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Regeneration of diploid annual medics via direct somatic embryogenesis promoted by thidiazuron and benzylaminopurine

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Abstract The development of a simple and rapid procedure for direct somatic embryogenesis from wild Medicago spp. (*M. truncatula*, *M. littoralis*, *M. murex*, *M. polymorpha*) has exploited various explants including meristematic zones. Phytogel-solidified medium supplemented with thidiazuron or 6-benzylaminopurine at different concentrations effectively promoted this process. The first somatic structures emerged within 20 days of culture initiation. Histological analyses confirmed the nature of the directly formed embryos. Secondary embryogenesis was also observed. Cuttings of clusters of primary and secondary embryos were used for cyclic production of new embryo generations. Regenerated plants with well-developed root systems on medium with reduced levels of macroelements and sucrose were easily adapted to a greenhouse.

Key words Diploid Medicago · Somatic embryogenesies · Regeneration

Abbreviations *BAP* 6-Benzylaminopurine · *MS medium* Murashige and Skoog medium · *TDZ* Thidiazuron

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Introduction

Classical approaches to crop improvement may be augmented by modern techniques of cellular and molecular biology, once reliable procedures are defined for stable and efficient regeneration of the plants in vitro. Somatic embryogenesis has been used for in vitro regeneration of a wide range of phylogenetically distinct species from both the Angiospermae and Gymnospermae (Ammirato 1983; De Jong et al. 1993; Firoozabady and Boer 1993; Mercle 1995). Direct somatic embryogenesis is the preferred pathway for regeneration because it tends to yield genetically stable plants, since there is no callus phase (Larkin and Scowcroft 1981) and the typically single-cell origin of the induced embryos facilitates the production of non-chimeric transformants (Vasil 1988; Pedroso and Pais 1995).

Although perennial *Medicago* species may be regenerated in vitro via indirect (Brown and Atanassov 1985) or direct (Denchev et al. 1991) somatic embryogenesis, these species cannot be used as models for legumes because of their high degree of heterozygosity and large genome size.

Autogamous annual medics are closely related to alfalfa but they are diploid, self-pollinating and possess short life cycles. Hence they are more suitable for molecular and genetic investigations than are allogamous, perennial *Medicago* species such as the cultivated *M. sativa*. Wild medics may also serve as a pool for genes conferring useful agronomic traits such as resistance to biotic and abiotic stress (Schmiedernnecht and Lesin 1968; Diwan et al. 1994; Krall et al. 1996) so long as clones with high morphogenic capacity are available.

Unfortunately, regeneration of annual medics is more difficult than regeneration of perennials from the same genus, and has been reported for only a few species, e.g. *M. lupulina* (Li and Demarly 1995), *M. truncatula* (Nolan et al.1989; Chabaud et al. 1996; Trien

and Harrison 1996; Hoffmann et al. 1997), *M. polymorpha* (Scarpa et al. 1993), *M. littoralis* (Zafar et al. 1995) and *M. suffruticosa* (Li and Demarly 1996).

The objective of the research reported in this paper was to develop a rapid and routine protocol for direct somatic embryogenesis of annual medics which is species independent and useful for genetic and molecular biological studies.

Materials and methods

The experiments were carried out with four diploid *Medicago* species: *M. truncatula* BG (Bulgarian ecotype) and *M. truncatula* cv. Jemalong, *M. littoralis*, *M. murex* and *M. polymorpha.* Seeds were sterilised with 70% ethanol for 30 s, then with 0.2% HgCl2 solution for 3–4 min and rinsed five times with sterile distilled water. All plants were cultivated in growth chambers at 26 °C, 70% humidity, 2500–3000 lux light intensity and photoperiods of 16 h light/8 h darkness.

Hypocotyls, cotyledons and petiole bases were used as explants along with nodal stem segments. Explants were removed from 30- to 35-day-old seedlings or plants grown in vitro. All explants retained some meristematic tissue (even after, for example, adjacent axillary buds had been removed).

Plant-growth-regulator-free Murashige and Skoog (MS) medium (Murashige and Skoog 1962) was used for seed germination and maintenance of donor plants. Media for plant regeneration (including embryo induction and development) contained MS salts, Morel vitamins, 3% sucrose, 0.25% Phytogel and were supplemented with 6-benzylaminopurine (BAP; 0, 0.88, 2.22, 4.44 and 44.38 μm) or thidiazuron (TDZ; 0, 0.91, 2.27, 4.54 and 45.41 μm). The growth regulators were added after filter sterilisation (0.22 μm Millipore).

Clusters of regenerated embryos were separated for growth to plants on MS medium with half-strength macronutrients, Morel vitamins, 1.5% sucrose and 8% agar.

The pH of all media was adjusted to 5.8 with 1 n NaOH.

Regenerants with well-developed roots and four trifoliate leaves were planted in an autoclaved soil:perlite (1:1) mixture. The plants were adapted to ex vitro culture in a misted greenhouse.

Explants with induced somatic embryos were fixed in formalin/acetic acid/alcohol (2:1:5 by volume) for 24 h. After paraffin embedding, tissues were cut and stained with 1% toluidine blue for 30 s and observed microscopically.

The results summarise the data of three independent experiments with three replicates for each treatment. Analysis of variance (ANOVA) was used for data evaluation.

Results

Primary somatic embryos were first observed on the bases of the explants containing meristematic zones (Fig. 1a,b). The somatic embryos which developed without an intermediate callus phase could be observed within 20 days of cultivation (Fig. 1c–f). In the presence of BAP and TDZ, regeneration was completed in 35–40 days. The complete regeneration process is illustrated in Fig. 1. Both TDZ and BAP stimulated embryo formation. On MS medium containing various levels of TDZ (0.91, 2.27, 4.54 μm) or BAP (0.88, 2.22, 4.44 μm), all the explants formed embryos but the efficiency varied according to the species. Embryo induction was completely blocked on plant-growth-regulator-free medium or at the highest concentrations of TDZ (45.41 μm) or BAP (44.38 μm).

Histological observations strongly indicated that (primary and secondary) somatic embryos were formed directly, without an intervening callus phase. They grew on the explant surface without any vascular connection with the maternal tissue (Fig. 1c–h). Embryogenesis from somatic tissues progressed through the stages typical for zygotic embryos: globular, heart-shaped, torpedo and cotyledonary stages (Fig. 2).

After 25 days of cultivation, secondary embryos were observed on the surface of the primary embryos (Fig. 3). The cultivation of such structures for one further passage on used regeneration media significantly increased the number of secondary somatic embryos.

In the presence of TDZ (2.27 μm) it was possible to provoke de novo recycling of embryo induction. Clusters of primary and secondary embryos (Fig. 4) which had been cut into small pieces promoted the emergence of the new structures within 20 days through a slight callus (Fig. 5).

In the presence of TDZ (0.91–4.54 μm) or BAP (0.88–4.44 μm), the embryogenic response of *M. truncatula* cv. Jemalong was more pronounced than that of the other species tested irrespective of the explant (Table 1). The number of induced somatic embryos per explant varied within small limits. The variation shown by nodal stem segments was no higher than that for petioles. TDZ at 2.27 μm was used for successful regeneration of embryos from explants tested.

M. truncatula BG (Table 1) showed an explant-specific requirement for the nutrient medium while both plant bioregulants (2.27 μm TDZ and 2.22 μm BAP) promoted direct embryo formation from cotyledon explants at an equal rate. Media supplemented with 4.54 μm TDZ was optimal for regeneration from petioles, while for hypocotyls, the optimal concentration was 2.22 μm BAP.

All explants from *M. murex* had higher embryogenic capacity in the presence of TDZ (2.27–4.54 μm). No significant difference was found in embryogenic esponse of the explants on media supplemented with different concentrations of BAP (0.88, 2.22, 4.44 μm) (Table 1).

The embryogenic potential of *M. littoralis* explants was expressed in the presence of both growth regulators, but the best outcome was observed when nodal stem segments were cultured on medium with 4.54 μm TDZ (Table 1).

Similar variation in embryogenic efficiency on various media was demonstrated for explants of *M. polymorpha*. Media with 2.27 or 4.54 μm TDZ stimulated direct embryo formation from all explants (Table 1).

On the basis of these data, the embryogenic capacities of the annual medics was orderd as follows: *M. truncatula* cv. Jemalong>*M. truncatula* BG>*M. polymorpha*>*M. littoralis*>*M. murex*.

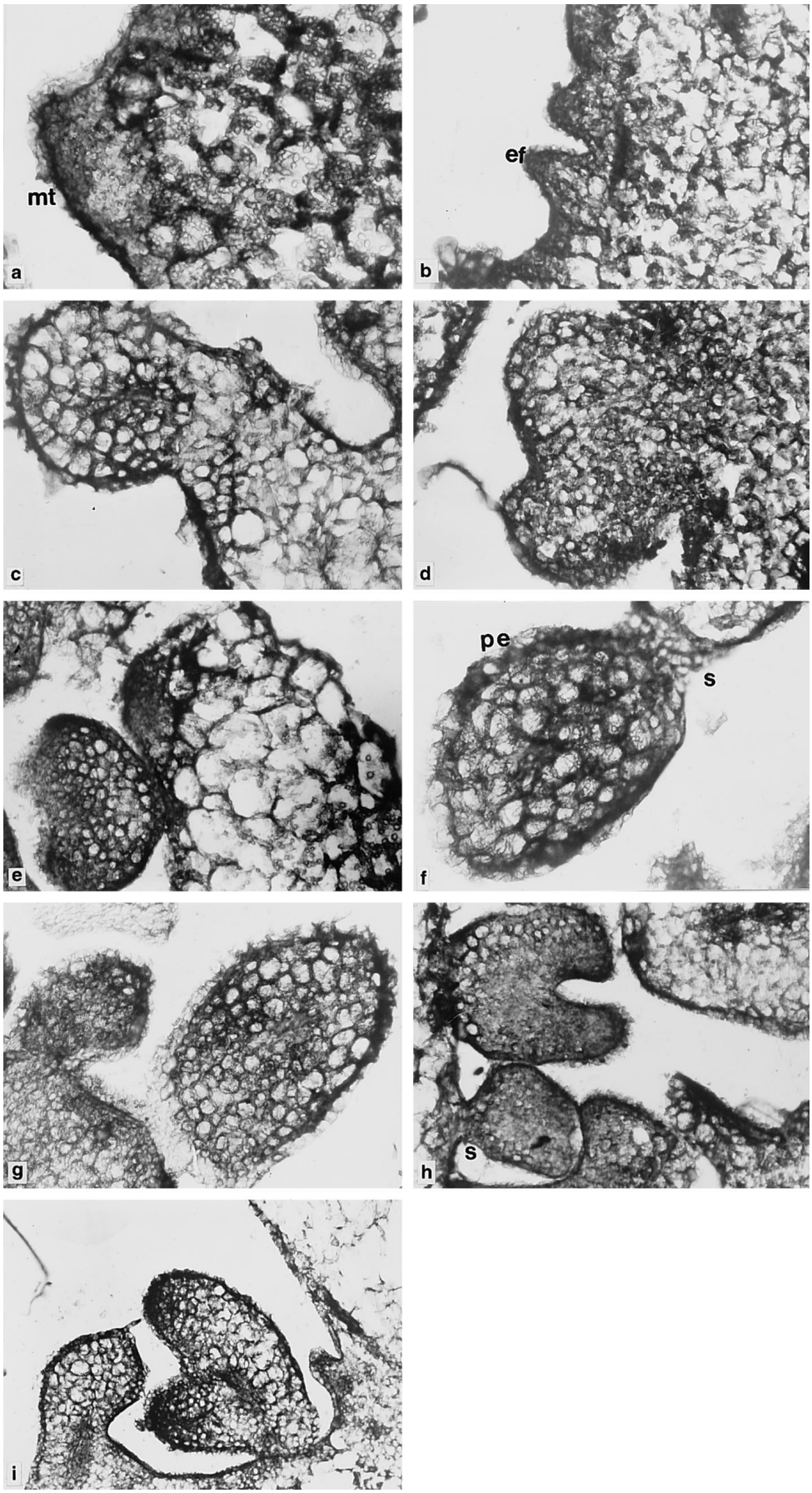

Fig. 1a–i Step-by-step process of embryo induction to embryo development (*mt* meristematic tissue, *ef* embryo formation, *pe* primary embryo, *s* suspensor)

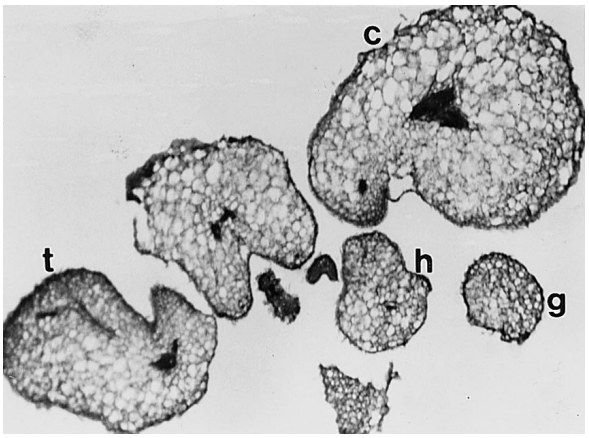

Fig. 2 Stages observed in somatic embryo formation (*g* globular, *h* heart, *t* torpedo, *c* cotyledonary)

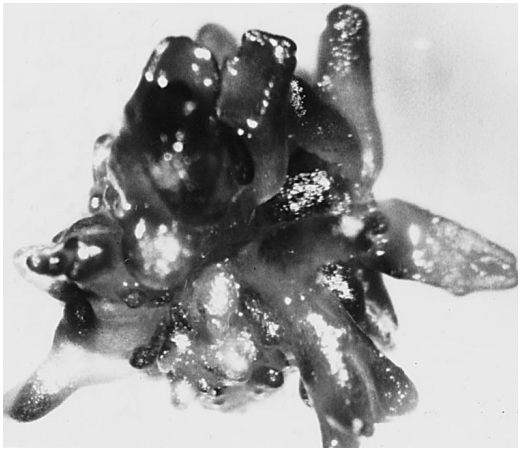

Fig. 4 Cotyledon base with cluster of somatic embryos

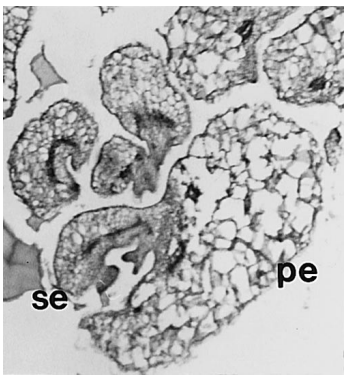

Fig. 3 Secondary embryo (*se*) formation (*pe* primary embryo)

Fig. 5 Recycling process (*es* embryo structure)

Embryo clusters occasionally hyperhydricity, which could be overcome when regenerated embryos were separated from the cluster and transferred to medium with reduced levels of macronutrients and sucrose, for two passages. About 80–85% of embryos on this medium grew to normal plants and developed very well formed root systems without special rooting conditions. Such plants were successfully transferred to a greenhouse without any losses, and all set fertile seeds (Fig. 6).

Discussion

This communication has outlined a novel, very efficient, rapid and species-independent protocol for the regeneration of plants via somatic embryogenesis from four diploid annual medics. The experiments showed that these species are more recalcitrant in vitro than cultivated alfalfa. To date, there are few reports on the regeneration of annual *Medicago* species, but most of the established protocols are characterised by long periods of cultivation, frequent media changes, an additional callus phase and relatively low regeneration frequency. In our procedure, these problems were resolved. We succeeded in obtaining somatic embryos directly from explants (avoiding a callus phase) and with a much shorter period (35 days) for regeneration. The whole process of regeneration (from embryo induction to development) was completed on one medium supplemented with TDZ or BAP. Obviously, the presence of growth regulators and the choice of explants with meristematic zones were crucial for induction of embryos in the medics tested. To date, in all reports with wild alfalfa, the induction of embryogenesis and organogenesis were accomplished on media supplemented with auxin (2.4-dichloroacetic acid or α-naphthaleneacetic acid) alone or in combination with cytokinin (*M. truncatula*: Nolan et al. 1989; Chabaud et al.1996; Trieu and Harrisson 1996; Hoffemann et al. 1997; *M. polymorpha*: Scarpa et al. 1993; *M. littoralis*: Zafar et al. 1995). With the exception of the report of Trieu and Harrisson (1996), the results of

Table 1 Assessment of direct somatic embryogenesis from various *Medicago* explants (number of embryos/explant)

Explant	TDZ			BAP		
	0.91 μM	2.27 μM	4.54 μM	0.88 μM	2.22 μM	4.44 μM
M. truncatula Jemalong						
Hypocotyl	20 ± 1.15	19.66 ± 1.45	14.33 ± 0.33	10.66 ± 2.33	16.33 ± 1.85	15.0 ± 1.73
Cotyledon	16.66 ± 0.88	22.33 ± 1.45	17.66 ± 1.45	8.0 ± 0.57	20.3 ± 0.33	11.33 ± 1.85
Petiole	16.66 ± 0.88	19.66 ± 3.17	12.0 ± 0.57	9.0 ± 1.52	14.0 ± 1	10.66 ± 0.66
Nodal stem	8.33 ± 0.88	13 ± 0.57	9.66 ± 0.88	9.33 ± 1.2	12.33 ± 1.45	11.33 ± 0.66
M. truncatula BG						
Hypocotyl	2 ± 0.1	1.33 ± 0.66	4.66 ± 0.66	2.66 ± 0.33	5.33 ± 0.33	2.33 ± 0.1
Cotyledon	3.33 ± 0.33	6 ± 1.52	3 ± 0.33	3.33 ± 0.33	6 ± 0.57	2.66 ± 0.33
Petiole	2.33 ± 0.33	3 ± 0.33	6.66 ± 1.66	2.33 ± 0.33	3.66 ± 0.33	3.66 ± 0.33
Nodal stem	3 ± 0.57	3 ± 0.57	3 ± 0.33	3 ± 0.33	5 ± 0.88	4 ± 0.1
M. murex						
Hypocotyl	2.0 ± 0.1	3.66 ± 0.66	2.66 ± 0.33	2.66 ± 0.33	2.33 ± 0.66	2.0 ± 0.01
Cotyledon	3.0 ± 0.33	5.0 ± 1.52	3.66 ± 0.33	2.0 ± 0.33	2.66 ± 0.33	3.0 ± 0.33
Petiole	3.33 ± 0.33	3.33 ± 0.33	3.33 ± 0.33	3.0 ± 0.33	3.0 ± 0.33	2.66 ± 0.33
Nodal stem	3.0 ± 0.88	3.33 ± 0.33	3.33 ± 0.33	1.66 ± 0.88	3.0 ± 0.33	2.0 ± 0.01
M. littoralis						
Hypocotyl	2.66 ± 0.66	2.0 ± 0.1	2.0 ± 0.1	2.66 ± 0.66	2.0 ± 0.1	3.0 ± 0.33
Cotyledon	3.0 ± 0.33	4.33 ± 0.33	3.66 ± 0.33	3.0 ± 0.33	4.66 ± 0.66	4.33 ± 0.88
Petiole	3.33 ± 0.33	4.0 ± 0.33	4.66 ± 0.88	2.66 ± 0.33	2.66 ± 0.33	4.33 ± 0.33
Nodal stem	3.33 ± 0.33	4.66 ± 0.33	5.0 ± 0.57	2.66 ± 0.33	3.0 ± 0.33	4.33 ± 0.33
M. polymorpha						
Hypocotyl	3.0 ± 0.57	4.66 ± 0.33	3.66 ± 0.33	2.33 ± 0.57	3.0 ± 0.57	3.0 ± 0.57
Cotyledon	3.33 ± 0.33	4.33 ± 0.33	3.66 ± 0.33	3.0 ± 0.57	4.33 ± 0.33	2.66 ± 0.88
Petiole	4.0 ± 0.57	3.66 ± 0.33	5.0 ± 0.57	2.33 ± 0.33	3.0 ± 0.57	4.33 ± 0.33
Nodal stem	3.33 ± 0.33	4.0 ± 0.57	7.0 ± 0.57	2.33 ± 0.33	3.0 ± 0.57	3.0 ± 0.57

Fig. 6 Regenerants from somatic embryos in the greenhouse

experiments with diploid medics involved prolonged callus phases before somatic embryos were induced. The auxins used are also potent activators of somatic embryogenesis in several leguminous species: pea (Kysely and Jacobsen 1987), alfalfa (Brown 1989; Parrott 1991), cotton (Firoozabady and De Boer 1993), and in a large number of cereals (Vasil 1988).

In our procedure for regeneration of annual medics, TDZ was tested for the first time. This synthetic compound is a derivative of phenyl urea and has been reported to possess strong cytokinin-like activity in a number of plants (Hutteman and Preece 1993). It has been used successfully for shoot regeneration in diverse plant species including woody plants such as apple (Sriskandarajah et al. 1990) and eastern cottonwood (Prakash and Thielges 1989), green ash (Kim et al. 1997) and in the leguminous plants, beans and peanut (Gill and Saxena 1992; Malik and Saxena 1992; Matand et al. 1994).

The high frequency of somatic embryogenesis induced by TDZ in our results suggests that it might influence the endogenous level of cytokinins, auxins and abscisic acid so as to induce the positive embryogenic response of the cultivated tissue as already reported by Murthy et al. (1995) and Hutchinson et al. (1996).

The application of BAP as the sole growth regulator also promoted embryo formation in our study. The induction of direct somatic embryogenesis and organo-

genesis in the presence of BAP (alone or in combination with auxin) has been observed in intact seedlings of bean (Malik and Saxena 1992), in cotyledon explants from *M. truncatula* (Trieu and Harrisson 1996) and leaf explants from *Helianthus smithii* (Lapara et al. 1997).

That the embryogenic potential of many *Medicago* species is genotype specific is well documented (Brown and Atanassov 1985). Our study confirmed that the morphogenic capacity of annual medics is slightly dependent on the species used. However, the stable and positive regeneration response of the explants used in our experiments could be due to the presence of meristematic tissues which are morphologically and physiologically more similar to each other than are differentiated somatic tissues. Thus, the slight species dependence of the embryogenic response in diploid medics is reduced. This is a particular advantage of our regeneration system which can be applied with equal success to various annual, diploid medics.

The ability to apply this protocol for a wide range of *Medicago* species is important when considering the use of in vitro techniques for the genetic improvement of alfalfa. The efficient protocols previously established for somatic embryogenesis of *M. truncatula* cv. Jemalong (Nolan et al. 1989), and *M. truncatula* R108 (Hoffman et al. 1997) could not be applied to other wild alfalfa species. In contrast, our procedure induced embryo formation in all the explants and species which were tested. The species tested, with the exception of *M. truncatula* cv. Jemalong, which possessed remarkable embryogenic potential, showed similar embryogenic responses.

Histological observations confirmed that somatic embryos were formed directly as independent units organised in clusters, some of them connected to the explant loosely by suspensors. The appearance of independent vascular systems in the embryos indicated, additionally, that the structures possessed the ability to form normal somatic embryos and plantlets.

The recycling procedure which we have established opens up the important possibility of scaling up embryo and plantlet formation and maintaining the embryogenic potential of the plant material for an unlimited period. This cyclic regeneration system is ideally suited for gene manipulation research.

We believe that this is the most optimised protocol for direct somatic embryogenesis from annual diploid medics. Three features of our results have been emphasised: (1) the ability to express the morphogenic potential of all the annual medics tested, (2) the positive embryogenic response of explants containing small meristematic zones and (3) the essential role of TDZ or BAP as growth regulators inducing high-frequency somatic embryogenesis.

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