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Responses of peanut somatic embryos to thidiazuron

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Abstract

Induction of both somatic embryogenesis and organogenesis in presence of thidiazuron is reported in peanut tissues. However the histological evidence of thidiazuron induced somatic embryogenesis was unclear. Thidiazuron triggered multiple shoot differentiation in the plumule of the embryos. Keeping in view the ability of thidiazuron to induce both organogenesis and embryogenesis in peanut tissues, experiments were conducted to define the pathway of morphogenesis in the plumule of rooted somatic embryos. On exposure to thidiazuron, projections appeared from the plumule. These projections closely resemble the somatic embryos. However histological examination revealed that these are caulogenic buds and not somatic embryos. In concurrence with the earlier reports on thidiazuron induced organogenesis it is concluded that this growth regulator induces organogenic response in peanut tissues.

Additional key words: *Arachis hypogaea*, caulogenesis, organogenesis.

Introduction

In peanut (*Arachis hypogaea* L.), several protocols for somatic embryogenesis have been developed, but plant recovery has been limited (Ozias-Akins 1989, McKently 1991, Baker and Wetzstein 1992, Durham and Parrot 1992, Ozias-Akins *et al.* 1992, Wetzstein and Baker 1993, Eapen *et al.* 1993, Reddy and Reddy 1993, Chengalrayan *et al.* 1994, McKently 1995, Sabitha Rani and Reddy 1996). The failure and/or low frequency of conversion are often attributed to morphological abnormalities or immaturity of somatic embryos (Ammirato 1987, Wetzstein and Baker 1993). To increase the frequency of plant recovery, attempts have been made to obtain normal somatic embryos by manipulating the culture medium prior to germination (Ozias-Akins *et al.* 1992), by modifying the germinating medium (Trigiano *et al.* 1988), or by exposing the embryos to the germination medium and subsequently by triggering shoot formation from the plumule of rooted embryos in presence of kinetin (KIN) and benzylaminopurine (BAP) (Chengalrayan *et al.* 1995). Somatic embryo conversion was also achieved in presence of BAP and naphthalene acetic acid (NAA) (Venkatachalam *et al.* 1997, 1999). Thidiazuron (TDZ) was effective in inducing differentiation in the plumule of the somatic embryos (Chengalrayan *et al.* 1997). Multiple shoot emergences were observed in 92 % of the rooted somatic embryos in eight weeks. However, the pathway of morphogenesis of multiple shoots in presence of TDZ remained unclear.

In previous experiments, attempt to trigger plumule differentiation in abnormal but rooted, somatic embryos of peanut, resulted in induction of multiple shoots in presence of 22.7 μM TDZ. Present experiment was designed to define whether the multiple shoot formation was due to *1*) differentiation of the meristems of the fasciated embryos, or *2*) induction and proliferation of the shoot meristem, or *3*) induction and subsequent differentiation of secondary embryos.

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Abbreviations: BAP - N^6-benzylaminopurine; 2,4 D - 2,4-dichlorophenoxyacetic acid; KIN - kinetin; MS medium - Murashige and Skoog medium; TDZ - thidiazuron.
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Materials and methods

Mature pods of a high yielding cultivar of peanut (*Arachis hypogaea* L. cv. JL-24) were collected from the Agriculture College, Pune, India. Embryo axes were excised from the cotyledons, washed with sterile water and treated with 0.1 % $HgCl_2$ for 4 - 5 min and washed with sterile water. These were then soaked in sterile double distilled water for 12 - 16 h. Immature leaflets were dissected from swollen embryo axes and were used as explants. Somatic embryos were obtained from these leaflets using the method described by Chengalrayan *et al.* (1994). In brief, the leaflets were cultured in Murashige and Skoog's (MS) basal medium (Murashige and Skoog 1962) supplemented with 90.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 6 % sucrose for induction of embryogenic masses. Somatic embryos developed from these masses in MS medium with 13.6 μM 2,4-D and 6 % sucrose. These were rooted in MS basal medium containing 2 % sucrose. The rooted embryos were transferred to agar gelled MS medium supplemented with 22.7 μM TDZ and 2 % sucrose for conversion to plantlets (Chengalrayan *et al.* 1997). After incubation for 7 d the cultures were studied under the microscope. After incubation for 10 - 12 d in same medium the rooted embryos were randomly divided into four groups. One group was retained in the conversion medium (MS + 22.7 μM TDZ and 2 % sucrose) for further incubation of four weeks. The upper 4 - 6 mm part of embryos (*i.e* plumule) in other three groups were isolated and cultured in three different media: *1*) MS + 2 % sucrose, *2*) MS + 2.22 μM BAP + 2.32 μM KIN + 2 % sucrose, *3*) MS + 13.32 μM BAP + 2 % sucrose. The pH of all the media was adjusted to 5.8 and media were solidified with 0.6 % agar. The cultures were incubated at temperature of 25 ± 2 °C, irradiance of 32 μmol m^{-2} s^{-1}, and 16-h photoperiod for 4 weeks. The developmental changes at the plumule were noted in all the groups. The experiments were repeated three times with 20 - 30 explants in each medium.

The morphology of the structures in the plumule of the rooted somatic embryos was studied at various stages of development. The plumule end (3 - 5 mm) was isolated after 7, 21, and 28 d in TDZ medium and was fixed in FAA (formaldehyde : glacial acetic acid : alcohol, 5:5:90, v/v) for 48 h at room temperature. The tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin using the procedure described (Sharma and Sharma 1980). Serial sections of 10 μm, were cut using a *Reichert-Jung 2050* (Wien, Austria) rotary microtome. Sections were double stained with haematoxylin-eosin and mounted with *DPX* (*Loba Chemie*, Mumbai, India).

Results and discussion

The objective of the present investigation was to identify the structures, which appear in the plumule end of the rooted somatic embryos of peanut on exposure to TDZ containing medium. Microscopic examination after 7 d incubation revealed that often the cotyledons were fused and expanded to form a cup shaped structure. Small nodule like projections appeared from the central depression of cup shaped structure indicating that the structures develop from the plumule of the somatic embryos. Due to their position in the depression of the cup shaped structure, the origin of these was not clearly visible under stereo microscope. From histological studies (Fig. 1*A*) it is apparent that instead of a single meristematic dome, there are several caulogenic buds. TDZ induces proliferation of the meristematic cells in the plumule of the somatic embryos. This results in formation of a meristematic zone spread over between the cotyledons. Several meristematic buds appear from this zone. Proliferation of meristem in response to TDZ was noted in *Matteuccia struthiopteris* L. Masses resembling miniature nodules were identified as meristematic nodule (Thakur *et al.* 1998). In the present experiment extended incubation of the cultures resulted in further expansion of the cotyledons. The meristematic buds grow and elongate to form structures (Fig. 1*B*), which resemble the somatic embryos described earlier (Gill and Saxena 1992, Victor *et al.* 1999b). Following further incubation for two weeks (total 21 d), more structures appear and elongate whereas the cotyledons expand and become fan shaped (Fig. 1*C*). On histological examination (Fig. 1*D*) these projections appear as meristematic buds. Presumably due to rapid and uneven cell proliferation under the influence of TDZ, the meristematic center in some of the structures is shifted giving the appearance of cellular projections with meristematic patches. However none of the projections had the characteristic morphology of the early somatic embryo of peanut (Hazra *et al.* 1989, Chengalrayan *et al.* 1994, 2001). The morphology of somatic embryo is defined at a very early stage (Raghavan 1976, 1986, Maheswaran and Williams 1985). Occasionally these projections (Fig. 1*E*) were similar to the structures, induced by TDZ at the cotyledonary node, petiole base, and on the rachis of the peanut leaf, and identified as "emergences" (Kanyand *et al.* 1997). Like the "emergences" these structures differentiated into shoots. In contrast to the characteristic white somatic embryos of peanut, all the structures developed in presence of TDZ in the present study were bright green from the tip to base.

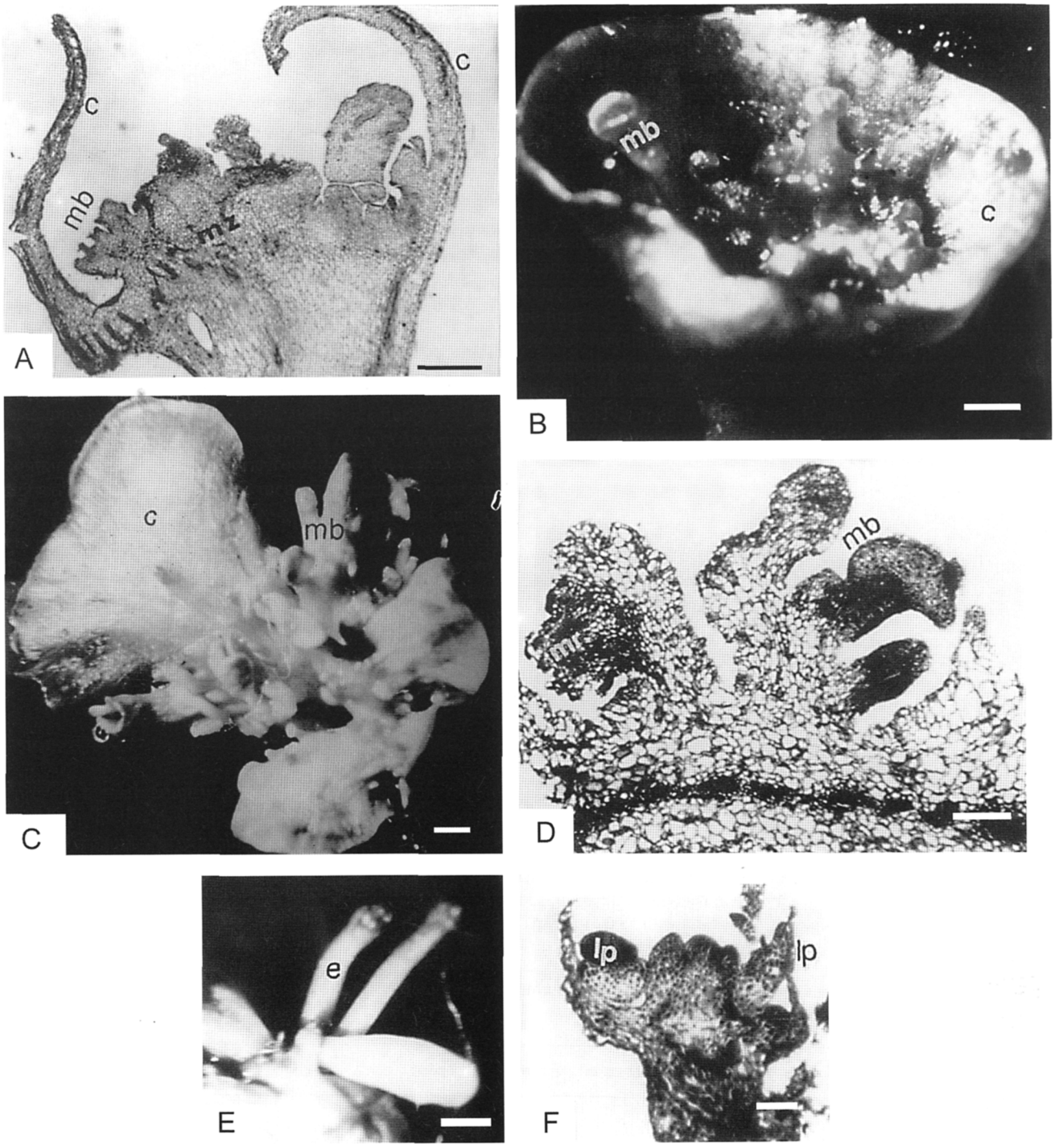

Fig. 1. Morphology of TDZ induced meristematic buds from the plumule of peanut somatic embryos:
A - longitudinal section of somatic embryo after 7 d in 22.7 μM TDZ containing medium (*bar* = 500 μm), meristematic zone (mz) is formed due to proliferation of the cells from the plumule surrounded by the cotyledons (c), meristematic buds (mb) developed from the meristematic zone;
B - somatic embryo like meristematic buds (mb), appeared from central depression of cup shaped fused cotyledons (c) (*bar* = 1 mm);
C - meristematic buds (mb) and projections between the fan shaped cotyledons (c) after 21 d of culture (*bar* = 1 mm);
D - section of cluster of meristematic buds (mb). Bud with the meristematic region (mr) in one side (*bar* = 200 μm);
E - elongated meristematic buds appear as emergences (e) (*bar* =1 mm);
F - stunted shoot with multiple leaf primordia (lp) (*bar* = 100 μm). Differentiation of the internodal region between the leaves is restricted.

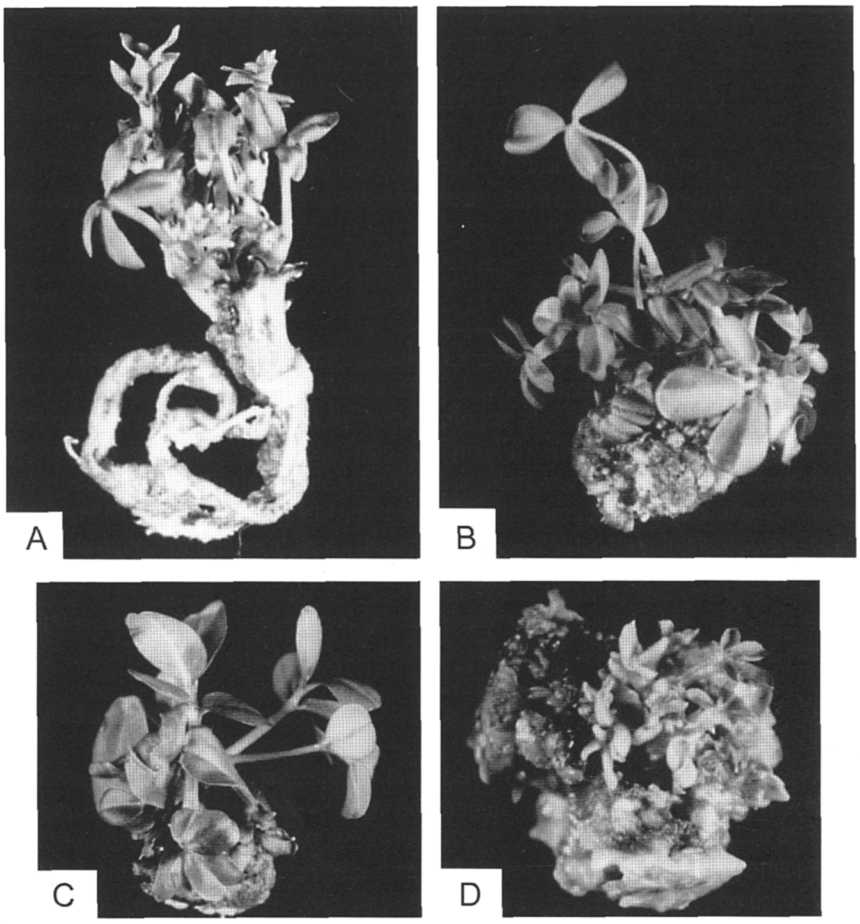

Fig. 2. Response of the plumule derived meristematic buds in various culture conditions optimized for specific response of peanut somatic embryos and meristematic buds:
A - plantlet with stunted multiple shoots developed in 22.7 μM TDZ containing medium;
B - shoot induction and elongation in the MS medium containing 2.22 μM BAP and 2.32 μM kinetin;
C - leaves differentiated from the meristematic buds cultured in medium devoid of growth regulator;
D - proliferation of caulogenic buds in medium with 13.32 μM BAP.

Table 1. Morphogenic responses in the TDZ induced somatic embryo like structures in the plumule of peanut somatic embryos in MS medium with various growth regulators. Means ± SD.

Growth regulator	Number of explants used	Response observed	Frequency of response [%]
0	59	no rooting, leaves	40.66 ± 3.78
22.70 μM TDZ	83	multiple shoots	79.21 ± 9.65
2.22 μM BAP + 2.32 μM KIN	61	differentiation and elongation of shoots	54.29 ± 11.09
13.32 μM BAP	58	caulogenic bud proliferation	89.63 ± 0.64

Unlike somatic embryos these were inseparable either individually or in cluster from each other or from the explant without injuring the base. All these evidences indicate that the structures developed from the plumule are not somatic embryos but projections with meristematic activity, which eventually differentiate to form shoots.

Similar to our earlier observation (Chengalrayan *et al.* 1997) the projections with meristematic activity differentiated to form cluster of small shoots on

incubation of the rooted somatic embryos for an extended period of four weeks in the same medium containing TDZ. The root differentiation of the embryo was restricted. The shoot buds elongated to a limited extent and the leaves opened. Histological studies conducted after 28 d of culture shows development of stunted shoot (Fig. 1*F*) due to restricted differentiation of the internodal region. Several such shoots appeared from the plumule of each rooted somatic embryo (Fig. 2*A*) giving the appearance of plantlets with stunted shoots and roots. This response was noted in 79 % of the embryos (Table 1). Similar plants were formed during conversion of somatic embryos (Chengalrayan *et al.* 1997). The responses of the somatic embryo like structures in the three media, indicated that these are organogenic buds and not somatic embryos. In the PGR free medium, the somatic embryos of peanut cultivar JL-24 germinate to form root (Chengalrayan *et al.* 1994). In the present study, the TDZ induced somatic embryo like structures failed to develop roots. On the contrary, in 41 % of the explants the structures differentiated into leaves (Fig. 2*B*). In MS medium containing BAP 2.22 μM + KIN 2.32 μM + 2 % sucrose 54 % of the somatic embryo like caulogenic buds differentiated into shoots of varying height (Fig. 2*C*). This is in concurrence with our earlier report describing shoot elongation in the rooted embryos following transfer to this medium (Chengalrayan *et al.* 1994). In medium containing 13.32 μM BAP, 90 % of the plumule with the structures converted into dense mass (Fig. 2*D*) of caulogenic buds. The efficiency of this medium in proliferation of caulogenic buds to form large number of buds was demonstrated earlier (Chengalrayan *et al.* 1995).

It is apparent from the above evidences that TDZ triggers meristematic activity at the plumule of peanut somatic embryos. This result in formation of caulogenic buds, which resemble the structures, identified as somatic embryos (Gill and Saxena 1992, Victor *et al.* 1999b). These buds differentiate to form shoots. The shoots are stunted due to restricted elongation of the internodal region. Therefore high frequency conversion of abnormal peanut somatic embryos (Chengalrayan *et al.* 1997) is due to TDZ aided caulogenic activity at the plumule.

In peanut tissue we used both adenine type cytokinins (KIN and BAP) and TDZ to induce caulogenic buds. Unlike the organogenic buds induced in presence of cytokinins, the bud induced by TDZ resembles the somatic embryos. Other researcher (Akasaka *et al.* 2000) reports similar observation. Induction of direct somatic embryogenesis from the hypocotyl of intact seedlings of peanut in presence of 10 μM TDZ has been reported (Murthy *et al.* 1995, Gill and Saxena 1992). This is contradictory to the report describing induction of caulogenesis in the hypocotyl tissue of seedling (Li *et al.* 1994) in presence of similar concentration TDZ and following the same method.

Keeping in views the earlier reports and on the basis of our observations we propose that TDZ induce organogenesis in peanut tissues and not somatic embryogenesis.

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