INDUCTION OF EMBRYOIDS IN REPRODUCTIVE AND VEGETATIVE TISSUES OF RANUNCULUS SCELERATUS L. IN VITRO

K. NATARAJA[1] and R. N. KONAR[2]

Department of Botany, University of Delhi, Delhi 7, India

SUMMARY

Isolated sepals, petals and anthers grown on a basal medium supplemented with coconut milk and 2,4-D yielded callus and embryoids resulting in plantlets. Carpels, however, developed callus and embryoids on the basal medium alone.

Seeds germinated in vitro and produced seedlings but failed to produce embryoids on their stem, in contrast to plantlets raised from embryoids. Formation of callus and embryoid differentiation was also noticed in cultures of parts of seedlings, shoot tips, segments of root, stem, petiole and lamina of *in vivo* and *in vitro* plants.

1. INTRODUCTION

During the last decade several studies have been made on the morphogenetic plasticity of cells and tissues and reports are available on the induction of embryoids in calluses obtained from various parts of the plant body. Earlier we reported the differentiation of embryoids in tissues derived from isolated floral buds and from epidermal cells of the stem of in vitro plantlets of *Ranunculus sceleratus* (KONAR & NATARAJA 1969). This paper describes the formation of embryoids from callus of both reproductive and vegetative parts, leading to the formation of plants bearing flowers and fruits.

2. MATERIAL AND METHODS

The cultures were raised from the following organs: a. floral parts – sepals, petals anthers, and carpels; b. seeds; c. portions of seedlings, viz. radicular, hypocotylar, and plumular; d. segments of root, stem, petiole, and lamina.

The techniques of preparing media and raising aseptic cultures have already been recorded elsewhere (KONAR & NATARAJA 1964, 1969). The basal medium (BM) was a modified White's medium; in several experiments it was supplemented with 10% coconut milk (CM), IAA, or 2,4-D.

A set of 24 cultures was raised for each treatment and maintained in diffuse light (150–200 lux for *ca* 10 hrs daily) at 25 ± 2 °C.

[1] Research Fellow, USDA Pl 480 Project 'Tissue and cell culture of Pines and allied Conifers' directed by Dr. R. N. Konar.

[2] Visiting scientist at the IVT, Wageningen.

3. RESULTS

3.1. Culture of floral organs

Sepals and Petals – The pale green sepals at the outset of culture measured about 3 mm in length, while the greenish-yellow petals were 2 mm, each petal bears a nectary cup at the junction of the claw and the limb. On basal medium (BM) alone or on BM supplemented with coconut milk (CM) or IAA (1 ppm) or both the sepals did not show any significant change. The petals proliferated slightly at the nectary cup, and the callus turned brown within 4 weeks. On BM + CM + 2,4-D (1 ppm), sepals (*fig. 1A*) as well as petals developed a mass of yellow, friable tissue within 6 weeks after culture. Several embryoids with variously lobed cotyledons were formed after a lapse of 10 weeks in culture (*fig. 1B*). The ontogeny of the embryoids was similar to that described for those originating in tissues of flower buds (KONAR & NATARAJA 1964). The mature embryoids developed into plantlets (*fig. 1C*).

The plantlets which ensued from the embryoids showed fasciation of the stem and proliferation of the root. However, if the callus from 6–7 week old cultures was isolated and subcultured in BM alone, numerous embryoids with normal cotyledons (2 or rarely 3) were formed within 2 weeks. The embryoids subsequently developed into plantlets. The latter bore numerous embryoids all along the surface of their stem (KONAR & NATARAJA, 1965).

Anther – Callusing and subsequent differentiation of embryoids and plantlets from the anthers cultured at pollen the grain stage has already been reported by us (1965b). Similar morphogenetic responses were noted in anthers implanted at the pollen mother cell stage. Rarely they completed microsporogenesis resulting in the formation of pollen grains (*fig. 1 D, E*). In the majority of the anthers, as evidenced by anatomical studies, the pollen mother cells either degenerated or produced callus.

Carpels – At the outset of culture these were pale green and each contained an ovule with well developed nucellus and integument. When reared on BM the carpels mostly turned brown and degenerated. However, a few proliferated from their cut ends in about 8 weeks after culture. The embryoids which differentiated from this callus were normal and developed into plantlets. These were studded with embryoids all along their stem surface within 2 weeks after they had been formed.

On supplementing BM with 2,4-D (1 ppm) alone or along with CM, the carpels showed a general increase in size, followed by proliferation yielding a soft, brownish-yellow tissue in 4–6 weeks (*fig. 1F*). Embryoids differentiated in about 8 weeks after culture on medium containing both CM and 1 ppm of 2,4-D (*fig. 1 G*). However, on BM + 2,4-D (1 ppm) the appearence of embryoids was delayed by 2–3 weeks. At 5 ppm of 2,4-D, the callus remained unorganized. Proliferation of carpels followed by the differentiation of embryoids also occurred on BM + CM + IAA (1 ppm). IAA (1 ppm) alone induced only rooting of the carpels.

Histological studies revealed that proliferation occurred from the wall and cut ends of the carpel (*fig. 2 H*). The ovules did not show any proliferation but

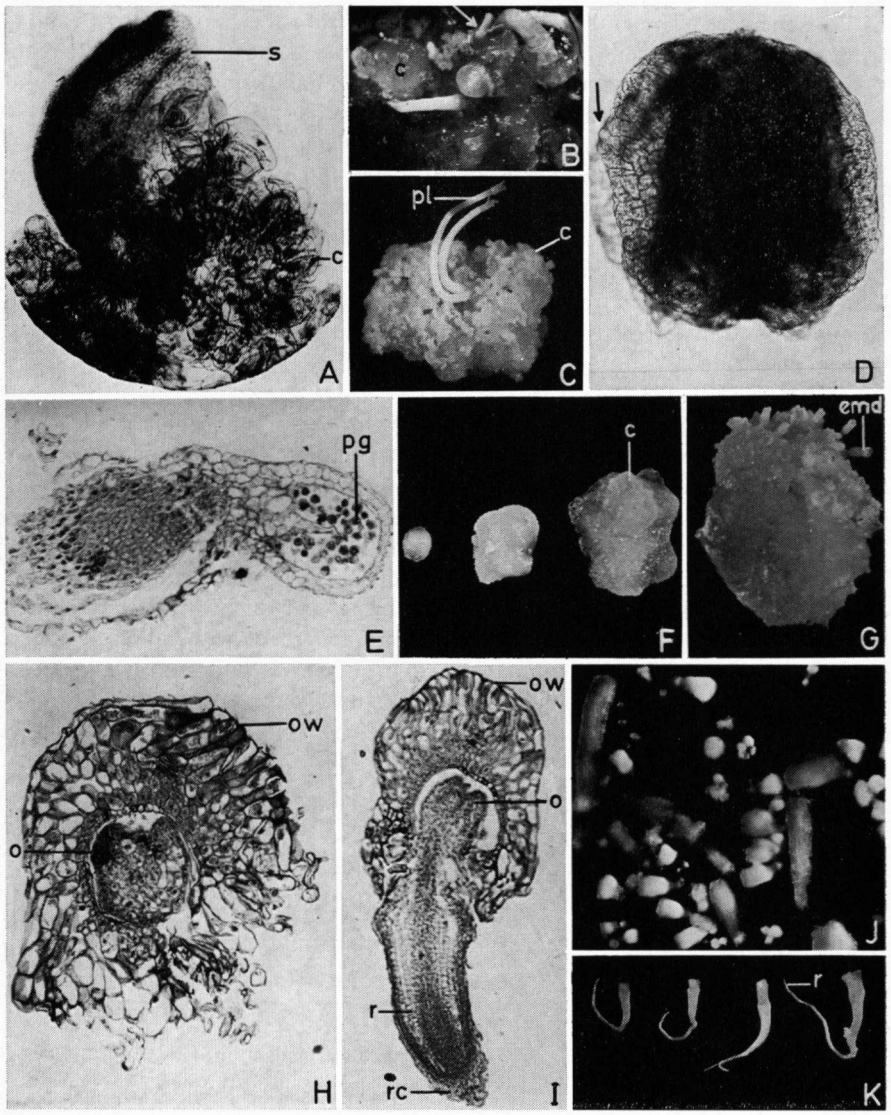

Fig. 1. A. Whole mount of a sepal after 4 weeks in culture. The cut end has proliferated. × 35. B, C. Callus masses obtained from petals with a dicotyledonous embryoid in B (arrow-marked) and young plantlets in C (10-week-old). B. × 3.5; C. × 3. D. Anther after one week of culture; note the bulged wall (arrow-marked). × 87. E. Same as D in l.s. showing the proliferating anther lobe at left and containing normal pollen grains at right. × 100. F. Two- (left), 4-(middle) and 6-(right) week-old cultures of carpels on BM+CM (10%) + 2,4-D (1. Omg/1) × 4. G. Embryoids differentiating from the callus in an 8-week-old culture. × 5. H. L. s. carpel (2-week-old) showing the proliferating wall. × 81. L. A. 2-week-old carpel in l.s. with a root from the cut end. × 58. J. Portion of suspension showing embryoids at various stages of development. × 10. K. Stages in the development of plantlets. × 2.

had degenerated. The embryoids differentiated from the peripheral as well as from the deep-seated cells of the callus. The roots had their origin directly from the carpellary wall or from the cut end (*Fig. 2I*), had a normal anatomy, and possessed a distinct root cap.

On transferring to a liquid medium in T-tubes and mounting the latter on a disc rotary shaker revolving at a speed of 3 rpm, the callus dissociated into free cells and small cell clusters. The morphogenetic responses were similar to those observed in callus of floral buds (KONAR & NATARAJA 1969). Within 6 weeks the tubes were filled with numerous embryoids of various stages, ranging from prelobular to mature ones (*fig. 1 J, K*). The embryoids eventually developed into plantlets. On transferring to agar medium the plantlests grew further, producing flowers and fruits.

3.2. Culture from seeds

Since the in vitro plantlets (developing from the embryoids) differentiated embryoids from the epidermal layer of their stems, an attempt was made to test if the seedlings formed by germinating seeds (obtained in nature) possess a similar potentiality. Germination of seeds occurred in all the media used (*table 1*). On BM the emergence of the radicle occurred during the first week. The germination was normal: the cotyledons elongated and turned green. Addition of yeast extract (500 ppm) to BM also favoured normal germination. The seedlings formed on BM + kinetin (1 ppm) were short and stout with intensely green cotyledonary leaves. The growth of the primary root was poor. On BM + GA_3 (1 ppm) the seedlings were long and slender; root growth was normal. Surprisingly, these seedlings failed to develop epidermal embryoids on any of the media tried (*table 1*).

Table 1. In vitro germination of seeds. On an average 200 seeds per treatment. Growth period 16 weeks.

Treatment	Percentage of seed germination	Remarks
BM	28.0	Seedlings normal, attained *ca* 5 cm height, no stem emb.
BM + kinetin		
0.5 ppm	37.0	seedlings small, stout; height from 0.5–
1.0 ppm	18.0	2.5 cm. Root growth poor, no stem emb.
5 0 ppm	11.0	
BM + GA_3		
0.5 ppm	24.0	Seedling slender, height 10 cm. Root
1.0 ppm	31.0	growth normal, No stem emb.
5.0 ppm	51.0	
BM + Yeast extract		
100 ppm	18.0	Seedling normal, attained a height of 5 cm.
200 ppm	22.0	No stem emb.
500 ppm	28.0	

\+ Growth period: 16 weeks
++ Average of 200 seeds

In nearly 30 per cent of the cultures on BM + 2,4-D (1 ppm), the seeds showed a slight swelling with the emergence of the radicle. The endosperm exhibited limited proliferation and then gradually turned brown. The radicular end produced a yellowish brown callus and the callusing later extended to the hypocotyl of the seedling. When transferred to BM, the callus produced several embryoids in 4 weeks. These subsequently matured into plantlets which bore embryoids on the surface of the stem.

Subculture of different parts of the seedling – The radicular, hypocotyl, and plumular segments of seedlings were reared in vitro with a view to study their potentialities for morphogenesis. The radicular and hypocotyl segments failed to respond to BM or BM supplemented with CM or IAA (1 ppm) or both. But on BM + CM + 2,4-D (1 ppm), they yielded a brownish friable callus from the cut ends in 2 weeks. The differentiation of embryoids from the callus occurred in the course of 8 weeks and the embryoids eventually developed into plantlets.

The plumular segment (*fig. 2A*, inset), comprising two green cotyledonary leaves, showed a slight enlargement on BM during the first week. The cotyledonary leaves became deep green and two or three fresh leaves emerged, accompanied by the initiation of roots from the cut end and from the cotyledonary node. The latter feature is common in the seedlings of *Ranunculus sceleratus in vivo* (TAMURA 1963). Further, growth of shoot and roots was normal (*fig. 2A*) and mature plants were formed. Similar responses were noticed in the presence of CM. On BM + CM + IAA (1 ppm) the shoot apices continued to grow further, but the cut end and the cotyledonary leaves produced callus and several embryoids from the later plantlets were formed in 3–4 weeks after culture (*fig. 2B*).

3.3. Culture of shoot tip, segments of root, stem, petiole and lamina

Finding that the isolated flower buds, the individual floral organs, and portions of seedlings possess a high degree of plasticity for callus formation and embryoid differentiation, it was thought worthwhile to test the morphogenetic potentialities of the vegetative organs of older plants. The responses of shoot tips comprising 2 or 3 leaf primordia were similar to those described for plumular portions of the seedlings.

Root – The segments of roots on BM, on BM + CM, or BM + IAA (1 ppm), or BM + CM + IAA turned brown and eventually dried. The cut end proliferated when the explants were grown on BM + CM + 2,4-D (1 ppm). The callus differentiated embryoids.

Stem and Petiole – Rapid proliferation (*fig. 2C*) from the cut surfaces was noted even on BM alone, but gradually these cells turned brown. On BM + CM + IAA (1 ppm) clusters of roots originated all around the cut surfaces of explants (*fig. 2D*). While the callus mostly originated from the cortical cells (*fig. 2E*), roots had their origin in the pericycle. The roots were robust, possessed abundant root hairs, and grew into the medium. With age they became pale green. Differentiation of shoots or embryoids did not occur. On replacing IAA

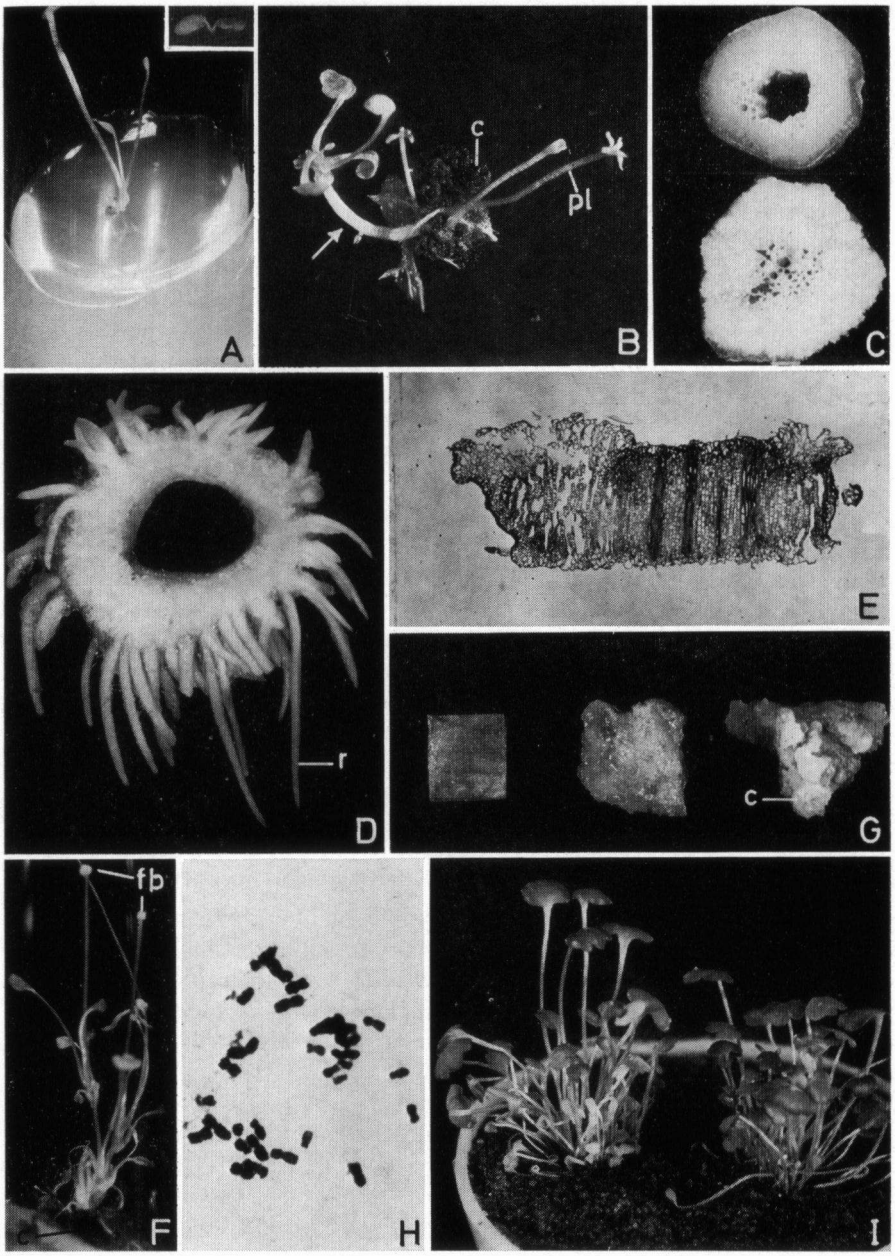

Fig. 2. A. Plumular portion after 4 weeks culture on BM. Insert, the explant at culture × 1.6; insert 3.5. B. Three new plantlets differentiating from the callus. The original shoot tip (arrow-marked) is also elongating. × 2. C. Surface view of stem segments in culture (above), and after 2-weeks on BM (below) showing proliferation. × 3. D. Stem segment from BM + CM (10%) + IAA (1.0 mg/l) showing numerous roots (4-week-old). × 3.3. E. L.s. of a proliferating stem segment (1-week-old) × 10.5. F. Plant with flower buds on BM. × 0.8. G. Segments of lamina in culture (left) and after 2-weeks growth on BM + CN + (10%) + 2,4-D (1.0 mg/1) showing proliferation (middle and right). × 2. H. Metaphase chromosomes (2n = 32) from a squash preparation of the root tip of an in vitro plant. × 1250. I. Plantlets photographed 2 weeks after transference to pots. Many new leaves have been formed. × 2.

by 2,4-D (1 ppm), the explants continued to proliferate and a yellowish, soft callus was formed. Even after 12 weeks no embryoids were formed from the callus. However, if transferred to BM, callus differentiated embryoids. The plants which ensued from the embryoids within 10 weeks produced flowers and fruits (*fig. 2F*).

Lamina – Callusing occurred all over the surface of the lamina segments (*fig. 2G*), followed by the differentiation of several roots from the cut regions when the explants were cultured on BM + CM + IAA (1 ppm). Addition of 2,4-D (1 ppm) in place of IAA favoured continuous callus growth followed by the organization of several embryoids leading to the formation of plantlets. The *in vitro* differentiated plants were diploid (2n = 32) as checked from chromosome counts of root tip squashes (*fig. 2J*).

3.4. Morphogenesis in explants isolated from in vitro plants

Since the explants obtained from the *in vitro* plants showed callusing and subsequently organogenesis, it was of interest to test if comparable responses could be obtained with in vitro plant parts. Flower buds, segments of root, stem, petiole, and lamina were reared on nutrient media.

3.5. Transplantation of in vitro plants to soil

The plantlets were first transferred to BM alone. After a week these were again planted in sterile sand and finally in soil maintained at 25° ± 2 °C. The plants were irrigated with half diluted White's medium. They remained fresh, forming new leaves during a period of 4 weeks (*fig. 2I*).

Thus, the explants from various parts (*see* KONAR & NATARAJA 1969) of in vitro as well as in vivo plants could yield callus capable of producing embryoids leading to the formation of plantlets bearing flowers and fruits.

4. DISCUSSION

The morphogenetic potentialities of flower buds of *Ranunculus sceleratus* have been discussed in a recent publication by KONAR & NATARAJA (1969). Among the floral parts, growth and regeneration in isolated sepals and petals has hardly been explored. In the present study, both sepals and petals callused readily, and the embryoids organized on a medium containing CM and 2,4-D.

The cultivation of anthers *in vitro* has been carried out extensively, mainly to understand the phenomenon of meiosis (VASIL 1967). Nevertheless, formation of callus from the sporophytic tissue of the anther has been reported in *Cajanus cajan* (VASIL 1963); callus, roots, and shoot buds in *Tradescantia paludosa* (YAMADA 1963); embryoids from the pollen of *Datura innoxia* (GUHA & MAHESHWARI 1966) and *Nicotiana tabacum* (NAKATA & TANAKA 1968; NITSCH & NITSCH 1968). Tissue formation from pollen has also been reported in *Ginkgo biloba, Taxus* sp. (TULECKE 1957; 1959), and *Ephedra foliata* (KONAR 1963). In *Ranunculus sceleratus* the anthers cultured at the pollen mother cell stage on a medium containing CM and 2,4-D produced callus from wall layers and mi-

crospore mother cells, but more readily from the connective. The callus eventually differentiated embryoids and plantlets. Callusing and embryoid formation from mature anthers was already reported (KONAR & NATARAJA 1965b). Thus the cells of the anthers, which are otherwise senescent, can revert to meristematic activity and even undergo morphogenesis if grown on a suitable medium.

Reports of the occurrence of embryoids in cultured post-pollinated ovaries of *Ranunculus sceleratus* (SACHER & GUHA 1962), *Anethum graveolens* and *Foeniculum vulgare* (JOHRI & SEHGAL 1966) on a medium having casein hydrolysate as a supplement are available. In these instances it is a case of zygotic polyembryony where the proembryo present in the ovules has proliferated and from which embryoids have organized. In the present study, however, the very young carpels yielded callus and embryoids on a simple medium.

In nature the seeds of *Ranunculus sceleratus* possess a dormancy period of about a year. But planting the seeds on a nutrient medium shows the absence of any dormancy period, and the germination starts after two weeks of culture. A feature of interest recorded was the striking difference in morphogenetic potentialities of the plantlets obtained through seed germination and those formed by the embryoids *in vitro*. Even though morphologically similar, the former failed to differentiate epidermal embryos whereas the latter showed their presence. What is (are) the causative factor(s) for this dissimilar behaviour is not clear. Embryoids from the epidermal cells have also been reported in carrot culture after we recorded it (KATO & TAKEUCHI 1966; KATO 1968). However, in *R. sceleratus*, if the callus is induced in cultured seeds or from parts of a seedling, the embryoids and plantlets readily differentiate in it. The plantlets also bear stem embryoids.

To date formation of embryoids has been reported in cultures of root and hypocotyl of carrot (STEWARD *et al.* 1958, 1966; KATO & TAKEUCHI 1963, 1966) and *Apium graveolens* (REINERT *et al.* 1966); stem of *Foeniculum vulgare* (MAHESHWARI & GUPTA 1965) and *Nicotiana tabacum* (HACCIUS & KAKSHMANAN 1965); leaf of carrot (HALPERIN & WETHERELL 1964), *Kalanchoë pinnata* (WADHI & MOHAN RAM 1964), *Macleaya cordata* (KÖHLENBACH 1965) and *Petroselinum hortense* (VASIL & HILDEBRANDT 1966) on a medium containing either one or many growth adjuvants like CM, 2,4-D, adenine, yeast extract, casein hydrolysate, IAA and kinetin. In the present study the tissues of roots, shoot tip, stem, petiole and lamina produced callus on media containing CM and 2,4-D. However, growth adjuvants were not indispensable for the differentiation of embryoids either from the callus or from plantlets.

Thus, in *Ranunculus sceleratus*, callus and subsequently embryoids could be induced from almost any part of the plant body, leading to the formation of plants bearing flowers and fruits.

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