cultures leaves resembling those *in vivo* were formed.

The above observations permit the following generalizations: (i) an isolated embryoid continues normal ontogeny and matures only if it is past stage I at the time of subculture; (ii) the parent callus (from which the embryoids differentiate) influences embryoid development because only the globular embryoids left *in situ* (*i.e.*, in contact with the parent callus) developed further, and not those isolated and grown singly; (iii) the formation of leaves on plantlets from embryoid, of stage II and IV in the majority of cultures, especially on CH-supplemented medium, supports the contention that cotyledons may contain some factor(s) which affect plumular growth, and these factor(s) become operative at specific stages of embryoid development. This is further strengthened by the fact that in cultures of mature embryoids (stage III) leaf formation is inhibited.

Discussion

In *Eschscholzia californica* the embryoids though frequent seldom develop into normal plantlets with all the constituent parts. Some plantlets have roots, others hypocotyl, still others cotyledons, and plumular leaves. As an analogy to germination of seeds, this indicated that there are 'blocks' in the transition of embryoids to plantlets. Germination, according to the definition accepted by the International Seed Testing Association (1966), is the emergence and development from the seed-embryo of such essential structures which for the kind of seed tested indicate the ability to develop into a normal plant under appropriate conditions. By this rationale the prerequisites for further development of an embryoid in *E. californica* could be: (a) development of primary root, (b) elongation of hypocotyl, (c) expansion of cotyledons, and (d) plumular leaves. A continuity of morphological variants of plantlets was encountered in the present study. Therefore it called for an analysis of the factors involved in embryos developing into seedlings and their application to embryoids.

A survey of literature reveals that specific requirements for each stage in the development of embryo to seedling, or embryoid to plantlet have not been worked out. It may be presumed that a series of factors operate at different stages of the transition. Such factors are also operative in the release of dormancy of seeds *in vivo*. Thus, it indirectly suggests that the embryoids which fail to form normal plantlets may be like 'dormant embryos' within the seeds. The environmental as well as chemical factors which lead

to the release of dormancy in seeds (*see* KHAN 1975) were consequently studied with respect to embryoids. Chilling treatments of the embryoids produce partially developed plantlets (KAVATHEKAR *et al.* 1977). Auxins, gibberellic acid, and cytokinins do not stimulate normal development of plantlets. Gibberellic acid and cytokinins produce partially developed plantlets.

The cotyledons have been reported to be sources of promoters as well as inhibitors of germination (KAMIASKA 1973, ABOU-ZEID and NEUMANN 1973, THEVENOT and COME 1973). It is noteworthy that in *E. californica* the cotyledons, in the majority of plantlets, neither turned green, nor elongated. Instead, they gave rise to secondary embryoids. This aberrant behaviour of cotyledons, in some way, prevents formation of normal plantlets. This assumption together with the reports of ABOU-ZEID and NEUMANN (1973) and AUNG (1972) indicates the need for the study of the effect of decotylization. The results of our experiments (on NB) suggest that mere removal of cotyledons does not lead to leaf production, nor does it significantly influence the behaviour of radicular pole and hypocotyl. Moreover, the presence of cotyledons does not necessarily promote embryoid differentiation. The cotyledons exert an inhibitory effect on the differentiation of leaves on CH-supplemented medium. This reflects that some factor(s) responsible for leaf-initiation on NB + CH is/are rendered inoperative when the cotyledons are present (*i.e.*, a possible inhibitor in the cotyledons overcoming promotery factor(s) in the embryoids).

Concerning the presence of endogenous inhibitor in embryoids in *Carum carvi* AMMIRATO (1973) reported that the omission of abscisic acid from the medium resulted in plantlet formation from the embryoids. But, in *E. californica* there is no possibility of leachable endogenous water-soluble inhibitor as a causative factor because of the inability of embryoids to develop into plantlets even on repeated transfer to liquid media (KAVATHEKAR 1974). We took care of the possibility of chemical inhibitors being present in the cotyledons of embryoids (*ab initio*). These may be present at variable levels, at different stages and age of embryoids. An analysis of Table 2 reveals that only embryoids isolated and cultured with cotyledons were capable of further development. The differential responses of embryoids at stages II, III and IV suggest that inhibitory factor(s) may be present in the cotyledons. Such factor(s) may be entirely absent, or at a lower than the threshold level at stage II, and above the threshold level at stage III. At stage IV the inhibitor(s) may be absent or even if present, it/they is/are rendered ineffective by some promotery factor(s). At stage IV, the embryoid is characterized by an elongated hypocotyl.

There is urgent need for extraction and identification of the promoters/inhibitors in the embryoids not only at different stages of development but also during plantlet formation.

The embryoids of *E. californica* lack an organized root apical meristem which, in some way, may be responsible for the failure of primary root formation. Of the 3600 embryoids carefully screened, a little less than 100 formed primary root. During later stages the root pole callused and this response persists in spite of physical and chemical manipulations.

Our study indicates the presence of one or more 'blocks' during the development of embryoids into normal plantlets. It also highlights the complicated

integrated correlative relations that exist when a seed germinates to a seedling and the lack of such integrated correlative steps in embryoid to plantlet transition.

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BOOK REVIEW

FORD, E. B.: **Genetics and Adaptation.** — Studies in Biology No. 69, Edward Arnold (Publishers) Ltd., London 1977. 58 pp. £ 1.30.

This new book in the popular series is characterized by two very valuable approaches. First, it is the selection of themes usually omitted from the textbooks of basic genetics (*e.g.* principles of polymorphism and super-genes, mutation rate and the factors influencing it). Second, a large amount of facts and data, from both plant and animal kingdom, with which the content of some chapters is documented (Isolation and Adaptation, Selection). If it was the author's intention to evoke in the reader admiration of the dynamics, continuity and multiplicity of evolution processes, then he was successful. The chapters demonstrating the active contribution of mankind and of his activity (industrialization, exploitation of minerals) in producing selection pressures against plant species in nature (Pollution, Melanism, Genetic isolation and the flora of mine tips) belong among the most interesting. The only drawback of the book from the point of view of popularization is its assignation to readers acquainted with the principles of genetics; inclusion of a brief terminological vocabulary would be a positive contribution in this respect.

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