MORPHOGENESIS OF ISOLATED FLORAL BUDS OF *RANUNCULUS SCELERATUS* L. IN VITRO

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SUMMARY

Morphogenesis of floral buds excised at various stages of development was followed *in vitro*. The buds comprising the primordia of sepals and stamens (Stage I) failed to complete normal development on any of the nutrient media tried. However, the initiation and further development of carpels occurred even on a medium containing mineral elements, glycine, vitamins and sucrose. On the other hand, the buds having the anthers at the pollen mother cell stage (Stage II) completed microsporogenesis and 2-celled pollen grains were formed in the anthers. The torus *i.e.* the central dome bearing the carpels, of Stage II, and III (buds having anthers at pollen grain stage) elongated enormously and emerged through the folded sepals. Regeneration of roots and shoot buds (especially in Stage II & III buds) was common.

In addition, the floral buds of all stages formed callus which subsequently differentiated roots, shoot buds and embryoids leading to the formation of plantlets. The latter, in turn, developed embryoids from the epidermal and pith cells of the stem. On subculturing, the hypocotyl portion of the *in vitro* plantlet was capable of developing embryoids directly from the epidermal cells, whereas the radicular and plumular portions of the same plantlet first formed callus and subsequently embryoids.

The growth of callus and the differentiation of embryoids could be maintained through repeated subculturing. Embryoids could be induced even on a simple medium having only mineral elements and sucrose. Among the several growth adjuvants used, a combination of coconut milk and IAA supported best callus growth and normal embryoid differentiation.

1. INTRODUCTION

In Angiosperms, the flowers in spite of their short span of life are the seat of intense morphogenic activities. Although a large amount of data has accumulated on the physiology of flower initiation (LANG 1965), little is known about the growth and development of floral buds and their nutritional requirements. In recent years with the use of *in vitro* culture technique, isolated floral buds have been subjected to experimentation to understand the factors which cause the orderly development of flowers and also to study precisely their potentialities for morphogenesis.

This paper presents the results of our investigations on *Ranunculus sceleratus* regarding the growth behaviour of floral buds (*see also* KONAR & NATARAJA 1964), excised at various stages of development, on nutrient media and their capacity for unlimited proliferation with a high propensity for the cells to organize whole plants by direct organ formation or through stages reminiscent of embryogenesis.

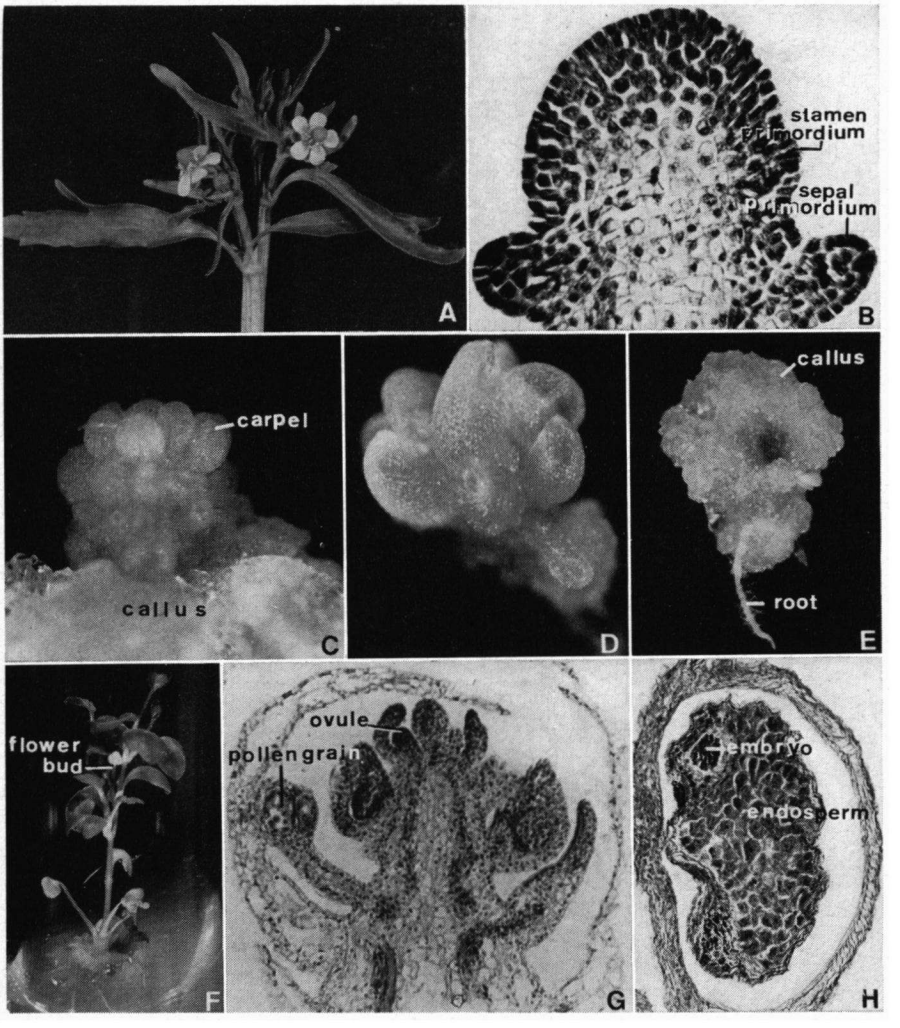

Fig. 1. A-H. A. A portion of a flowering twig. × 0.8 B. L.s. flower bud stage I at culture. × 218. C. The young spirally arranged carpels and profuse callus from the cut end on BM (3-weeks-old). × 22. D. Well developed carpels on BM (5-weeks-old). × 20. E. Three-weeks-old culture with a callus mass and roots on BM + CM (10%) + IAA (1 ppm). ×4. F. *In vitro* differentiated plant with a flower bud on BM + CM (10%) + IAA (1 ppm). × 1.5 G. L.s. *in vitro* developed flower bud at anthesis; note the anther sacs with pollen grains. × 88. H. *In vitro* seed in L.s. showing embryo and endosperm. × 83.

2. MATERIAL AND METHODS

Ranunculus sceleratus L., an annual herb, grows to a height of *ca* 90 cm and the main axis terminates in a flower (*fig. 1A*). The flowers (6–12 mm in diameter) are

bisexual, pentamerous with numerous stamens and carpels which are arranged spirally on an elongated torus. The following are the stages at which the floral buds were cultured:

a. *Stage I* – Buds with the primordia of sepals, stamens and a meristematic dome;
b. *Stage II* – Buds with anthers containing microspore mother cells. Of the carpels, the lower ones had the ovules with massive nucelli whereas the upper had only ovular primordia;
c. *Stage III* – Buds with anthers having 2-celled pollen grains and the carpels at mature embryo sac stage.

A modified White's medium with 2 per cent sucrose was used as basal medium (BM). Difco Bacto-agar (0.7%) was added to gel the medium and the pH was adjusted to 5.8. Casein hydrolysate (CH), coconut milk (CM), 2,4-D, GA_3, IAA, kinetin and yeast extract (YE) were added either alone or in various combinations to BM before autoclaving. In addition, supplements of H_1 medium of TEPFER *c.s.* (1963) were also tried.

Both tubes (15 × 2.5 cm) and petri dishes (8–9 cm diameter) were employed for cultures; each contained about 15 ml of the agar medium. For raising suspension cultures, the following quantities of liquid media were used: 10 ml for "tumble tubes", 100 ml for "nipple flasks" (500 ml capacity) and 50 ml for Erlenmeyer flasks (250 ml capacity).

To effect dissociation of the callus the "tumble tubes" and "nipple flasks" were mounted on a rotary shaker adjusted to 3 rpm, while the Erlenmeyer flasks were kept on a reciprocating action shaker at 90 strokes per minute.

The technique of raising cultures has already been described elsewhere (KONAR & NATARAJA 1964, 1965a and b). For each treatment a set of 24 cultures was raised and each experiment was repeated at least twice. All cultures were maintained in diffuse light (150–200 Lux for *ca* 10 hrs daily) at 25 ± 2 °C. In a few experiments one set of cultures was grown in dark (0 Lux) and another under comparatively intense continuous light (4000–6000 Lux).

3. RESULTS

3.1. Development of floral organs

Stage I: The flower buds of stage I at the time of culture were pale white to yellow and measured about 300 μ in width at the region of sepal primordia (*fig. 1B*). On BM they remained unchanged during the first week. The sepal primordia enlarged slightly and showed proliferation at the basal region. The cut end of the pedicel callused profusely and masked the sepals. Petal and stamen primordia failed to develop in any of the cultured buds. However, the torus enlarged considerably accompanied by the initiation and further development of the carpels in a spiral manner (*fig. 1C*). The number of carpels, with an ovule each, varied from 10–15 per flower bud as compared to about 100 *in vivo*. Each carpel was spherical, small (*ca* 0.5 mm) with a minute stigma. With

the advancement in age they elongated, slightly tapering towards the tip (*fig. 1D*) and gradually shrivelled; a few abscissed after 8 weeks of culture.

CM (10%) slightly accelerated the growth and development of callus as well as carpels. The latter attained normal size in the presence of IAA (1 ppm) alone or in combination with CM. The cut end of the pedicel formed 5–10 roots which bore numerous root hairs. Profuse callusing of explants was observed on medium containing both CM and IAA. One or two buds out of a set of 24 cultures showed complete differentiation of sepals in addition to carpels. These were small and did not enclose the torus during bud growth. The development of petals or stamens was not observed. Observations on responses of flower buds to various nutrient media are summarized in *table 1.*

Stage II: The flower buds of stage II cultured on BM, BM + CM (10%) and BM + CM (10%) + IAA (1 ppm) showed a general enlargement during the first week of culture. The sepals and petals failed to grow further and turned brown. Microsporogenesis in the anthers was normal and bi-celled pollen grains were formed, but the anthers failed to dehisce. The torus elongated considerably and emerged through the folded sepals. The carpels grew slightly, but shrivelled in 8 weeks. Roots were frequently formed from the pedicel. In about 25 per cent of the buds on BM and a majority in others proliferated rapidly from the cut end into a yellowish-white, friable callus.

In rare cases (2 or 3 out of 24 explants), on BM + CM (10%) + kinetin (1 ppm), the torus enlarged rapidly and branched to form several (10–15) spherical structures producing a fresh crop of carpels on them. Roots and shoot buds differentiated from the cut end of the pedicel. The flower buds showed only slight proliferation (*table 1*).

On BM + supplements of Tepfer's H_1 medium the explants showed slight proliferation and both roots and shoot buds originated from the pedicel. 2,4-D (1 ppm) alone or in combination with CM (10%) caused the proliferation of all the floral parts and within a week a copious amount of amorphous tissue was formed.

Stage III: As observed in cultures of stage II buds, the sepals, petals and stamens showed no morphogenesis on BM; the torus merely elongated and emerged through the folded sepals. Roots originated directly from the pedicel and torus in about 10 per cent of the cultures. Addition of IAA (1 ppm) to BM enhanced root production. On BM + CM (10%) + IAA (1 ppm) or 2,4-D (1 ppm), the buds produced roots, and also callused.

There was no proliferation of buds on BM containing CM (10%) and kinetin (1 ppm) or supplements of Tepfer's H_1 medium. Shoot buds emerged from the pedicel (*table 1*).

3.2. Differentiation of root and shoot buds in the callus

On BM alone or supplemented with CM (10%) the cut end of flower buds (of all stages) continued to proliferate rapidly resulting in a mass of whitish, friable callus after 2 weeks of culture. In a few cultures the shape of the original explants

Table 1. The response of floral parts to various nutrient media in cultures of floral buds of stage I, II and III. Growth period: 8 weeks.

Nutrient media	Sepals			Petals			Stamens			Carpels			General responses of entire buds
	I	II	III	I	II	III	I	II	III	I	II	III	
BM	*	–	–	–	–	–	–	***p	–	***	***	–	callusing of cut ends, rooting of II and III buds
BM + CM (10%)	*	–	–	–	–	–	–	***p	–	***	***	–	callusing of cut ends, rooting of II and III buds
BM + IAA (1 ppm)	*	–	–	–	–	–	–	***p	–	***	***	–	Slight proliferation and rooting of cut end
BM + CM (10%) + IAA (1 ppm)	*	c	c	–	c	c	–	**p	–	*	**c	c	Profuse callusing and rooting of cut end
BM + kinetin (1 ppm)	–			–			–			***			
BM + CM (10%) + kinetin (1 ppm)	–	–	–	–	–	–	–	***p	–	***	***	–	Callusing and shoot buds formed in II and III buds
BM + GA_3 (1 ppm)	*			–			–			***			
BM + CM (10%) + GA_3 (1 ppm)	*			–			–			***			Callusing of cut end
BM + TEPFER'S supplements	c	–	–	–	–	–	–	**p	–	–	–	–	Profuse callusing of I buds
BM + 2,4-D (1 ppm) and BM + CM (10%) + 2,4-D (1 ppm)	c	c	c	–	c	c	c	c	c	–	c	c	Profuse callusing of I buds

– = No growth, * = Poor growth, ** = Fair growth, *** = Good growth, p = Pollen grains formed, c = Callused but no normal growth.

was completely distorted due to the proliferation all over including the apex. In about 4 weeks, roots (1–3 per culture) differentiated from the callus. Shoot buds were formed independent of roots and eventually developed into slender shoots (8 cm in height). The radical leaves were formed in whorls of 3–6 per shoot. These were entire or slightly notched and had rudimentary stomata on the lower surface. The main axis terminated in a flower bud in about 10 weeks.

On BM + CM (10%) + IAA (1 ppm), roots (*fig. 1E*) and shoot buds appeared within 3 weeks of culture. A whorl of 5–15 radical leaves with long, thick petioles developed on the shoot and the latter terminated in a flower bud (*fig. 1F*). The flower buds were normal except for their miniature size. The number of stamens and carpels was very much reduced (8–10 and 10–20 respectively). Microsporogenesis in the anthers was normal and resulted in 2-celled pollen grains (*fig. 1G*); the anthers, however, failed to dehisce. The pollen grains were viable (tested by germinating them *in vitro*). A week after anthesis, all the floral organs except the carpels abscised. The latter increased in size and developed into achenes. However, the achenes were empty since no pollination could take place and only in an isolated case the seed contained a globular embryo and cellular endosperm (*fig. 1H*).

When the concentration of IAA was increased from 1 ppm to 2 or 5 ppm, the growth of callus and the size of the plants developed from it were proportionately reduced. Under continuous light (4000–6000 Lux) the shoots were short, and robust with several dense green leaves. In dark the plants were etiolated. However, callus formation and its subsequent differentiation into roots and shoot buds in cultures kept in continuous light and in dark were similar to those grown under diffuse day light conditions.

The callus originating from flower buds implanted on BM containing supplements of Tepfer's H_1 medium produced roots and shoot buds after 6 weeks of culture. On the contrary, neither roots nor shoot buds were formed on BM supplemented with 2,4-D (1, 2 or 5 ppm).

3.3. Differentiation of embryoids* in the callus

Besides root and shoot buds, numerous embryoids invariably differentiated in the callus. On BM the embryoids were visible in 6-week-old cultures (*fig. 2A, B*), but on BM + CM (10%) + IAA (1 ppm) they appeared earlier (3–4 weeks) and eventually developed into plantlets.

Stages in embryoid development resembling normal embryogeny were often encountered (*fig. 2C, D; see* KONAR & NATARAJA 1964 for further details). In a 4-week-old culture grown on BM + CM (10%) + IAA (1 ppm), various stages of embryoid formation were noticed. These could be easily separated

* In literature several terms have been designated for the embryo-like structures originating in tissue cultures. WARDLAW (1968) refers to these as *embryoids* or *pseudoembryos*. VASIL & HILDEBRANDT (1966a) have suggested that the term *embryo* could be used for all sexually produced structures and *embryoid* for all asexually or vegetatively produced embryo-like structures. In the present study also the term *embryoid* suggested by VASIL & HILDEBRANDT has been followed. Further, the term *proembryoid* has been used to describe the early stages of embryoid development.

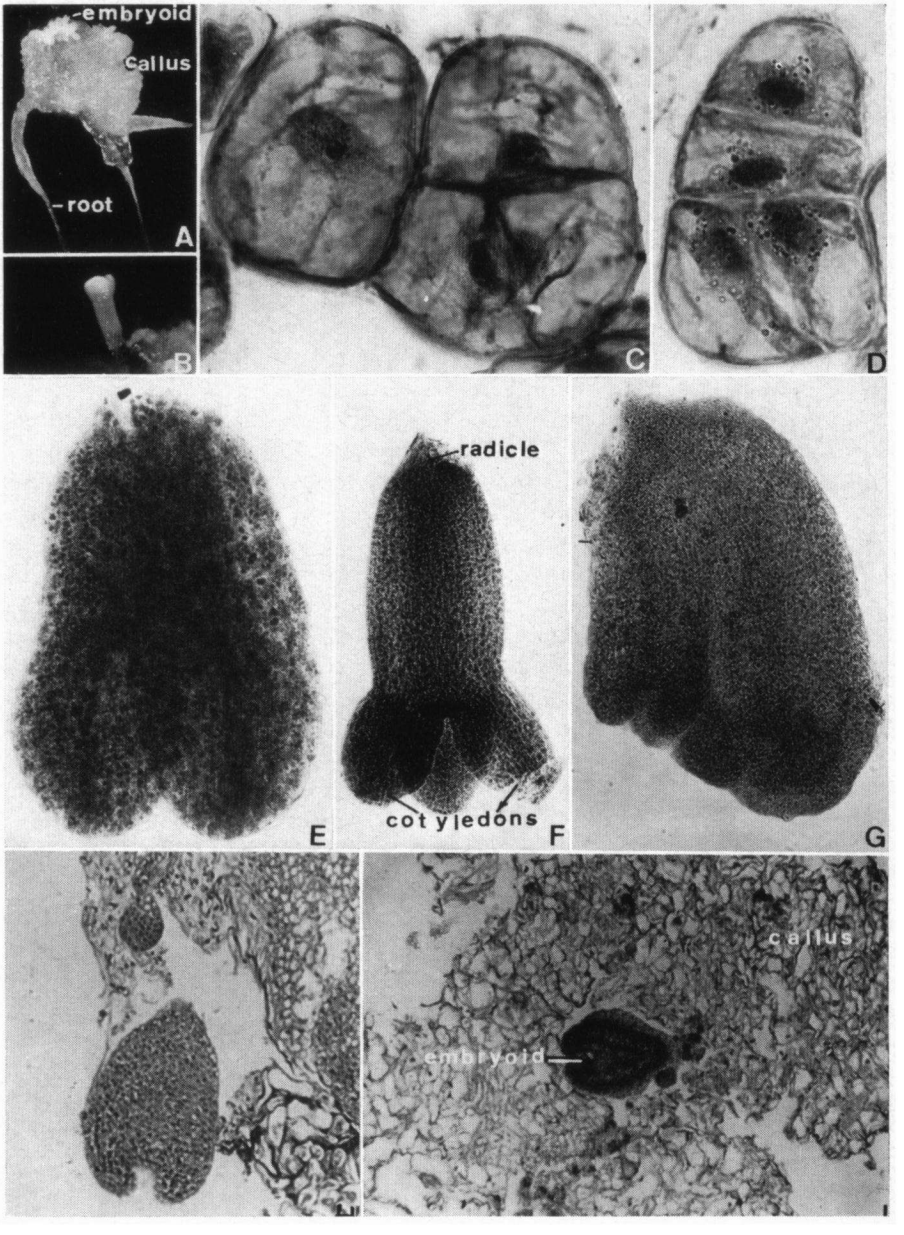

Fig. 2. A-I. A. A six-week-old culture on BM showing rooted callus. An aggregate of embryoids is seen on top. ×3. B. An embryoid magnified. × 11. C, D. Acetocarmine squash preparation of callus to show the early ontogeny of embryoid. C. × 135, D. × 60. E-G. Whole mount of mature di-, tricotyledonous and twin embryoids. E. × 130; F. ×128; G. ×50. H. Section through a portion of callus showing a globular and a heart-shaped embryoid. × 96. I. An embryoid differentiating deep within the callus. × 104.

from the friable callus. The embryoids were connected to the callus mass by suspensor-like structures. The mature embryoids were virtually indistinguishable from the seed embryos except for their larger size and better differentiation of the cotyledons. Each embryoid possessed a radicle-plumule axis and two (rarely three or four) well-developed cotyledons (*fig. 2E, F*). In general there was no definite orientation of the embryoids with respect to the surface of the callus.

The development of mature embryoids into plantlets resembled the stages in germination of a dicotyledonous embryo in nature. Occasionally, twin embryoids were also noticed which were fused all along the axis and had a common radicle (*fig. 2G*). Each of the twin embryoids generally showed 2 or 3 cotyledons with distinct vascular strands. Plantlets obtained from such embryoids had twin shoots.

Histological studies of the callus revealed that the embryoids originated either from the periphery or from the deep seated cells of the callus (*fig. 2H, I*). In globular embryoids, the protoderm and the ground meristem were easily distinguishable, whereas the late heart-shaped embryoids showed differentiation of provascular strands.

Contrastingly, on BM containing 2,4-D (1 or 2 ppm) alone, the differentiation of embryoids occurred after 10–12 weeks of culture. The majority of them possessed multiple or lobed cotyledons. The mature embryoids developed into plantlets, which ceased to grow beyond 2 cm in length due to the fasciation and proliferation of the radicular end and the hypocotyl. Nonetheless, normal plants were obtained when the embryoids were isolated and transferred to BM. In about 12 weeks, these *in vitro* plants flowered. At 5 ppm the embryoids failed to appear even though the callus continued to proliferate.

Addition of CM (10%) to the 2,4-D medium resulted in the differentiation of embryoids in about 8 weeks. Embryoids with 2 or 3 cotyledons were rare compared to those with multiple cotyledons. Although the callus comprised several proembryoids in cultures grown on a medium containing supplements of Tepfer's H_1 medium, they did not mature. But when the callus with the associated proembryoids was transferred to BM, mature embryoids and plantlets were obtained.

Subculture of embryoids: Heart-shaped and mature embryoids developed into plantlets when isolated and transferred to a fresh medium. However, isolated embryoids on transference to BM + CM (10%) + IAA (1 ppm) showed proliferation at the radicular end to yield a fresh mound of callus from which numerous embryoids differentiated. If 2,4-D (0.5 or 1.0 ppm) was used instead of IAA, the isolated embryoids failed to develop into plantlets but yielded a yellowish-white, friable callus. The latter gave a fresh crop of embryoids in about 10 weeks time thus repeating the cycle of dedifferentiation and redifferentiation.

3.4. Differentiation of embryoids from the epidermal cells

The plantlets developed from the embryoids reached a length of about 4 cm in 4–5 weeks in all media except the one containing 2,4-D. A feature of interest

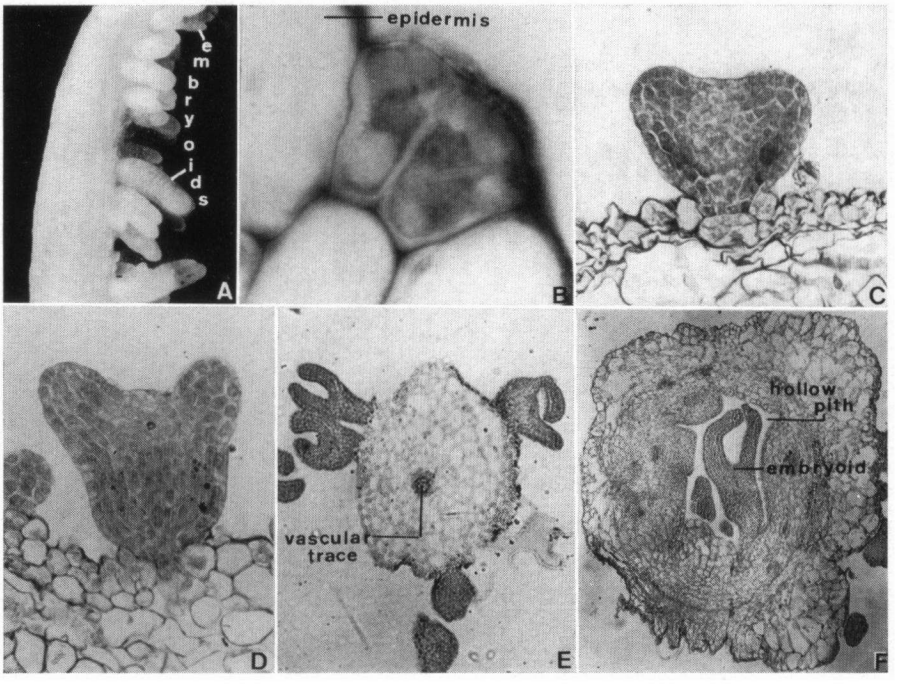

Fig. 3. A-F. A. Portion of stem of *in vitro* plantlet enlarged to show embryoids. × 13. B. A portion of stem in T.s. magnified to show an epidermal cell which has divided anticlinally. × 1270. C, D. Stages in the development of stem embryoids. C. × 205; D. × 108. E. T.s. stem of a plantlet developed *in vitro*. Note several embryoids. × 70. F. T.s. stem to show a cavity with a mature embryoid with well developed cotyledons. × 36.

was that the plantlets showed the development of embryoids all along the surface of the stem (*fig. 3A*). The number of embryoids on each plantlet was variable (5–50) and they were irregularly distributed. Six generations of these epidermal embryoids were studied and the cycle of embryoid – plantlet – embryoid could be successfully repeated.

Histological preparations showed that the embryoids originated from the epidermal cells of the stem (*fig. 3B–D; see* KONAR & NATARAJA 1965a). In no instance did the embryoids show vascular connection with the parent stem (*fig. 3E*). Surprisingly, embryoids were also found in the hollow region of the pith (*fig. 3F*). The appearance of stem embryoids, their distribution and ontogeny were similar in both dark and light grown cultures.

Subculture of different parts of the plantlet: Plantlets obtained from the embryoids before they developed stem embryoids, were divided into different parts and reared separately on nutrient media with a view to study their potentialities for morphogenesis and these responses are summarized in *table 2* (see also *fig. 4A–C*).

Table 2. Morphogenetic responses of explanted portions of the plantlet.

Media	Source of explants		
	Root	Hypocotyl	Plumule
BM	No growth, turned brown.	No growth, turned brown.	Several leaves were formed accompanied by roots from cut end and cotyledonary node.
BM + CM (10%) or BM + CM (10%) + IAA (1 ppm)	Proliferated into a white, friable callus in about 2 weeks. Embryoids and plantlets eventually differentiated, the latter in turn bore embryoids on stem surface.	Slightly swelled and bore embryoids directly on the exposed surface, the portion in contact with the medium formed roots and callus. Embryoids developed into plantlets which differentiated embryoids on stem surface.	Several leaves with long petioles were formed, the cut end and cotyledonary nodes developed roots. Profuse callusing was also noted on IAA medium which produced embryoids, plantlets and stem embryoids.
BM + CM (10%) + 2,4-D (1 ppm)	Callus brownish-yellow and friable embryoids developed after 10 weeks.	Soft, brownish-yellow, friable callus was formed, embryoids developed after 10 weeks.	Soft, brownish-yellow and friable callus, no leaves were developed, embryoids arose on the callus after 10 weeks.

The different portions of the same plantlet exhibited varied morphogenic responses. The radicular portion did not differentiate embryoids directly but on BM + CM (10%) or BM + CM (10%) + IAA (1 ppm), the cortical and epidermal cells proliferated to form a tissue from which embryoids and plantlets differentiated. The part of the hypocotyl which was in contact with the nutrient medium produced roots and callus, whereas the portion away from it developed epidermal embryoids. The plumular portion continued to grow further producing roots and new leaves on BM or BM + CM (10%); callus and subsequently embryoids on BM + CM (10%) + IAA (1 ppm). However, on BM + CM (10%) + 2,4-D (1 ppm), all the three types of explants yielded a mass of brownish yellow callus, which after 10 weeks gave rise to embryoids.

3.5. Subculture of the callus*

For the majority of experiments, portions of calli (*ca* 50 mg) growing on BM + CM (10%) + IAA or 2,4-D (1 ppm each) were used. When subcultured on BM, the callus showed proliferation followed by the differentiation of embryoids and plantlets in 2 weeks (*fig. 4D, E*).

Effect of sucrose: Stock cultures of callus were maintained on a medium containing 2 per cent sucrose. In the absence of sucrose, growth was inhibited. The differentiation of embryoids occurred at all concentrations of sucrose (0.2,

* The callus at subculture contained mostly parenchymatous cells.

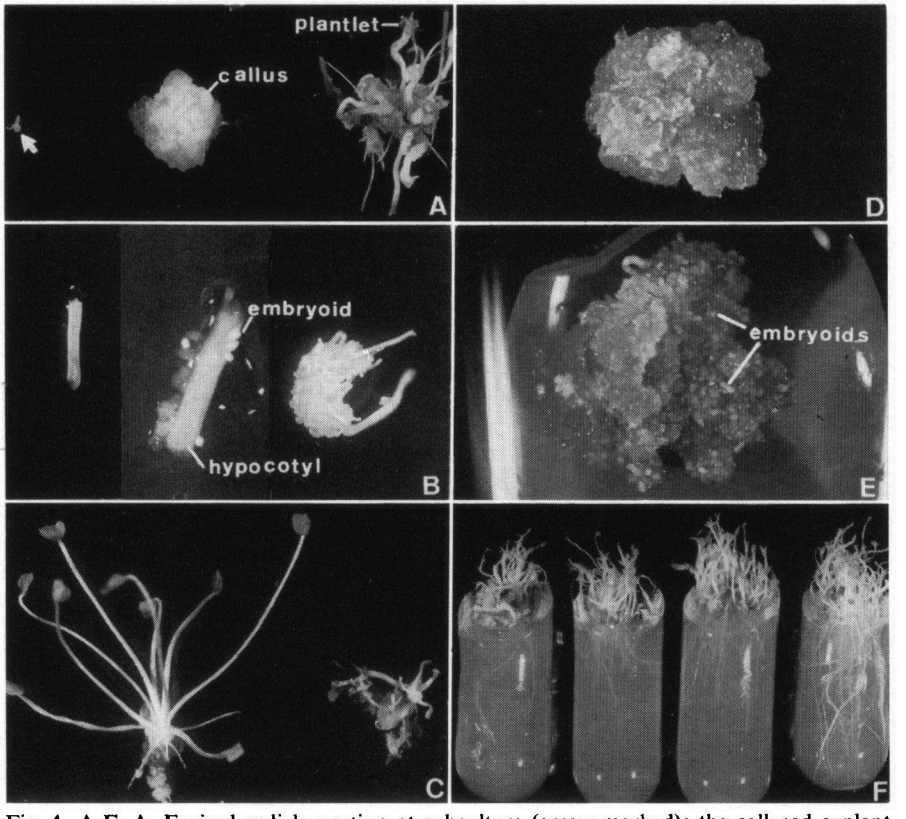

Fig. 4. A-F. A. Excised radicle portion at subculture (arrow-marked); the callused explant (middle, 2-weeks-old); and the differentiated callus showing embryoids and plantlets (right, 4-weeks-old). × 3. B. Excised hypocotyl at culture (left); with embryoids on its surface (middle, 2-weeks-old) with embryoids and plantlets (right, 3-weeks-old). × 2.5, 3.5 and 2 respectively. C. Plumular portion after 4 weeks in culture showing proliferated cut end and several newly formed leaves (left) on BM + CM (10%). On right, callus with embryoids and plantlets on BM + CM (10%) + IAA (1 ppm). × 3 D. Unorganized callus obtained on BM. × 3. E. Subcultured callus (2-weeks-old) on BM with numerous embryoids and plantlets. × 3.1. F. Callus 3 weeks after subculture on: (from left to right) BM without vitamins and glycine, BM without gylcine, BM without vitamins and BM. Note, that differentiation of plantlets occurs even in the absence of glycine and vitamins. × 0.5.

0.5, 1.0, 2.0 and 5.0%) but with increase in concentration to a certain limit there was an increase in the number of embryoids (*table 3*). However, at 5 per cent the callus showed prolific growth and the embryoid differentiation was considerably reduced.

Effect of glycine and vitamins: As the BM contained glycine and vitamins (calcium pantothenate, niacin, pyridoxine hydrochloride and thiamin hydrochloride) in addition to mineral elements and sucrose, an attempt was made to find out whether these were essential for callus growth and embryoid

Table 3. Effect of various concentrations of sucrose on the differentiation of embryoids and plantlets.*

Growth period: 4 weeks	Inoculum: *ca* 50 mg callus culture				
Concentration of sucrose (%)	No. of embryoids			No. of plantlets	Remarks
	Globular	Heart-shaped	Mature		
0	Nil	Nil	Nil	Nil	Callus turned brown
0.2	256	115	61	77	Plantlets slender, stunted, pale green with *ca* 0.5 cm long roots; about 20% showed stem embryoids
0.5	289	165	80	66	Plantlets robust, green with *ca* 3 cm long roots; about 25% showed stem embryoids
1.0	329	205	146	92	
2.0	362	229	152	98	
5.0	114	72	55	50	Plantlets stout with *ca* 7–9 cm long roots; about 5% showed stem embryoids

* Average of 6 cultures.

differentiation. Portions of unorganized callus growing on BM were implanted on BM from which either glycine or vitamins or both were excluded. Although the callus growth was greatly affected on BM without glycine and vitamins, the embryoids and plantlets continued to appear in 3–4 weeks (*fig. 4F*).

On exclusion of glycine alone the callus grew slightly and embryoids as well as plantlets appeared in almost the same time as above. The plantlets formed were pale green and root growth was better. On BM minus vitamins, the growth of callus, differentiation of embryoids and their subsequent development into plantlets was comparable to that observed on BM. The growth and differentiation of embryoids on BM without glycine and vitamins were similar in spite of three subcultures.

Effect of CH and YE: On BM with CH or YE (50, 100, 500 or 1000 ppm), the growth of the callus was initially profuse. Normal embryoids differentiated at all the concentrations within 3 weeks after subculture. The plantlets were brownish and developed secondary embryoids on their stem surface.

Effects of IAA, 2,4-D, kinetin and CM: Portions of unorganized callus (*ca* 20 mg each) were subcultured on BM alone or on BM supplemented with different concentrations of IAA (1, 2 or 5 ppm), 2,4-D (1, 2 or 5 ppm), kinetin (0.1, 0.5 or 1.0 ppm) or CM (5, 10 or 20%) individually or a combination of CM and IAA or 2,4-D or kinetin. After 4 weeks of growth, the cultures were scored for the number of embryoids and plantlets (*fig. 5*).

On BM + IAA (1, 2 and 5 ppm), the callus grew rapidly and embryoids appeared within 2 weeks after subculture. However, at 2 and 5 ppm of IAA, the

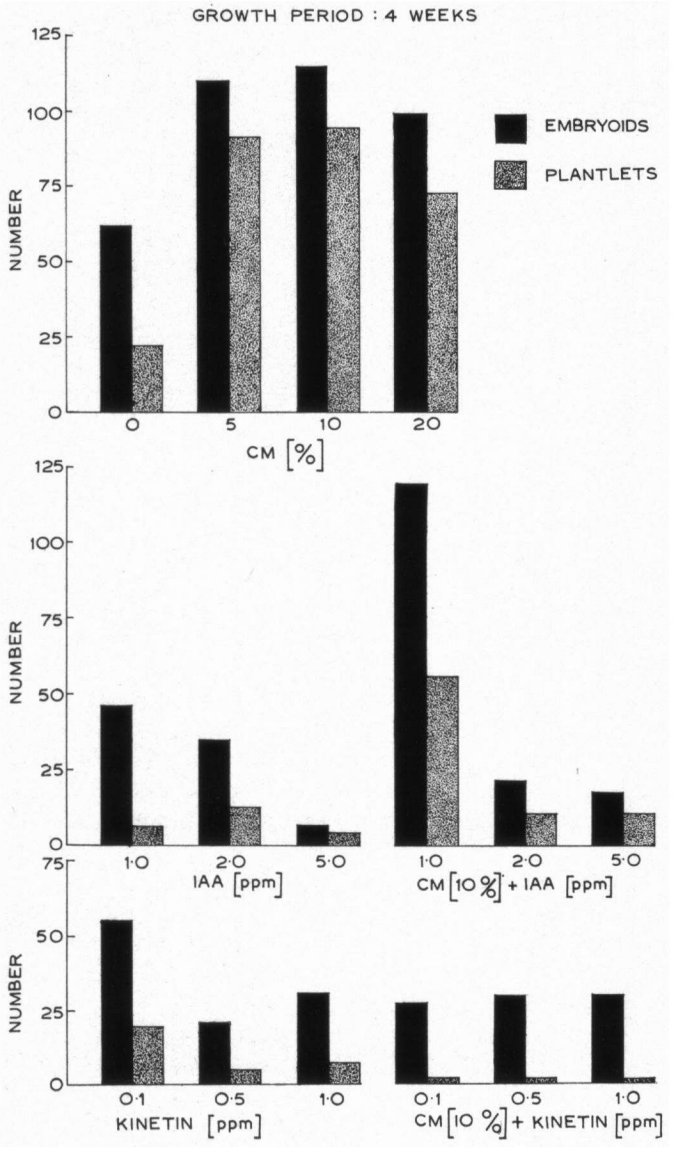

Fig. 5. Histogram showing the total number of embryoids and plantlets formed per culture (average of 6 cultures) on BM and BM with growth adjuvants.

radicular end and the hypocotyl showed fasciation and proliferation. On BM + 2,4-D (1, 2 and 5 ppm), embryoids were not formed even after 4 weeks, while on BM + kinetin (0.1, 0.5 and 1.0 ppm) embryoids differentiated. Root growth in the plantlets was poor on kinetin medium. On BM + CM (5, 10 and 20%)

embryoid differentiation was enhanced and within 2 weeks mature embryoids and several plantlets were formed. At 10 per cent CM the number of embryoids formed was maximal (*fig. 5*).

In the presence of CM (10%) + IAA (1, 2 and 5 ppm), the callus became yellowish-white, friable and mature embryoids were formed on BM + CM (10%) + IAA (1 ppm). Although there was a gradual decrease in the number of embryoids produced at higher concentrations of IAA (2 and 5 ppm) along with CM (10%), there was an increase in the growth and differentiation of embryoids and plantlets that were formed on BM + IAA (1, 2 and 5 ppm) alone. CM (10%) along with kinetin (0.1, 0.5 or 1.0 ppm) did not show any special feature over BM containing kinetin alone. A combination of CM (10%) and 2,4-D (1, 2 or 5 ppm) promoted only callus growth during the first 4 weeks (*fig. 5*).

3.6. Morphogenesis in suspension culture and on agar plates

In our earlier paper we reported briefly the formation of large numbers of embryoids in suspension cultures (KONAR & NATARAJA 1965b). The various stages in the development of embryoids leading to the formation of plantlets and flowering are illustrated in *fig. 6A-I*. The mature embryoids were snowy-white to pale green and had 2 or 3 cotyledons. The differentiation of cotyledons was poorer than that seen in embryoids on agar media. The presence of multiple and twin embryoids was common. The number and size of various stages of embryoids and plantlets formed in suspension cultures are given in *table 4*. Even when the flasks were removed from the shaker and kept on culture shelves, the plantlets continued to grow and flower buds were produced in 8 weeks (*fig. 6-I*). Fruit set was also observed.

A small quantity of suspension, consisting of free cells and clumps of cells when spread on the agar surface of the semi-solid basal medium, filled the entire surface with several cell colonies in about 4 weeks. However, the growth of cell colonies and embryoid differentiation was better on BM + CM (10%) + IAA + kinetin (1.5 ppm each).

Table 4. Number and size of embryoids formed in suspension cultures.

Growth period: 6 weeks Inoculum: 100 mg of callus

Stage	Number*	Size (μ)**		
		diameter	length	breadth
Pre-globular	1310	68.5	–	–
Globular	2720	157	–	–
Heart-shaped	1000	–	357	238
Mature	1200	–	260	277
Plantlets	800	–	2800	450

* Average of 10 flasks ** Average of 200

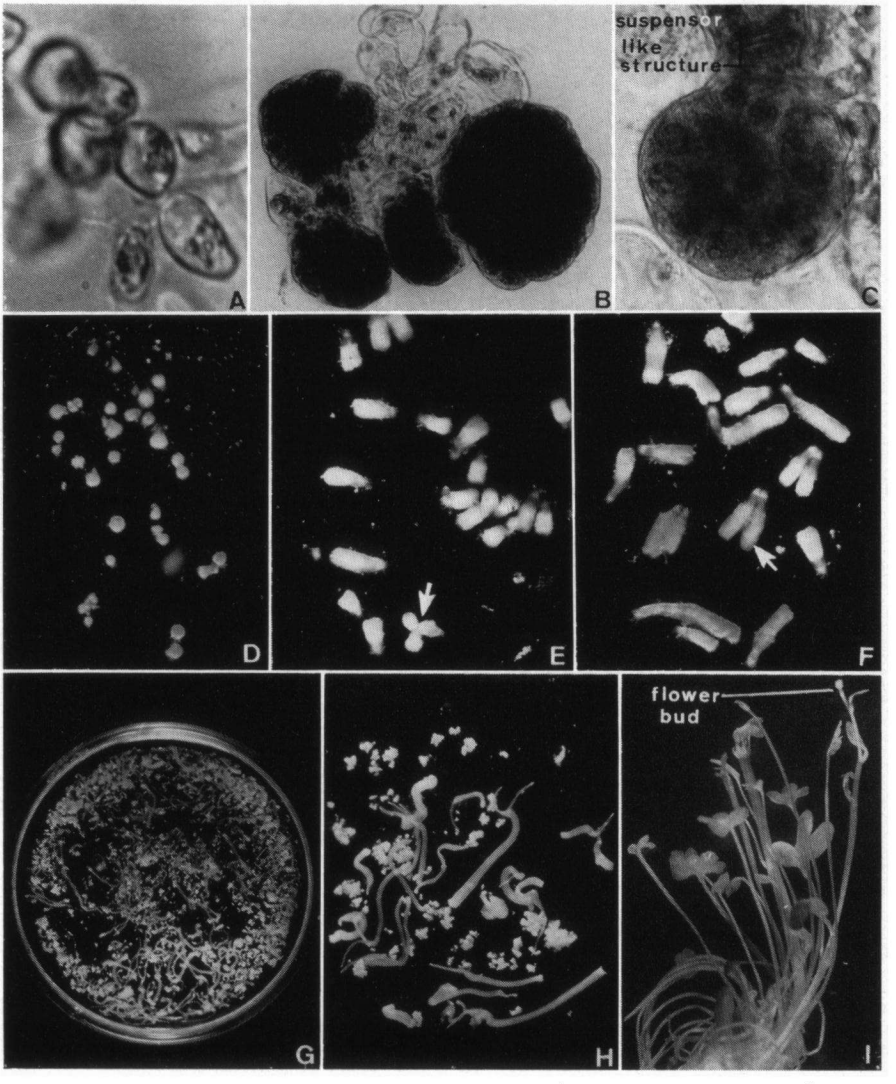

Fig. 6. A-I. A. Free cells from a-week-old culture on reciprocal shaker. ×300. B, C. Smear preparations of cell aggregates showing proembryoids. In the C globular embryoid has a clear suspensor-like structure. B. × 520; C. × 570. D-F. Globular, heart-shaped and mature embryoids respectively. The arrows in E and F indicate multiple and twin embryoids respectively. × 5. G. A 6-week-old culture with numerous embryoids and plantlets. ×0.8. H. A portion from G enlarged to show details. × 2.5. I. Mature plant with flower buds obtained in liquid media (the flasks were kept stationary after the formation of plantlets). × 1.25.

4. DISCUSSION

4.1. Growth of flower buds

The present study has revealed that the isolated flower buds of stage I failed to develop normally even in the presence of CM or other growth adjuvants. The initiation and normal development of carpels, however, occurred even on BM alone, though they were slightly smaller than those *in vivo*. In the presence of IAA (1 ppm) the carpels reached the normal size. IAA is known to promote ovary development in excised flower buds of *Cucumis* (GALUN *c.s.* 1962, 1963). Further, in *Ranunculus sceleratus*, ovules differentiated in the carpels. This is in contrast to the findings of TEPFER *c.s.* (1963) in *Aquilegia* (also a member of the *Ranunculaceae* where the ovules failed to differentiate even on a complex medium. The development of ovules is also reported in flower bud cultures of *Cucumis melo* (PORATH & GALUN 1967), *Viscaria candida* and *V. cardinalis* (BLAKE 1966). Thus, the nutritional requirements for normal development of flowers appear to be complex and the present study indicates that the flower buds, for some of their requirements, depend on the vegetative parts of the plant body which are not available in the medium.

Earlier studies have indicated that the flower buds excised at premeiotic stages failed to complete microsporogenesis (LARUE 1942; TEPFER *c.s.* 1963). But in *Cucumis* (PORATH & GALUN 1967) and *Viscaria* (BLAKE 1966) as well as in the present study (stage II buds) completion of microsporogenesis and pollen grain formation in buds excised at the premeiotic stage has been clearly observed. In these cases, probably the "meiotic stimulus" is already present in the explants before excision.

4.2. Regenerative capacities of flower buds

LARUE (1942) observed the failure of immature buds to produce roots even in the presence of IAA in the medium. But the mature flowers developed roots on a simple medium. In the present study, stage I buds were able to produce roots only in the presence of IAA in the medium, whereas those of stages II and III formed roots even in the absence of IAA in the medium. Auxin requirement for rooting of immature buds has been seen in *Kalanchoe* also (MOHAN RAM & WAHDHI 1968). Correlation between the age of flower bud and its auxin content has been noted in several plants (*see* LANG 1961). These observations are in agreement with the fact that auxin-like substances increase during floral development.

In *Kalanchoe globulifera* and *Nemesia strumosa* shoot bud formation was noted in cultured flower buds (LARUE 1942). The flower buds of stages II and III of *R. sceleratus* developed shoot buds in the presence of CM and kinetin or supplements of H_1 medium of TEPFER *c.s.* (1963). MOHAN RAM & WADHI (1968) have also reported shoot formation in excised flower buds of *Kalanchoe pinnata* in the presence of kinetin and 2,4-D.

4.3. Callusing and organogenesis

Callusing is a common phenomenon in tissue or organs cultured *in vitro*.

LARUE (1942) observed callus formation in isolated flower buds of *Hibiscus* and *Kalanchoe* on a simple medium. Similarly, the flower buds of *R. sceleratus* even on BM developed callus capable of differentiating into plants bearing flowers and fruits. CM and IAA promoted callus growth and differentiation of plants, but 2,4-D (1, 2 or 5 ppm) although it enhanced callusing, inhibited organogenesis. In *Aquilegia* (TEPFER *c.s.* 1963) high concentrations of IAA, kinetin or 2,4-D inhibited the growth of floral organs resulting in disorganization of the apical meristem of flower buds into a mass of callus.

4.4. Differentiation of embryoids

A landmark in the study of plant morphogenesis has been the induction of embryo-like structures from many parts of the plant body in tissue cultures (*see* STEWARD *c.s.* 1966). The ability of a somatic cell to develop into an entire plant simulating the stages in normal embryogeny is no longer a theoretical problem. The present study has demonstrated that callus obtained from the excised flower buds or from almost any part of the plant body (KONAR & NATARAJA unpublished) is capable of producing embryoids leading to the formation of plantlets. The latter in turn have the potentiality to develop embryoids from epidermal and pith cells. Thus, any diploid cell of this species, regardless of its position in the plant body is totipotent.

Resemblance of embryoids to stages in embryogeny: The embryogenesis in *Ranunculus sceleratus* is of the Crucifer type (MAHESHWARI 1950). Stages in embryoid formation starting from a single cell to di- or tricotyledonous embryoids has been traced in the callus, as well as from the epidermal cells of *in vitro* plantlets. Although the ontogeny of embryoids simulated the stages in normal embryogeny there was also considerable deviation from the normal pattern during early stages of their development (*fig. 3B*). These minor variations, however, did not affect the normal morphogenesis of embryoids and from the globular stage onwards they showed normal histological organization. JENSEN (1963) also considers that irregularities in cellular arrangement in zygotic embryos do not alter the eventual embryo development. The mature embryoids in *R. sceleratus* were markedly larger than the mature zygotic embryos, yet the general morphology was similar. Such similarities of embryoids to zygotic embryos have also been noted in *Daucus carota* (STEWARD *c.s.* 1963; NAKAJIMA 1963; HALPERIN 1966a) and *Petroselinum hortense* (VASIL & HILDEBRANDT 1966b). They also showed the presence of a suspensor.

The mature embryoids in *R. sceleratus* possessed well-developed cotyledons except those formed on 2,4-D media where they showed supernumerary and lobed cotyledons. HALPERIN & WETHERELL (1964) noted the twinning of roots and shoots, as well as the development of abnormally small cotyledons in carrot embryoids. The latter condition did not improve even in the presence of IAA, kinetin or 2,4-D either singly or in combination. Abnormalities of the cotyledons and twinning of embryos were observed in *Eranthis hiemalis* in seeds treated with solutions of 2,4-D, 2,4,5-T or NAA (HACCIUS 1955). These have been attributed to the interference of the above chemicals with the normal

dominance of the apical regions.

Is cell isolation a necessity for expression of totipotency?: Daucus carota has been extensively investigated in various laboratories for differentiation of embryoids (STEWARD *c.s.* 1966; KATO & TAKEUCHI 1963, 1966; HALPERIN & WETHERELL 1964; REINERT *c.s.* 1967). STEWARD *c.s.* (1963) emphasized the need for separation of cells from their neighbours and provision of special metabolites drawn from the environment of the zygote for the release of totipotency of any diploid cell in carrot. However, there is no report of embryoid differentiation from a single isolated cell or free cells in suspension. Suspension cultures of carrot and *Ranunculus* contain a mass of cells or cell groups besides free cells (STEWARD *c.s.* 1958, 1963; HALPERIN 1966a and b). But the potentiality of a somatic cell to develop into an embryoid simulating the stages in embryogeny has been established beyond doubt in the present study and also in carrot (STEWARD *c.s.* 1966). Soon after our report (KONAR & NATARAJA 1965a) differentiation of embryoids was also reported from the intact epidermal cells of excised hypocotyl segments of carrot (KATO & TAKEUCHI 1966) and from the cortical cells of *in vitro* differentiated roots of *Petroselinum hortense* (VASIL & HILDEBRANDT 1966b). CROOKS (1933), LINK & EGGERS (1946) described adventitious bud formation from the intact epidermal cells of seedlings of *Linum usitatissimum*. Thus, the need for cell isolation to express the intrinsic potentialities do not appear to be necessary.

Role of coconut milk and other growth adjuvants: The requirement of coconut milk for the initiation of embryoids has been much emphasized (STEWARD *c.s.* 1958, 1963, 1966). The investigations of KATO & TAKEUCHI (1963, 1966) on carrot and of YAMADA *c.s.* (1967) on *Solanum melongena* have revealed that embryoids can differentiate even in the absence of coconut milk in the medium. Also coconut milk has been shown to inhibit embryoid formation partially or completely depending upon the cultural conditions (HALPERIN 1966a, b). The present study demonstrates the initiation of embryoids even on a simple medium containing mineral elements and sucrose. The growth of callus and the differentiation of embryoids is accelerated by coconut milk, but embryoids can be initiated without it. Further, the present work shows that the role of reduced nitrogen (ammonium) in the formation of embryoids as pointed out by HALPERIN & WETHERELL (1965b) and KATO & TAKEUCHI (1966) does not hold true for *Ranunculus*.

In carrot, early stages of embryoid formation were obtained even at 1.0 and 0.1 ppm of 2,4-D, but, for their further development it was necessary to reduce the concentration to 0.01 ppm (HALPERIN 1964; HALPERIN & WETHERELL 1965a) or even delete it from the medium (NAKAJIMA 1963). The capacity for embryoid formation was lost if the callus was maintained through several passages on a medium containing 2,4-D, but could be reversed either by lowering the concentration of 2,4-D (0.01 ppm) or supplementing coconut milk or kinetin to the medium (HALPERIN 1966a). KATO & TAKEUCHI (1966) could substitute the effect of 2,4-D with high concentrations of IAA. In *Nicotiana*, HACCIUS & LAKSHMANAN (1965) obtained mature embryoids under high light intensity in a

medium containing coconut milk and kinetin. According to them, high light intensity lowers the concentration of growth substances on the surface of the callus, thereby promoting the differentiation of embryoids. In the present investigation 2,4-D (0.5–4.0 ppm) alone or with coconut milk was not inhibitory to embryoid formation even in dark or under low light intensity (150–200 Lux); only a delay in their appearance was noticed (8–12 weeks as against 4 weeks on BM). However, 5 ppm of 2,4-D inhibited the differentiation of embryoids. As in the case of zygotic embryos (*see* RAGHAVAN 1966), in the embryoids of *R. sceleratus* high concentrations of IAA induced fasciation and proliferation of the hypocotyl, and kinetin inhibited the growth of the cotyledons and root. In wild carrot, IAA (1 ppm) or kinetin (1 ppm) induced abnormal organ formation in embryoids (HALPERIN 1964).

Thus, *R. sceleratus* exhibits a remarkable plasticity to produce callus and subsequently embryoids. But the studies carried out on some other ranunculaceous members viz. *Consolida orientalis*, *Delphinium brunonianum*, and *Clematis gouriana* NATARAJA unpublished) have demonstrated that callus cultures could be readily established, but the differentiation of embryoids could not be consummated.

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