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Absence of meristems in androgenic embryos of *Datura metel* (L.) induces secondary embryogenesis in vitro

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ABSTRACT

The induction of secondary embryos in androgenic and zygotic embryos of *Datura metel* (L.) (Solanaceae) was investigated by excising their shoot and root meristems and culturing the hypocotyls on Nitsch medium. Androgenic embryos produced secondary embryos on the hypocotyl. Histological sections showed secondary embryo development was independent of maternal tissue with no connection to the maternal vascular system. The zygotic embryos produced shoot buds and adventitious roots from the cut surface. Shoot buds had vascular connections with the maternal tissue. Excision of both meristems produced more secondary embryos than excision of the apical meristem alone. Excision of apical meristems in embryogenically competent immature embryos and their in vitro culture could potentially produce secondary embryos on the hypocotyls. The significance of these results is the clonal multiplication of the primary embryo at an early stage of embryo development. The meristem excision steps are easily performed under sterile conditions.

1. Introduction

The morphology of a dicotyledonous plant embryo consists of the shoot apical meristem enclosed by the cotyledons, the hypocotyl, the root system and the root meristem. The shoot and root meristems grow in polar directions, the shoot apical meristem (SAM) generating the aerial plant tissues and the root meristem producing the underground parts (Mayer et al., 1991; Jürgens et al., 1991). The cells in the meristem divide throughout the vegetative lifespan of the plant to produce stem tissues and leaves unless physically damaged. After the vegetative phase it is converted into an inflorescence meristem that bears flowers (Steeves and Sussex, 1989). The cells from the meristem differentiate during the life cycle into the various vegetative and floral organs. The meristems are initiated during embryogenesis and determine the basic morphology of the embryo.

In their seminal work to determine the morphology of plant embryos, Mayer et al. (1991) induced mutations in the seeds of *Arabidopsis thaliana* which produced abnormal embryo phenotypes lacking one or more elements from the embryo. The mutants showed the absence of apical (SAM and cotyledons), central (hypocotyl), basal (hypocotyls and radical) to terminal (SAM, cotyledons and root meristem) regions, from which genes determining these elements were identified.

In plants, zygotic embryogenesis following the fertilization of the egg cell by pollen is the norm. However, under specific in vitro conditions, embryos can develop from immature pollen grains (microspores). This development called androgenesis, was first discovered by Guha and Maheshwari (1964) in anther cultures of *Datura innoxia*. The development of embryos under in vitro conditions provides an opportunity to observe the different stages of embryogenesis. During seed germination in the soil, abnormal embryos would not survive and emerge and hence remain unknown. However, such abnormal embryos can be observed under in vitro conditions of embryogenesis. In our in vitro experiments on androgenesis, we observed abnormal phenotypes amongst androgenic embryos of *Datura metel* lacking one or more elements from the basic morphology similar to that described by Mayer et al. (1991). These defective androgenic embryos produced secondary embryos.

In mature dicotyledonous plants, apical dominance is a common phenomenon, which suppresses the development of axillary branches. This ability is due to a few pluripotent cells within the SAM and removal or damage to this meristem activates the axillary meristems and branching. In *Petunia* species three decreased apical dominance loci have been identified. Mutations in each of these loci produced plants whose gene loci have been identified with a decreased apical dominance gene, a mutation which causes an increase in branching (Napoli, 1996; Snowden and Napoli, 2003).

Abbreviations: SAM, shoot apical meristem; RAM, root apical meristem.

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In plant embryos, the apical and basal regions contain the primary meristems, which later serve as centers for the development of organs and secondary meristems. Thus the removal or absence of these primary meristems, at an early stage of embryogenesis, would drastically change post-embryonic development.

Embryos mechanically wounded by cutting them and cultured in vitro have induced somatic embryogenesis in carrot (Smith and Krikorian, 1988). Prabhudesai and Bhaskaran (1993) induced secondary embryos from microspore derived embryos of *Brassica juncea* by excision. In *Brassica napus* Nehlin et al. (1995) induced secondary embryos on transversely cut microspore derived embryos. In *Annonas squamosa*, Nagori and Purohit (2004), induced shoot bud differentiation on hypocotyl segments of zygotic embryos. These in vitro studies suggest that wounding or isolation of the embryo hypocotyl may be equated to removal of the embryo meristems, which is similar to meristem removal in mature plants and the activation of their axillary meristems.

In this study we investigated the hypothesis that removal or absence of apical meristems in embryos would induce secondary embryogenesis on the hypocotyls of *D. metel*. To test this hypothesis, the meristems of androgenic and zygotic embryos were excised and their hypocotyls cultured in vitro to determine if they underwent secondary embryogenesis.

2. Materials and methods

2.1. Donor plants for anther culture

Datura plants were collected in the Kandy district, and identified as *D. metel* L. from the description in the Flora of Ceylon Vol. IV (Dassanayake and Fosberg (1987) and comparison with herbarium specimens at the Royal Botanic Gardens, Peradeniya. The plants were established in the greenhouse, flowers were selfed and seeds from the dehisced pods were germinated and seedlings transferred to pots. A population of plants was maintained in the greenhouse in clay pots (dia. 30 cm). The plants were fertilized fortnightly with a commercial garden fertilizer (Bayer Chem. Co.) and watered daily. Daytime temperature was 26–30 °C and relative humidity 70%. The plants flowered at a height of one meter, three months after planting. Flowers were removed to avoid pod setting and to prevent the bushes from senescing.

2.2. Anther culture

Unopened flower buds (length 3.5–6 cm, corresponding to the mid-late uninucleate stage) were washed with two drops of a commercial detergent and rinsed in tap water. Under the laminar flow hood the washed buds were rinsed in 95% ethanol for two minutes in a sterile bottle followed by 2% (w/v) calcium hypochlorite for five minutes and finally rinsed in three changes of sterile distilled water. Flower buds were aseptically opened, the anthers separated from their filaments, and cultured in a Petri-dish containing Nitsch (1969) medium with 3% sucrose and 4.65 μM kinetin. The pH was adjusted to 5.8 using 1N NaOH or 1N HCl, the medium solidified with 0.42% agar-gel (Sigma Chem. Co.) and autoclaved at 121 °C for 20 min at 105 kPa pressure. Approximately 20 ml of medium was poured into 100 mm diameter glass Petri-dishes. The culture plates were incubated at 25 ± 2 °C under 16 h photoperiod. Light was provided by fluorescence bulbs (Thorn, 40 W tropical daylight).

2.3. Zygotic embryos

Flowers of *D. metel* were selfed and green immature pods were harvested. The pods were washed in running water with a detergent to remove surface dirt. The pods were surface sterilized with ethanol and calcium hypochlorite as described for the flowers above. The pod was cut under sterile conditions and the seeds removed and cut open carefully under a stereo-microscope to release the embryos into sterile water in a Petri-dish. From a pool of zygotic embryos, uniform embryos of 7–10 mm were selected for experiments.

2.4. Meristem excision

Well developed androgenic and zygotic embryos, 7–10 mm, in length were selected for meristem excision. Two types of cuts were made on the embryos with a sharp scalpel: (a) apical meristem excision – the shoot apical meristem and the cotyledons were excised, and (b) terminal meristem excision – the shoot apical meristem, cotyledons and the root meristem were excised (Fig. 1).

The meristem excised explants and control embryos (with meristems intact) were cultured on Nitsch medium (1969) with

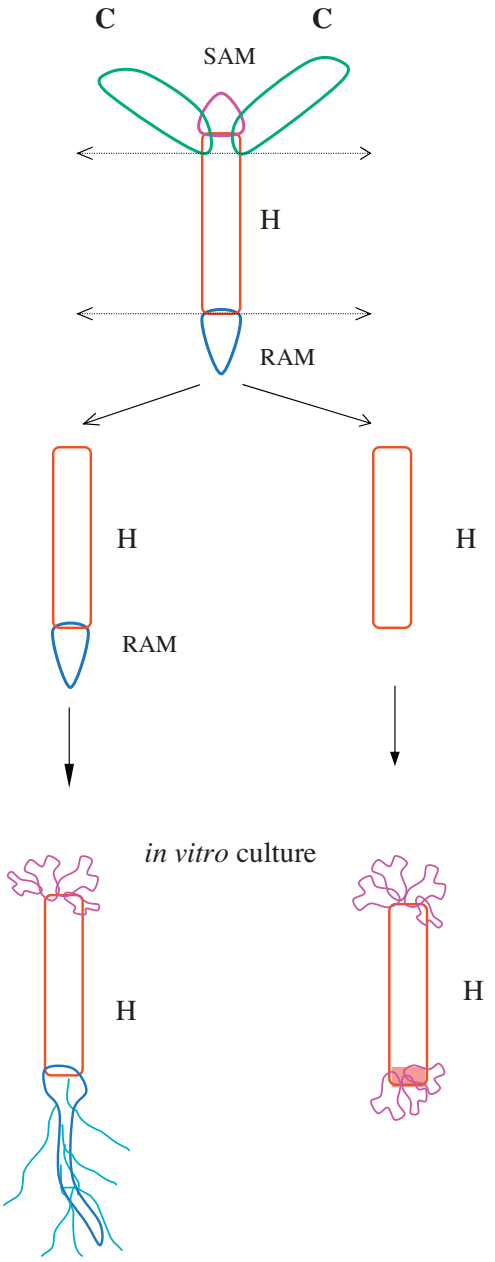

Fig. 1. Diagrammatic illustration of the two types of excision on the embryos of *Datura metel*: (a) apical meristem excision and (b) terminal meristem excision. (C-cotyledons, H-hypocotyl, SAM-shoot apical meristem, RAM-root apical meristem).

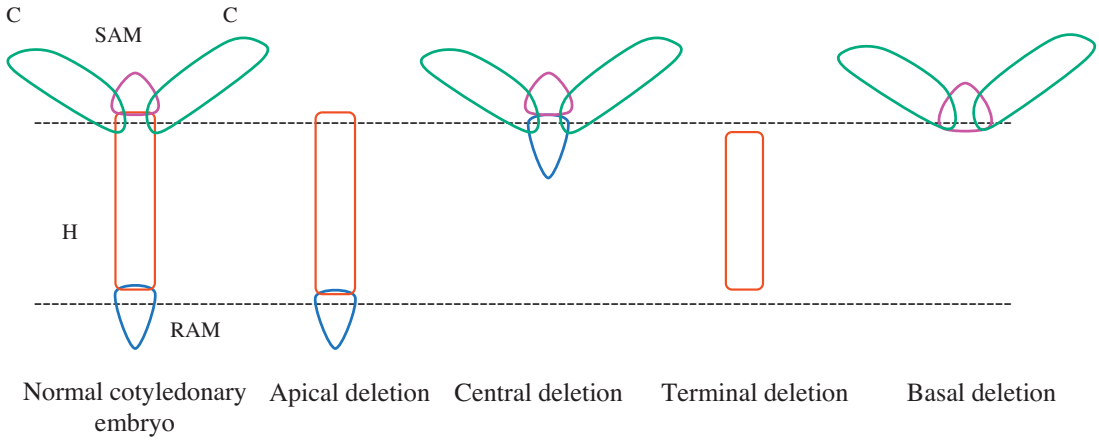

Fig. 2. Diagrammatic illustration of the basic pattern deletions observed in haploid embryos of *Datura metel*. (C-cotyledons, H-hypocotyl, SAM-shoot apical meristem, RAM-root apical meristem).

10^{-7} M kinetin, with five explants in a 100 mm Petri-dish. Half the cultures were kept at 25 ± 2 °C and 16 h photoperiod and the other half at the same temperature but continuous darkness. After six weeks of culture the number of secondary embryos emerging from the hypocotyl were determined.

The experimental unit for embryo cultures was a Petri-dish with 5 embryo explants. The effect of three treatments on the development of secondary embryos were studied: (1) mechanical wounding of the embryos by excision of (i) apical meristems, and (ii) terminal meristems; (2) kinetin (i) with and (ii) without, kinetin in the culture medium; (3) illumination (i) 16 h photoperiod and (ii) continuous darkness, of the cultures. This gave eight treatment combinations (see Fig. 4). Each treatment combination was replicated thrice, which formed an experiment. Three independent experiments were performed.

2.5. Abnormal androgenic embryos

All the androgenic embryos emerging from the anthers were pooled together. They were examined under a stereo-microscope and the defective embryos were classified according to the proposed model by Mayer et al. (1991) (Fig. 2). Over 1500 embryos were assessed and each deletion pattern expressed as a percentage of the total defective embryos.

2.6. Histological analysis

Embryos were fixed in FAA (formalin:acetic-acid:alcohol) and dehydrated in a graded ethanol series, infiltrated with 1:1 mixture of butanol/Technovit 7100 resin (Heraeus) overnight and finally embedded in Technovit 7100 resin blocks. Serial sections (10 μm thick) were cut with a Rotary microtome (Kyoto Scientific Specimens Co. Japan), transferred to microscope slides and stained with Toluidine-blue (0.05%) in 0.2 M aqueous solution of sodium acetate and glacial acetic-acid. Photographs were taken with an Olympus camera attached to an Olympus BH2 stereoscope using Kodacolour 100 Gold film.

2.7. Statistical analysis

All the treatments were arranged in a completely randomized design. The mean number of secondary embryos for each culture plate was determined. The count data was analyzed by categorical data analysis procedures of PC-SAS programming language (SAS Institute 2000). The fitted model for data was that of a log-linear type and treatment combinations were compared by a *Z*-test based on maximum likelihood predicted values for frequencies.

3. Results

The anthers produced embryos after 6 weeks of culture on Nitsch (1969) medium. Some of these embryos showed abnormal phenotypes with one or more elements of their morphology missing.

3.1. Excision of meristems and embryogenic response

3.1.1. Androgenic embryos

To verify the phenomenon of secondary embryogenesis of meristemless embryos, shoot and root meristem were excised from normal androgenic and zygotic embryos of *D. metel*. The androgenic embryos after meristem excision underwent secondary embryogenesis on their hypocotyls (Fig. 3C). The treatment, meristem excision, significantly enhanced secondary embryogenesis over the control embryos with intact meristems (Table 2). Embryos with both meristems excised produced a higher number of secondary embryos than embryos with only an apical cut (Fig. 4). Callusing was also observed on the meristem deleted embryos although at a lower frequency (data not shown).

Secondary embryos appeared on the meristem excised androgenic embryos after two weeks. The secondary embryos were initiated after the cessation of growth of the primary embryo. Translucent humps appeared on the hypocotyls in and around the cut surface. The growth and development of secondary embryos were through a series of stages characteristic for zygotic embryogenesis (Fig. 3D). However, their development was asynchronised (Fig. 3C). Histological examination showed that secondary embryos originate from the epidermal or sub-epidermal surface cells of the hypocotyls (Fig. 5A). Mature secondary embryos were bipolar structures with distinguishable elements such as cotyledons, hypocotyls and radical along the apical–basal axis of the embryo (Fig. 5B and D). At the heart stage of embryo development, both SAM and RAM were observed on the secondary embryos (Fig. 5C). There was no vascular connection between the secondary embryo and parental tissue (Fig. 5E). A successful conversion of detached secondary embryos into plantlets was achieved when detached and cultured on hormone free solid MS medium (Fig. 3G).

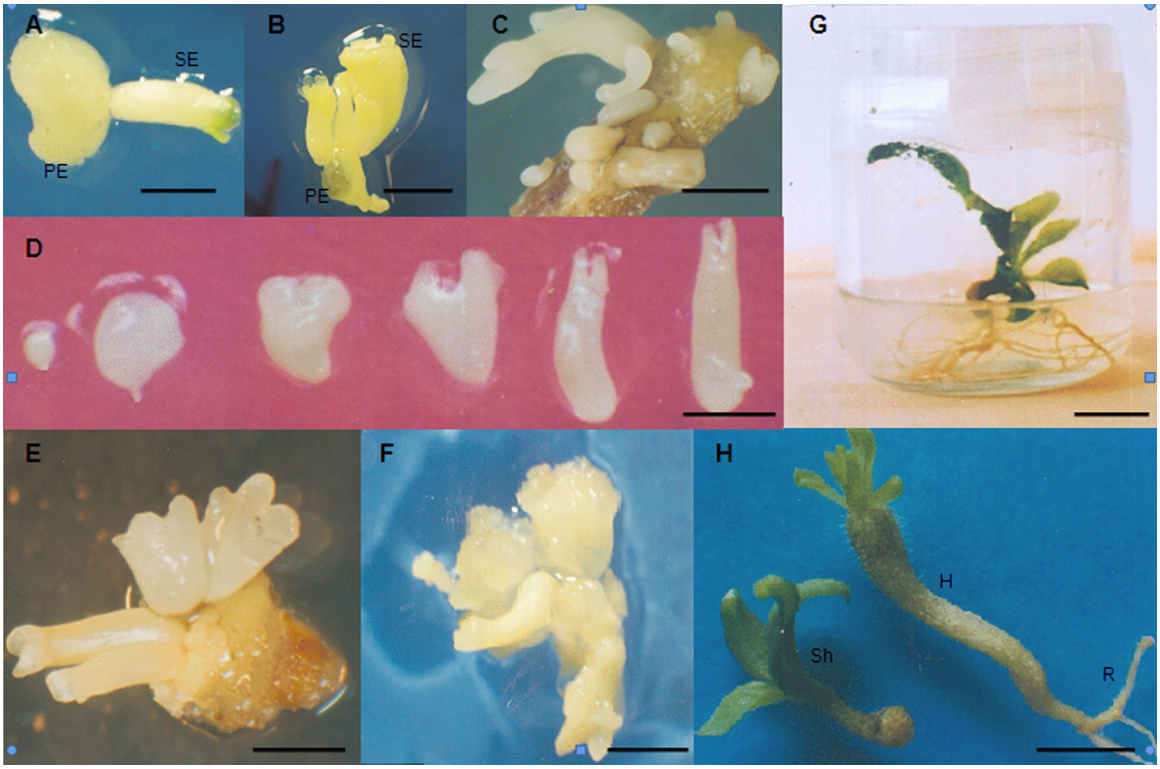

Fig. 3. Secondary embryogenesis in meristem deleted embryos. (A) Embryo formation on terminal deleted haploid embryo. (B) Apical deleted haploid embryo with spontaneous secondary embryo development. (C) Asynchronised emergence of secondary embryos on hypocotyl. (D) Sequential development of secondary embryos (from left to right, globular, early heart, heart, torpedo, early coteledonary and coteledonary stage). (E) Secondary embryos on excised haploid embryo cultured on Kinetin containing MS medium and under light. (F) Abnormal embryos developed on excised haploid embryos cultured on Kinetin free MS medium and kept in darkness. (G) Plantlet from a secondary embryo. (H) Organogenesis on the cut surface of zygotic embryo. (PE-primary embryo, SE-secondary embryo, H-hypocotyl, R-root, SH-shoot). *Scale bar* = 5 mm.

3.2. Abnormal androgenic embryos

Under natural conditions, seeds with abnormal embryos would have a reduced fitness and not survive. The abnormalities would hence remain unknown. However, under in vitro conditions it was possible to observe such abnormalities in androgenic embryos. In anther cultures of *D. metel*, four deletion patterns along the apical–basal axis of the embryo were observed (Fig. 2). The abnormalities were deletions of the major elements of the embryo morphology: apical deletion (cotyledons and SAM), central deletion (hypocotyl), terminal deletion (cotyledons, SAM and RAM), and basal deletion (hypocotyl and RAM).

Embryos with an apical deletion did not develop cotyledons and a SAM. There were differences in the extent of the abnormality. In strongly affected embryos the apical region and part of the upper hypocotyl were missing (Fig. 6A) and in less affected embryos, remnants of the cotyledons were present but the embryo failed to develop a shoot meristem (Fig. 6B). The hypocotyls and root were unaffected even in the strongly defective embryos. A longitudinal section through the embryo shows the absence of cotyledons and the SAM (Fig. 6C) with the hypocotyl and root intact. The root is similar to a normal embryo with a root apical meristem and root cap. In centrally deleted embryos, their hypocotyl was absent (Fig. 6D and F). The cotyledons are directly attached to the root causing distorted cotyledons. The internal anatomy and the vasculature of the embryo (Fig. 6E) reflects the external morphology: in the absence of the hypocotyl the interface between cotyledons and root is confined to a narrow collar region and the vascular strands from the upper region of the root immediately separates into two and enters the cotyledons. In embryos with a basal deletion, the radicle did not elongate and roots were not produced. The apical region and hypocotyl were intact (Fig. 6G and H). Embryos without the apical and basal elements possess only the hypocotyl (Fig. 6I). Based on their appearance, the remnant hypocotyl was cylindrical (Fig. 6Ia) or ball-shaped (Fig. 6Ib). The ball-shaped embryo had no recognizable apical–basal polarity. In both forms the internal tissues showed the three basic radial tissues, epidermis, cortex and central vascular strand confined to a narrow region in the center (Fig. 6J). Besides these deletion patterns, embryos with abnormal cotyledons were observed. These included mono (Fig. 6K), supernumerary (Fig. 6L) and funnel shaped or fused cotyledons (Fig. 6M).

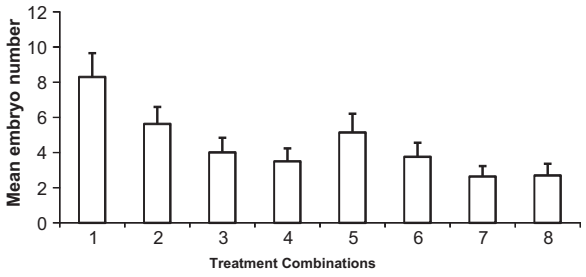

Fig. 4. Mean embryo number of direct secondary embryos produced on haploid embryo explants after six weeks on induction medium. A = apical meristem cut, T = apical and root meristem cut, K = kinetin, L = light. (1) T + K + L (2) T + K-L (3) T-K + L (4) T-K-L (5) A + K + L (6) A + K-L (7) A-K + L (8) A-K-L (vertical bars represent standard error).

3.3. Regeneration and secondary embryogenesis of abnormal embryos

Amongst the abnormal androgenic embryos with terminal and apical deletion, secondary embryos developed on the hypocotyls. From 100 randomly chosen primary embryos, 10% showed

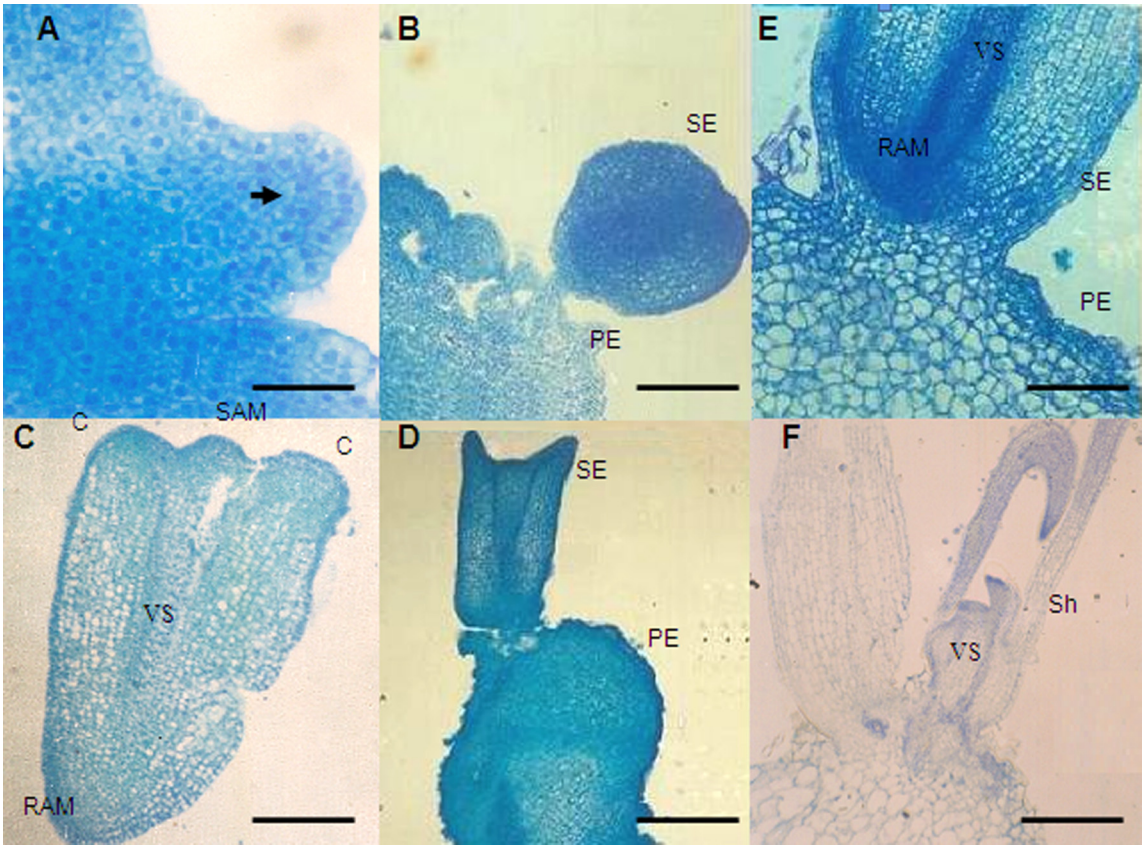

Fig. 5. Longitudinal sections showing: (A) Origin of a secondary embryo from sub epidermal cells of the hypocotyl (arrow). (B) Early heart stage secondary embryo. (C) Heart shaped secondary embryo with SAM and RAM. (D) Section of an apical deleted primary embryo with a bipolar secondary embryo. (E) Section through secondary embryo and hypocotyl showing independent vascular system of embryo. (F) Section of a shoot developed on the cut surface of zygotic embryo hypocotyls showing the vascular connection with parental tissue. (PE-primary embryo, SE-secondary embryo, Sh-shoot, VS-vascular strand, RAM-root apical meristem, SAM-shoot apical meristem). *Scale bar* = 1 mm.

spontaneous secondary embryogenesis. The abnormalities in all the androgenic embryos were, however, not permanent. On transfer to a hormone free MS (Murashige and Skoog, 1962) regeneration medium, some of the apical and basal deleted embryos regenerated to plantlets (Table 1). Embryos without both meristems (terminal deletion) did not regenerate to plantlets. Embryos with a central deletion elongated in the regeneration medium and developed shoots and roots. This abnormality was of the lowest frequency (Table 1). Of the basal deleted embryos, 50% regenerated to plantlets. Since they had a shoot meristem, shoots developed initially. After two weeks, adventitious roots developed from the base of the hypocotyls. The rest of the embryos with a basal deletion did not develop roots while callusing and swelling of the base was observed in some.

Some of the defective embryos with an apical or terminal deletion produced secondary embryos on their hypocotyls (Table 1) and did not regenerate to plantlets (Fig. 3A and B.). These were initiated after the cessation of growth of the primary embryo. Secondary embryogenesis was observed only on embryos where secondary shoots or roots did not develop. Roots did not develop on the terminal deleted embryos while some developed root hairs on their hypocotyls. Embryos with a central and basal deletion did not develop secondary embryos (Table 1).

Table 1
Abnormal phenotypes among androgenic embryos, their conversion to plantlets and occurrence of secondary embryogenesis.

Embryo phenotype	Occurrence (%)	Conversion to plantlets (%)	Secondary embryogenesis (%)
Apical deletion	15	42.5	30
Terminal deletion	33	–	52.7
Central deletion	0.5	66.7	–
Basal deletion	3.5	50	–
Abnormal cotyledon	19.5	–	–
Normal embryos	12	–	–
Unclassified	16.5	–	–

3.3.1. *Zygotic embryos*

Zygotic embryos of *D. metel* whose meristems were excised underwent organogenesis from the cut surface (Fig. 3H). They did not produce secondary embryos. Elongated leafy structures either individually or as multiple shoot buds were produced after 14 days. In the apical meristem deleted embryos roots developed from the intact root meristem followed by shoot buds from the cut surface. In the terminal cut embryos, shoot buds developed on the cut surface at the apical end with adventitious roots appearing later. Cytological examinations showed that those shoot buds developed on the cut surface were having vascular connections with the parental tissue (Fig. 5F).

3.4. *Photoperiod and phytohormones*

A 16 h photoperiod had a significant positive effect (Table 2) on the induction of secondary embryogenesis and their development. The explants treated with light and kinetin produced good quality embryos which were large and bipolar (Fig. 3E). However, the beneficial effect of light on embryogenesis was not observed in the absence of kinetin (Fig. 4). Absence of light

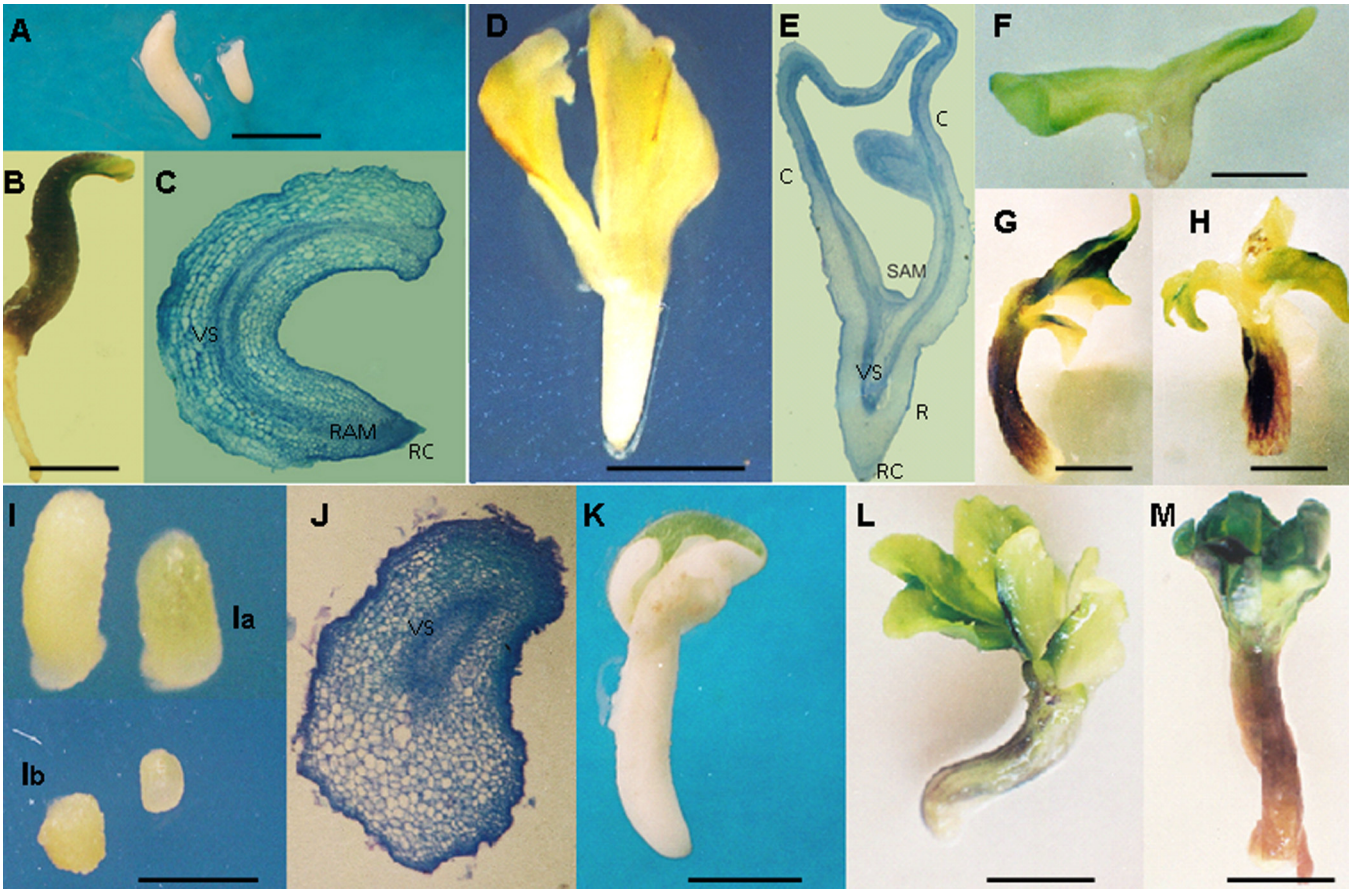

Fig. 6. Abnormal androgenic embryos of *Datura metel*. (A) Apical deleted embryos. (B) Germinating apical deleted embryo showing only root growth (arrow: remains of cotyledons). (C) LS of an apical deleted embryo. Note the lack of cotyledons and apical meristem. (D) Central deleted embryo (note the missing hypocotyl). (E) Internal tissue arrangement of central deleted embryo. (F–H) Embryo showing basal deletion without a hypocotyl/or a root. (I) Terminal deletion ((Ia) oblong shaped embryo, (Ib) ball shaped embryo). (J) Internal tissue arrangement of terminal deleted embryo. (K) Monocotyledon embryo. (L) Multiple cotyledonary embryo. (M) Embryo with fused cotyledons to form a funnel. (LS-longitudinal section, SAM-shoot apical meristem, RAM-root apical meristem, VS-vascular strand, C-cotyledons, R-root). *Scale bar* = 5 mm (C, E, and J *scale bar* = 1 mm).

and kinetin produced deformed embryos which were not easily detachable from the primary embryos (Fig. 3F).

4. Discussion

Unlike in animals, abnormalities in plant embryo body organization do not manifest themselves: they perish below ground on germination. Thus to detect and screen for mutations disrupting development of the embryo in plants Mayer et al. (1991) induced mutations in *A. thaliana* seeds and screened them for mutants. They identified distinct classes of embryo mutants which determine specific elements of the embryo morphology: cotyledons, SAM, hypocotyls, RAM and root. In androgenic embryos of *D. metel*, we found the similar spectrum of naturally occurring abnormalities in vitro. These androgenic mutants were similar in phenotype to that described for *A. thaliana*. However, abnormal embryo phenotypes from androgenic embryos have not been studied in the context of embryo body organization, to our knowledge. In their first report of androgenesis from *D. innoxia*, Guha and Maheshwari (1964) reported embryo abnormalities where most were poly-cotyledenous. Similar, cotyledenory abnormalities were also observed in androgenic embryos of Nicotiana (Nitsch and Nitsch, 1969). Abnormalities in androgenic embryos – hypertrophy, abnormal cotyledons, absent hypocotyl – were also reported in *Aesculus hippocastanum* (Calic et al., 2005).

In this study, the subsequent development of abnormal embryos of *D. metel* did not imply a complete loss of function for the genes responsible for body organization. In the absence of the shoot and root meristems, the embryo cannot develop shoots or develop into a plantlet. Under in vitro conditions, however, secondary embryogenesis occurred on abnormal embryos with a hypocotyl. These secondary somatic embryos had a normal morphology. Thus the abnormalities observed in the primary androgenic embryos were apparently transient. To test our hypothesis that the absence of meristems in embryos would induce somatic embryos, meristems were excised from normal androgenic and zygotic embryos of *D. metel* under in vitro conditions. Somatic embryos developed on their hypocotyls after two weeks. Although meristem excision per se was not investigated, Nehlin et al. (1995) mechanically wounded androgenic embryos of *B. napus* to induce secondary embryogenesis. Epidermal and sub-epidermal

Table 2
Maximum likelihood analysis of variance for meristem excision, kinetin and photoperiod on secondary embryogenesis in androgenic embryos of *Datura metel*. Significance of the main effects was tested by Chi-Square values at 5% significance level.

Source of variation	DF	Chi-square	Probability
Meristem excision	1	21.42	0.0000
Kinetin	1	40.70	0.0000
Photoperiod	1	8.45	0.0037
Likelihood ratio	116	271.11	0.0000

cells of cotyledons and hypocotyls of androgenically derived plantlets of *Aesculus hippocastanum* underwent direct secondary embryogenesis (Radojević, 1995). When embryos were transversely cut, keeping the hypocotyl-root intact (i.e. removal of cotyledons and SAM), a high number of somatic embryos were formed on the hypocotyl. Wounding of immature cotyledons of soybean (*Glycine max*) with a scalpel resulted in an earlier induction of somatic embryos with embryo initials observed after seven days (Santarem et al., 1997). In *Bixa orellano*, somatic embryos were induced on the hypocotyl; the authors suggest they may be due to wounding of the embryos during extraction from the seed tissues (Neto et al., 2003). In cereals, wounding of the caryopses of *Hordeum vulgare* induced somatic embryos (Bouamama et al., 2011).

In other studies, somatic embryos were induced when the primary embryos were wounded or broken. Smith and Krikorian (1988) reported that zygotic embryos of carrot, formed somatic embryos only when broken in half. Similarly Choi and Soh (1996) cultured wounded zygotic embryos of ginseng on MS medium which formed somatic embryos. Besides secondary embryogenesis, wounded zygotic embryos in some species underwent organogenesis. This was confined to the cut surfaces with no response from the cells on the hypocotyls as observed in zygotic embryos in *D. metel* in this study. This phenomenon can be species dependent since species have responded differently to wounding whether by embryogenesis or by organogenesis. Seed derived hypocotyl segments of *Annona squamosa* produced shoot buds (Nagori and Purohit, 2004) whereas wounded zygotic embryos of carrot (Smith and Krikorian, 1988) and ginseng (Choi and Soh, 1996) produced somatic embryos. In zygotic embryos of chickpea *Cicer arietinum*, shoot and root apices were removed from the embryo axis and used as explants by Singh et al. (2002). Multiple shoots were regenerated from the cut plumular ends as also observed in the zygotic embryos of *D. metel* in this study (Fig. 6F). In celandine (*Chelidonium majus*), Vinterhalter and Vinterhalter (2002) speculate that wounding per se could be the inductive stimulus for somatic embryogenesis from epicotyls explants. In *Feronia limonia* in vitro green shoot buds developed from the cut ends of the hypocotyls (Vyas et al., 2005). They were vascularly connected to the mother tissue as in our study. Such breakages or wounding of embryos can be considered as equivalent to the removal or damage to the shoot apical meristem and thereby interfering with hormone translocation from the meristem to the hypocotyl.

In plants, auxins promote the dormant state of axillary buds, although its mode of action remains obscure. An indirect mode of action through cytokinin has been suggested (Leyser, 2003). Cytokinins promote the outgrowth of axillary buds in plants when directly applied and their levels increase during the release from apical dominance (Turnbull et al., 1997). This agrees with our observations where induction of secondary embryos increased with kinetin. Besides deletion of the meristem, kinetin in the medium significantly increased the number of somatic embryos on the embryo. From the different treatment combinations in our experiments, those with kinetin produced significantly more embryos than those without. Similarly in androgenic embryos of *B. juncea*, somatic embryogenesis had an obligate requirement for benzyladenine (Prabhudesai and Bhaskaran, 1993). A source of cytokinin is necessary or enhances the induction of embryogenesis and organogenesis. Benzyl-adenine was a more effective cytokinin than kinetin as shown by Mao et al. (2000), Tawfik and Noga (2001), and Vyas et al. (2005). The addition of the cytokinin TDZ to the culture medium also enhanced multiple shoots in *C. arietinum* whereas kinetic enhanced shoot regeneration of *D. metel* in this study. These studies of cytokinins enhancing the induction of somatic embryogenesis suggest that, it is similar to the release of apical dominance in mature plants and increase in cytokinin levels in the tissues, resulting in axillary bud break.

In their study of the role of hormones in apical dominance, Sachs and Thimann (1967) showed that auxins produced in the apex suppress the growth of lateral buds. The application of cytokinins on the lateral buds released them from apical dominance. Bangerth (1994) and Li et al. (1995) have further shown that removal of the SAM dramatically increases the cytokinin content in the xylem exudates. This increase was eliminated by application of NAA to the apex of decapitated plants. This interaction indicates that auxin inhibits the synthesis or action of cytokinin, which is manifested as apical dominance. Our observations at a very early stage in embryogenesis are in agreement with these studies.

An embryogenic response was possible only in embryos that did not have the ability to produce a primary shoot (absent or excised meristems). We did not observe secondary embryos on androgenic or zygotic embryos of *D. metel* that developed into plantlets. In androgenic embryos of *B. juncea*, Prabhudesai and Bhaskaran (1993) also observed that once shoots differentiated, somatic embryos were no longer produced. Thus the absence or inability of the embryo to produce a shoot would be similar to the loss of "apical dominance" in mature plants followed by axillary bud growth.

Androgenesis is a commonly applied technique in horticultural cops. This study shows induction of further secondary embryos on the hypocotyl of primary haploid embryos is possible by meristem excision of the haploid embryos. This enables a rapid multiplication at an early stage of in vitro culture, to increase the availability of embryos for subsequent diploidization and regeneration of genetically homozygous dihaploid plants. Homozygous dihaploid plants are fertile and have the potential to become pure breeding new cultivars or the parents for producing uniform hybrids of horticultural crops.

5. Conclusion

Androgenesis is a useful technique to study the fundamental aspects of plant morphology through the manifestation of abnormal embryos. The phenomenon of branching after removal of apical meristem in mature dicotyledonous plants is shown in this study at the embryo level by secondary embryogenesis. Excision of apical meristems in embryogenically competent immature embryos and their in vitro culture could potentially produce secondary embryos on the hypocotyls and enhanced by exogenous cytokinins. Excision of meristems and in vitro culture of hypocotyls at an embryogenically competent stage could promote secondary embryogenesis in recalcitrant species for multiplication of elite genotypes. The promotion of secondary embryos on the hypocotyls of meristem excised embryos, which can be independently regenerated to plantlets, provides a horticultural application for rapid multiplication of recalcitrant species and elite genotypes at the in vitro stage of embryo development.

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