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Somatic Embryogenesis in Genera *Medicago*: an Overview

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Abstract This chapter outlines the details of somatic embryogenesis in genera *Medicago*. Various factors that influence the process of somatic embryo induction, development, maturation and conversion are discussed. The role of genotype, explant choice and preparation hormonal compositions and the origin of somatic embryos are also reviewed. Brief attention is paid to the regenerant's phenotype and fertility.

1 Introduction

The genus *Medicago* is composed of annual and perennial species. They are diploid, tetraploid and polyploid; wild and cultivated. The perennial species *M. sativa*, *M. falcata*, *M. varia*, *M. coerulea*, *M. arborea* and *M. glutinosa* are generally grouped as *M. sativa* complex. Alfalfa (*M. sativa*) is the most important forage crop cultivated on over 32 million hectares in the world (Michaud et al. 1988). For a long time it has been the object of genetic, cellular and molecular studies because of its good regeneration capacity in vitro. The first report of regeneration of *M. sativa* (Sanders and Bingham 1972) was via somatic embryogenesis. Since then, many reports on regeneration of this perennial species have been published, mostly by indirect somatic embryogenesis (Bingham et al. 1988; Arcioni et al. 1990; McKersier and Brown 1996; Barbulova et al. 2002). Regeneration via direct somatic embryogenesis was also reported in *M. sativa* (Maheswaran and Williams 1984) and *M. falcata* (Denchev et al. 1991). Annual *Medicagos* are closely related to alfalfa but they are diploid, self-pollinated and possess a short life cycle. The regeneration of annual *Medicagos* is more difficult than that of perennials. The first regeneration protocol of annual *M. truncatula* via indirect somatic embryogenesis was achieved by Nolan et al. (1989) and a few more have been reported since (Chabaud et al. 1996; Hoffmann et al. 1997; Trinh et al. 1998). Protocols for regeneration of other annuals have also been made in *M. polymorpha* (Scarpa et al. 1993), *M. littoralis* (Zafar et al. 1995), *M. suffruticosa* (Li and Demarly 1996) and *M. lupulina* (Li and Demarly 1995). Regeneration via direct somatic embryogenesis in liquid and solid media for *M. truncatula* (Iantcheva et al. 2001; Iantcheva et al. 2005) and for *M. littoralis*, *M. murex* and *M. polymorpha* also has been established (Iantcheva et al. 1999).

In this chapter, various factors that affect the process of somatic embryo induction, development, maturation and conversion are discussed. The genotype, explant choice and preparation, origin of somatic embryos and hormonal composition of culture media are also described. Brief attention is paid to the phenotype and fertility of the obtained regenerants.

2
Induction of Somatic Embryogenesis

2.1
Type of Somatic Embryogenesis

Somatic embryogenesis is a process whereby a cell or group of cells from somatic tissue forms an embryo. The development of somatic embryos nearly replicates the process of zygotic embryo formation. Somatic embryogenesis mostly occurs indirectly via an intervening callus phase or directly, i.e. embryos develop on the explant surface like epidermal or sub-epidermal layers, as in *M. falcata* (Denchev et al. 1991) and *M. truncatula* (Iantcheva et al. 2001). See Table 1 for a list of references on the induction of somatic embryogenesis.

2.2
Genotype, Choice of Explant and Type of Preparation

Genotype is the most important factor influencing embryogenic response. Variability in the induction and frequency of the obtained embryos is observed among different species of genera *Medicago* and within the cultivars (Brown and Atanassov 1985; Chen et al. 1987). Considerable variations in embryogenic capacity were also observed between individuals of one cultivar or species. Genotype-dependent embryogenic capability was widely reported especially in *M. sativa* (Seitz Kris and Bingham 1988; Mitten et al. 1984; Chen et al. 1987; Nagaradjan et al. 1986; Barbulova et al. 2002; Ivanova et al. 1994). The use of this species for in vitro experiments requires the isolation of a highly embryogenic genotype. In general, a regenerative genotype could be found in any alfalfa germplasm if enough genotypes are screened (Brown and Atanassov 1985; Mitten et al. 1984). Together with genotype there are other factors affecting embryogenic response: explant, culture condition and medium composition. Removal of the explant from the mother plant is a prerequisite for the acquisition of embryogenic competence (Finstad et al. 1993). Choice of explant is a factor that determines success in establishing embryogenic protocol. Besides, somatic embryo regeneration was achieved from different explant sources in perennial and annual *Medicagos*. Indirect somatic embryogenesis of *M. sativa* was induced from a broad range of explants such as leaf (Meijer and Brown 1987; Barbulova et al. 2002), petiole (Lai

and McKersie 1994), internode (Parrott and Bailey 1993), immature embryo (Ninkovic et al. 1995), hypocotyl (Meijer and Brown 1987; Kim et al. 2004), suspension culture and mesophyll protoplast (Atanassov and Brown 1984). For this perennial species, direct embryo formation was achieved from immature embryos (Maheswaran and Williams 1984) and from leaves in *M. falcata* (Denchev et al. 1991). Immature inflorescence is a suitable explant source for embryo induction in *M. lupulina* (Li and Demarly 1995). Explants including meristematic active zones such as hypocotyl, cotyledon, petiole base and nodal stem segments are used for direct embryo formation in *M. truncatula*, *M. littoralis*, *M. murex* and *M. polymorpha* (Iantcheva et al. 1999). Recently, direct embryo formation from root explants was reported in *M. truncatula* (Iantcheva et al. 2005).

In order to select the appropriate explant as an initial material for induction of embryogenic potential, donor tissue has to be tested for ploidy level. Different tissues are mixtures of cells with different ploidy levels (polysomaty). Moreover, given that tissue polysomaty predisposes to ploidy variation in regenerants, possible sources of explant have to be checked for polysomaty. In the study of Iantcheva et al. (2001) the ploidy levels of leaf and petioles are examined to select more uniform monosomatic tissue, dominated by 2C nuclei as an initial explant for induction of embryogenic potential.

The age of explants, size, preparation and culture environment are important factors for the type of somatic embryogenesis—indirect or direct. The age of the in vitro plant and physiological stage are of great importance for induction of somatic embryogenesis. In a direct somatic embryogenesis system (in liquid medium) of *M. falcata* (Denchev et al. 1991) and *M. truncatula* (Iantcheva et al. 2001), the leaf explants were excised from 30-day-old in vitro plant material. The explants were chopped into small pieces by razor blade to a size of 2–4 mm. Such explant preparation with severe wounding and small size, together with liquid culture conditions and agitation on a rotary shaker (100 rpm), led to direct embryo formation on the surface of the explants, with the period of induction shortened to 15–20 days. Embryos emerged first on the cut edges of the explants. In perennial *M. falcata*, indirect embryo formation was noted when leaf squares were cultured on a solid medium with the same composition, which was reported earlier by Denchev et al. (1991). Obviously the age of the explant, preparation (severe wounding) and culture environment (liquid media, agitation) are of great importance for the type of somatic embryogenesis induced. Wounding of explants on a small scale probably triggers the expression of specific genes (wound-inducible genes), which were already identified and cloned (Dudits et al. 1995) for further acquisition of embryogenic competence towards cell division and differentiation. Wounding of the explants was found to be a key factor in *M. sativa* A70-34 embryogenesis (Piccioni and Valecchi 1996).

Application of an osmoticum as stress stimulus can also lead to acquisition of embryogenic competence. Osmotic pre-treatment with 1 M sucrose of

the initial root explant of *M. truncatula* is important for the shortening of the regeneration period (induction, maturation and conversion) and a higher percentage conversion of somatic embryos to plants (Iantcheva et al. 2005). This information also confirmed that embryo induction and regeneration from root explant is also genotype specific, even after osmotic pre-treatment of the primary explant of *M. truncatula cv*. Jemalong and *cv*. R 108 1. Perhaps osmotic stress activates pre-determined embryogenic cells to switch them from the somatic to embryogenic type followed by cell division. Osmotic pre-treatment for only 1 h with 1 M sucrose activated cells for division in root tips of transgenic *M. falcata* plants expressing the *gus* gene under cell cycle promoters: *cyc* 3a (cyclin type A) and *cyc* 1a (cyclin type B) (Iantcheva et al. 2004). Short-term osmotic stress is found to be necessary for the accumulation of free proline (Gangopadhyay et al. 1997) and this could be connected with the improvement of somatic embryogenesis. The positive role of proline in the induction of somatic embryogenesis of alfalfa was similarly reported by Shetty and McKersie (1993).

The endogenous hormone level of the initial explant is essential for determining the ability of a particular genotype to induce somatic embryogenesis (Jiménez 2001). The performed comparative investigation (Ivanova et al. 1994) of two *M. falcata* lines (highly embryogenic 47/1/150 and non-embryogenic 47/1/165) confirms that the level of endogenous indole-3-acetic acid (IAA) in the initial explant was higher in the embryogenic line. In the same study, the negative correlation between endogenous ABA and acquisition of embryogenic potential was observed. The investigation of Pintos et al. (2002) on the endogenous cytokinin level of embryogenic and non-embryogenic calli of *M. arborea* established the higher endogenous cytokinin level in non-embryogenic than embryogenic callus. The above studies indicated that the processes of in vitro morphogenesis (organogenesis and somatic embryogenesis) are the results of a proper balance of plant growth regulators supplied to the culture medium and endogenous regulators in the tissue of the primary explant.

The acquisition of embryogenic competence and direct formation of somatic embryos are directly related to genome size. After examination of the genome size of several annual species of *Medicago*, it was found that the smallest genome size species formed somatic embryos for the shortest period of time, with a high number of embryos per explant compared to those with a larger genome size (Iantcheva et al. 2003).

2.3 Hormonal Composition of Culture Media

To date, in all reports of alfalfa and wild *Medicago*, the induction of somatic embryogenesis is accomplished on media supplemented with an auxin (2,4-D, dichlorophenoxyacetic acid, or NAA, α-naphthaleneacetic acid) alone or

in combination with cytokinin (Sanders and Bingham 1975; McKersie and Brown 1996; Brown and Atanassov 1985; Nolan et al. 1989; Chabaud et al. 1996; Pintos et al. 2002). The embryogenic effect of 2,4-D is well known in legumes and in genera *Medicago* (Denchev et al. 1991; Trinh et al. 1998; Zafar et al. 1995). 2,4-D can reach the highest intracellular concentration and usually results in high-frequency embryo formation. The concentration of 2,4-D also plays an important role in the processes of de-differentiation and differentiation in vitro (Denchev and Atanassov 1988). In the study of Barbulova et al. (2002), a 2,4-D concentration of 5 or 2 mg/l produced a more dense, necrotic and less embryogenic callus compared to the white, soft and highly embryogenic callus obtained in a medium with 1 mg/l 2,4-D. For these cultivars, the lowest concentration of 2,4-D is the optimum. According to Vergana et al. (1990) the higher concentrations of 2,4-D, at some point, block the cell division and inactivate the cells that already possess embryogenic potential. A high frequency of direct somatic embryo formation was observed in liquid medium in perennial *M. falcata* (Denchev et al. 1991) and annual species of *M. truncatula* and *M. polymorpha* (Iantcheva et al. 2001) in the presence of 4 mg/l 2,4-D. The concentrations up to 11 mg/l 2,4-D are able to induce somatic embryogenesis, while higher levels prevent induction.

The addition of NAA is essential for somatic embryogenesis initiation for some annual species like *M. polymorpha* (Scarpa et al. 1993), *M. rigidula* and *M. orbicularis* (Ibragimova and Smolenskaya 1997), and *M. truncatula* (Nolan et al. 1989). However, the molecular mechanisms involved in the induction of this process are still not fully understood. Recently a somatic embryogenesis receptor kinase (SERK) gene from *M. truncatula* (*Mt*SERK 1) was cloned and its expression examined in culture (Nolan et al. 2003). An auxin stimulates *Mt*SERK 1 expression, but its expression is much higher when both auxin (NAA) and cytokinin (6-benzylaminopurine (BAP)) are present in the medium. The effect of cytokinin appears to be more promotive in indirect somatic embryogenesis systems. Enhancement in the production of callus tissue with following embryo formation is observed in *M. truncatula* and *M. sativa* when the induction medium is supplemented with BAP (Trinh et al. 1998). For induction of embryogenic potential and its expression among different species of genera *Medicago*, different cytokinins (kinetin, BAP, zeatin, thidiazuron (TDZ) are required (Denchev et al. 1991; Nolan et al. 1998; Ding et al. 2003; Chabaud et al. 2004; Kim et al. 2004).

Induction of somatic embryogenesis by cytokinin alone is relatively rare among legumes and especially in genera *Medicago*. In legumes, somatic embryogenesis induced by cytokinin is established in *Trifolium repence* (Maheswaran and Williams 1985) and *Phaseolus* (Malik and Saxena 1992). In the annual *Medicago* species *M. truncatula*, *M. littoralis*, *M. murex* and *M. polymorpha*, direct induction of somatic embryos was achieved on solid media in the presence of TDZ or BAP (Iantcheva et al. 1999). In this system the whole process of embryogenesis from induction to maturation was completed on

a medium containing cytokinin as well, and this system was species independent. Actually, the embryogenic effect of TDZ was more pronounced than that of BAP in terms of embryo number. TDZ possesses cytokinin-like activity and induced high-frequency direct somatic embryogenesis in other legumes (Saxena et al. 1992; Murthy et al. 1995). This growth regulator is found to be more active than 2,4-D and BAP. After 1 h treatment with 1 mg/l TDZ, root-tip cells of transgenic *M. falcata* plants were activated for division and expressed the *gus* reporter gene (under promoters from cell cycle regulating genes—*cyc* A and *cyc* B) more strongly than 2,4-D and BAP (Iantcheva et al. 2004). The positive embryogenic response induced by TDZ suggests that it might influence the endogenous level of cytokinins, auxins and abscisic acid (ABA) (Murthy et al. 1995; Hutchinson et al. 1996). The above mentioned *Mt*SERK 1 gene (Nolan et al. 2003) was not expressed in the presence of cytokinin, or the cells that expressed *Mt*SERK 1 were few in number in the direct somatic embryogenesis system, as the level of *Mt*SERK 1 mRNA in the tissue was relatively low and was not detectable. In the direct somatic embryogenesis system of annual *Medicago* induced by TDZ (Iantcheva et al. 1999) the process started in a small number of meristematic cells.

2.4 Origin of Somatic Embryos in Direct Embryogenesis of Model M. falcata and M. truncatula Systems in Liquid and Solid Media

The indirect somatic embryogenesis systems in genera *Medicago* are characterized by a sequence of events that includes the stimulation of cell proliferation, dedifferentiation, acquisition of embryogenic competence and the induction of embryogenesis. Treatment with an auxin (usually 2,4-D) is a characteristic move for the early stages but subsequent embryo development requires removal of exogenous auxin. One feature of indirect systems is that the initial activation of cell proliferation is temporary and physically separated from the induction of embryo-specific cell division.

Direct somatic embryogenesis is characterized by the formation of embryos directly from differentiated tissue without the apparent requirement of the dedifferentiation stage involving disorganized cell proliferation. For example, the somatic embryogenesis system of *M. falcata* (Denchev et al. 1991) and *M. truncatula* (Iantcheva et al. 2001) involved direct formation of embryos from young alfalfa leaf explants and petioles in response to an induction treatment. There are two different models to explain this phenomenon. The first proposes that there are cells within the tissue that are already embryogenically competent and require the inductive signal to trigger direct embryo formation (Williams and Maheswaran 1986; Carman 1990). It has also been argued that in the direct system, embryogenesis does not differ significantly from the indirect procedure at the molecular level, and both proceed through similar stages of genetic re-programming at different rates

(De Jong et al. 1993). These models have different explanations for cell division activation in the process of direct somatic embryogenesis. In the first, the inductive signal acts as a mitotic trigger and re-activates cell division in cells that are already competent to switch from the somatic to embryogenic type and proceed into asymmetric cell division to form embryos. In the second model, the induction of cell proliferation is required for dedifferentiation which then permits the acquisition of embryogenic competence in certain cells, just as in the indirect system.

To distinguish these models, the investigation of induction of first cell division was studied in two single-cell suspension culture systems of *M. falcata* and *M. truncatula* for direct somatic embryogenesis. Initial embryogenic cell division and embryogenic competence might be linked to the expression of reporter *gus* gene under the control of promoters from cell cycle regulatory genes (*cyc* 3a, *cdc* 2a) and green fluorescent protein (*gfp*) reporter gene under 35 S promoters. The expression pattern of the studied reporter genes and the behaviour of single embryogenic cells in liquid culture confirm the asymmetry of first cell division, which starts the process of direct somatic embryogenesis.

Confocal microscopy observation of the 35 S *gfp M. truncatula* single-cell fraction confirmed that the fraction is composed of three types of cells: spheroid, ovoid (Fig. 1A) and elongated (Iantcheva et al. 2001). Transfer of these cells into a fresh induction medium supplemented with 2,4-D reactivates the cell for division. The *gfp* was detected strongly in the nucleus where it tends to accumulate slowly and the nucleus is situated at the cell periphery (Fig. 1B). The first asymmetric division is probably a consequence of nuclear migration from the central region to the periphery, which was also observed in *M. sativa* mesophyll protoplast (Dijak and Simmonds 1988). Further development of such asymmetrically divided cells (Fig. 1C) continued with the formation of a three-cell proembryo (Fig. 1E). Confocal software offers the possibility of depicting the *gfp* fluorescence profile in cells and structures. The peak indicated that the highest level of *gfp* expression is concentrated in the nucleus. Two peaks confirm the presence of two nuclei with separation of the cell into two unequal cell parts (Fig. 1D). Three peaks correspond to the three nuclei of a three-cell proembryo (Fig. 1F.)

In other systems of direct somatic embryogenesis (*M. falcata*), a single-cell fraction (expressing *gus* gene) is formed from the initial suspension culture after 10–15 days of induction (Iantcheva et al. 2004). These cells possess the potential to divide and form embryos and develop into a whole plant. In this fraction, three types of cells are also observed: spheroid, ovoid and elongated. Most of the spherical and ovoid cells are highly cytoplasmic, reduced in size and divided asymmetrically (Fig. 2A), and are capable of forming embryos and completing their development. In most cases, the smaller cell from this division tends to form a suspensor composed of two to five cells and the other cell continues to divide and form the embryo (Fig. 2B). In a later development, the suspensor aborts in well-shaped globular embryos (Fig. 2C). By

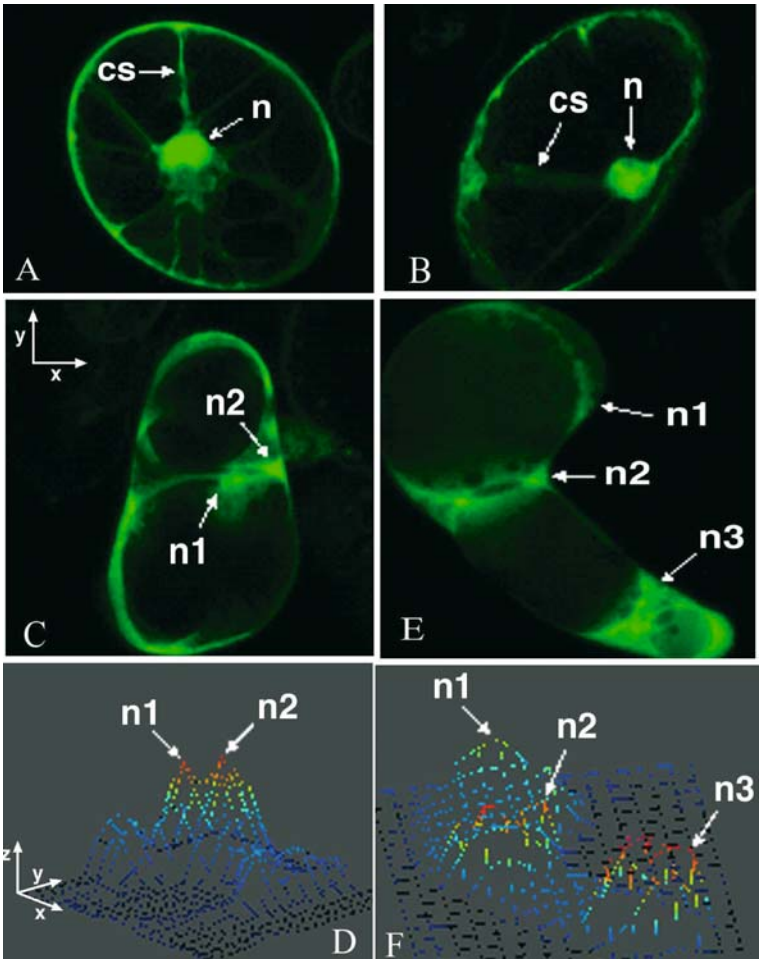

Fig. 1 Process of direct somatic embryo formation from a single cell in *Medicago truncatula*. **A** Spherical cell with a central nucleus (*n*) and cytoplasmic strands (*cs*) radiating to the cortical cytoplasm. **B** Ovoid cell with a nucleus in the cell periphery. **C** Asymmetric division with two nuclei *n1*, *n2*. **D** Level of fluorescence after first cell division; fishnet display of intensity (*z*) profiles for cell in (**C**). **E** Three-cell proembryo with nuclei *n1*, *n2*, *n3*. **F** Fishnet display of intensity profiles for proembryo in (**E**)

following the expression of *gus* gene under cyclin promoter it is possible to observe the aborted cells of suspensor which are not coloured blue, in contrast to cells of globular embryo that are still active for division (Fig. 2C). Further development of such a structure continued with the formation of torpedo and cotyledonary stage embryos, which eventually developed into plantlets and also formed secondary embryos on the surface of the primary structure (Fig. 2D,E).

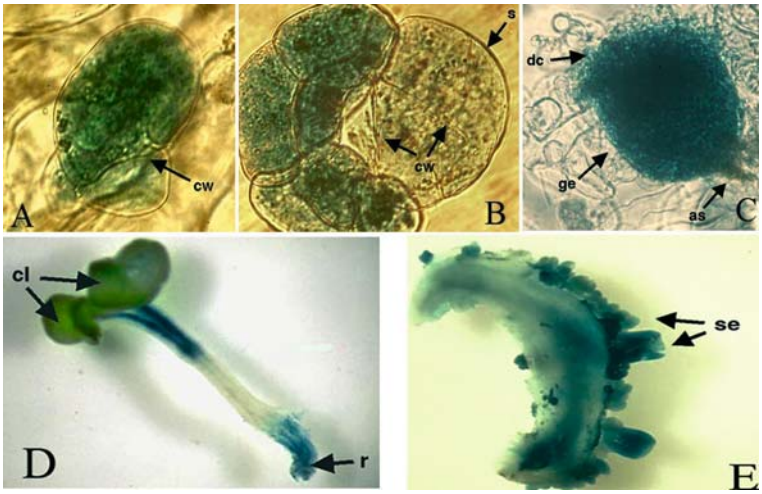

Fig. 2 Process of embryo formation from single cell to plantlet in *Medicago falcata*; *gus* activity is revealed by blue staining. **A** Asymmetric division in ovoid cell (*cw*=cell wall). **B** Proglobular embryo (*cw*=cell wall, *s*=suspensor). **C** Well-shaped globular embryo (*ge*=globular embryo, *dc*=divided cell peripheral cells, *as*=aborted suspensor). **D** Plantlet (*cl*=cotyledonary leaves, *r*=root). **E** Secondary embryo formation (*se*=secondary embryo)

2,4-D in induction medium acts as a mitotic trigger, which re-activates cell division as an inductive signal for cells in *M. falcata* and *M. truncatula* cell suspension culture. Asymmetry of the first cell division and establishment of cell polarity are the prerequisites for further embryo development. Similar results were observed in *M. varia* genotype A2 mesophyll protoplasts (Dudits et al. 1995). It is unclear what function is played by the suspensor, which develops on somatic embryos even in liquid media. It is perhaps essential for embryo polarity and serves as a channel for the nutrients and growth regulators to the developing embryo; however, it aborts later (Fig. 2C).

Single-cell suspension cultures of *M. falcata* and *M. truncatula* are particularly suitable for studying primary division and the induction of embryogenic potential of direct somatic embryos from single cells. They also confirm the asymmetry of the first cell division which starts the process of embryo formation.

Direct somatic embryogenesis of annual diploid *Medicago* on solid media supplemented with TDZ or BAP is characterized by the formation of embryos directly on the explants containing meristematic zones (Iantcheva et al. 1999). These somatic embryos develop without an intermediate callus phase. They are formed as independent units organized in clusters. The origin of somatic embryos is single cell or multicellular. The zones of embryo formation are characterized by groups of small meristematic cells with dense cytoplasm. In order to determine cell division and to reveal mitotic activity, $4', 6$ – diamidino – 2 – phenylindole (DAPI) stained nuclei (unpublished

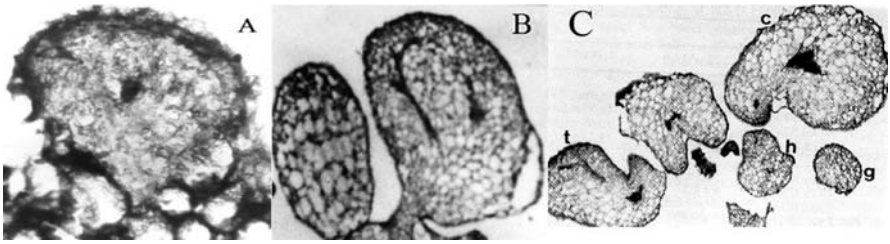

Fig. 3 Process of embryo formation promoted by TDZ in *Medicago truncatula*. **A** Primary late globular embryo. **B** Torpedo embryo connected by suspensor. **C** Stages in embryo formation (*g*=globular, *h*=heart, *t*=torpedo, *c*=cotyledonary)

data) confirmed that cells were highly divided and within 10 days of incubation globular embryos were observed. Histological observation indicated that somatic embryos develop without any connection with maternal tissue and in some cases they are connected with suspensor (Fig. 3A,B). Embryogenesis progresses through the stages typical of zygotic embryos: globular, heart, torpedo and cotyledonary (Fig. 3C). The appearance of an independent vascular system in the embryos indicated additionally that they develop as bipolar structures with apical and root parts, and possess the ability to convert to plantlets. The formation of secondary embryos on the surface of the primary structure is also detected. The origin of secondary embryos in most cases is a single cell that undergoes asymmetrical cell division. In this system and for direct induction of embryos, TDZ or BAP act as a mitotic trigger and start the process with activation of meristematic cells. Induction, development and maturation of somatic embryos proceed on the same medium in the presence of mitogene. The other advantage of the system is that it is species independent. The embryogenic capacities of the species used differ very slightly. The stable and positive embryogenic response could be due to the presence of meristematic cells in the explants, which are morphologically and physiologically more similar to each other than are differentiated somatic cells. The genotype-dependent embryogenic response which is typical for diploid *Medicago* is reduced. The ability to apply this system to a wide range of *Medicago* species is important when considering the use of gene transfer techniques.

3 Embryo Development and Maturation

Once obtained, the globular embryos from callus in indirect systems or on the explant surface in direct somatic embryogenesis proceed through the next stages, i.e. development and maturation. The formation of tissues and organs in globular, torpedo and cotyledonary embryos is a process that includes many factors and is genotype specific. In most of the *Medicago*, the require-

ments of growth regulators during the induction, development and maturation are specific. A significant decrease or complete elimination of auxin is necessary for further normal embryo development and maturation (Denchev et al. 1991; Trinh et al. 1998; Iantcheva et al. 2001; Barbulova et al. 2002). Reduced cytokinin concentration is essential for proper embryo development in *M. suffruticosa* (Li and Demarly 1996; Chabaud et al. 1996; Iantcheva et al. 2001). Elimination of growth regulators for successful embryo development is essential in *M. truncatula* R 108 1 (Trinh et al. 1998). In the *M. falcata* system for direct somatic embryogenesis, removal of both auxin and cytokinin are necessary for embryo development; PEG (polyethylene glycol) and maltose lead to conversion of globular embryos to high number of vigorous torpedoes. This treatment of somatic embryos with an osmotic agent such as PEG resulted in a high rate of embryo development to the next stage. In *M. truncatula cv*. Jemalong and *cv*. R 108 1, the PEG in the culture medium (Iantcheva et al. 2001) also resulted in a high number of embryos in the torpedo stage but without a normally developed vascular system. Apparently, an increased osmolality of the culture medium does not improve further development of somatic embryos as in *M. falcata*. The stage of embryo maturation is critical for embryo development, and it is mostly characterized by the reserve accumulation which determines successful conversion of embryos into a vigorous plant. Alfalfa has been intensively investigated for reserve deposition in somatic embryos, and different compounds such as abscisic acid, amino acid and different types of carbohydrates have been monitored. This issue is still not fully solved and is one of the crucial steps which limits large-scale utilization of somatic embryogenesis for speeding and improving the breeding programme in this forage crop.

In alfalfa, ABA is found to regulate storage food accumulation and prevent precocious germination (Fujii et al. 1990; Denchev et al. 1991) and it also promotes desiccation tolerance in somatic embryos (Senaratna et al. 1989). The effect of ammonium ion alone or in combination with amino acids on alfalfa somatic embryogenesis is well documented (Walker and Sato 1981; Stuart and Strickland 1984; Lai and McKersie 1994; Barbulova et al. 2002). L-Proline emerges as the most stimulatory amino acid; the optimal level of L-proline that enhances embryo yield and quality is around 100 mM. In some cases, the synergistic interaction of proline and ammonium showed a positive effect on the embryo (Stuart et al. 1985). Successful application of 3 g/l proline in the medium for embryo development and maturation was earlier reported for commercial alfalfa cultivar (Barbulova et al. 2002). Proline is known to stimulate auxin-induced somatic embryogenesis and elongates alfalfa somatic embryos in a hormone-free medium. This may be due to improved cell signalling as proline is always associated with various signal transduction pathways in plants (Phang 1985).

Amino acids such as glutamine, serine and adenine are often added either alone (Stuart and Strickland 1984) or as a component of a mixture, such as ca-

Table 1 References for induction of somatic embryogenesis in perennial and annual *Medicago*

Perennial *Medicago*			
Species	Explant	Growth regulators for induction of SE	References
Medicago sativa	MP, CS	2,4-D+Kin	Atanassov and Brown, 1984
	H, C	2,4-D+Kin	Brown and Atanassov, 1985
	L		Arcioni et al. 1989
	P		Finstad et al. 1993
			Shetty and McKersie, 1993
			Lecouteux et al. 1993
			Lai and McKersie, 1994
			Senaratna et al. 1995
			Horbowicz et al. 1995
	L, P, IN	NAA+IAA+Kin	Parrott and Bailey, 1993
	IE	BAP	Nincovic et al. 1995
	L, P	2,4-D+Kin+Lpro	Barbulova et al. 2002
	H	IAA+Z	Kim et al. 2004
Medicago falcata	L	2,4-D+Kin	Denchev et al. 1991
	L		Kuklin et al. 1994
	L		Shao et al. 2000
Medicago sativa (diploid)	L	2,4-D+BAP	Trinh et al. 1998
Medicago coerulea	L, MP	2,4-D+BAP	Arcioni et al. 1982
Medicago varia	S	2,4-D+Kin	Deak et al. 1986
Medicago lupulina	Ii	BAP	Li and Demarly, 1995
Medicago arborea	H, C, P, L	2,4-D+Kin/BAP/TDZ	Martin et al. 2000
Medicago marina	P	2,4-D+Kin	Walton and Brown, 1988
Medicago glutinosa	L, MP	2,4-D+Z	Arcioni et al. 1982
Annual *Medicago*			
Medicago suffruticosa	L	2,4-D+BAP	Li and Demarly, 1996
Medicago truncatula	L	NAA+BAP	Nolan et al. 1989
	L, P	2,4-D+Z	Chabaud et al. 1996
	L	2,4-D+BAP	Trinh et al. 1998
	L	2,4-D+Z	Das Neves et al. 1999
	H, CB, PB	TDZ/BAP	Iantcheva et al. 1999
	L, P	2,4-D+Kin	Iantcheva et al. 2001
	R	2,4-D+Kin	Iantcheva et al. 2005
Medicago littoralis	H	2,4-D+BAP	Zafar et al. 1995
	H, CB, PB	TDZ/BAP	Iantcheva et al. 1999
Medicago murex	H, CB, PB	TDZ/BAP	Iantcheva et al. 1999

Table 1 Continued

Annual *Medicago*			
Species	Explant	Growth regulators for induction of SE	References
Medicago polymorpha	H	2,4-D+IAA	Scarpa et al. 1993
	H, CB, PB	TDZ/BAP	Iantcheva et al. 1999
Medicago scutelata	H, C, P, R	2,4-D+Kin	Walton and Brown, 1988
	L, P, R	2,4-D+Kin	Iantcheva et al. 2003
Medicago arabica	L, P, R	2,4-D+Kin	Iantcheva et al. 2003
Medicago orbicularis	L, P, R	2,4-D+Kin	Iantcheva et al. 2003
Medicago rugosa	H, C, P, R	2,4-D+Kin	Walton and Brown, 1988

Abbreviations: SE, somatic embryogenesis; MP, mesophyll protoplast; CS, cell suspension; H, hypocotyl; C, cotyledon; CB, cotyledon base; P, petiole; PB, petiole base; L, leaf; S, stem; R, root; IN, internode; Ii, immature inflorescence; IE, immature embryos; BAP, 6-benzylaminopurine; 2,4-D, dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; IAA, indole-3-acetic acid; Kin, kinetin; TDZ, thidiazuron; Z, zeatin; L, pro-L-proline; GA3, gibberellic acid

sein hydrolysate or yeast extract (Chabaud et al. 1996) or in combination with cytokinin (Iantcheva et al. 2001) for a high rate of embryo conversion. Accumulation of proteins at the maturation stage is a key step and is a prerequisite to high-vigour conversion of somatic embryos (Krochko et al. 1992; Lai and McKersie 1994).

Secondary embryo formation is mostly observed at the embryo maturation stage. If primary embryos fail to accomplish development to plants or recallus, secondary embryos appear on their surface as observed in *M. falcata* (Denchev et al. 1991), *M. sativa* (Barbulova et al. 2002) and *M. truncatula* (Chabaud et al. 1996; Iantcheva et al. 2001; Das Neves et al. 1999). A few of the secondary embryos develop into plants, the rest are arrested at the globular or torpedo stage or give rise to an additional round of embryos. Therefore, secondary embryogenesis may be useful in the clonal multiplication of alfalfa. Secondary embryo formation was originally described by Lupotto (1983) for alfalfa and reported for the other tetraploid alfalfa genotypes (Parrott and Bailey 1993; Ninkovic et al. 1995). Repetitive formation of embryos was observed when primary embryos were transferred on hormone-free medium. The capacity for production of the new cycle of embryos of these cultures remained stable for at least 2 years but was strongly dependent on the presence of sugars in the medium (Parrott and Bailey 1993). Repetitive de novo recycling of embryos was also established for different diploid *Medicago* (*M. truncatula*, *M. littoralis*, *M. murex* and *M. polymorpha*). If one regenerated cluster of embryos and secondary embryos is isolated and trans-

ferred again on TDZ embryo induction medium, the emergence of the new embryos is visible within 20 days of culture (Iantcheva et al. 1999). This recycling procedure opens up the possibility of scaling up embryo and plantlet formation, and maintains the embryogenic potential for an unlimited period. Such a cycling regeneration system is an advantage for gene transfer research, especially in the model plant *M. truncatula* (Iantcheva et al. 2005). Repetitive embryogenesis could be obtained from a single embryogenic cell developed in liquid culture medium. Separation of such a fraction composed of highly embryogenic cells into a fresh embryo induction medium led to new embryo formation. The whole regeneration period is shorter and the embryogenic potential may be kept for four to five passes (Iantcheva et al. 2005).

4 Embryo Conversion

Embryo conversion is the last stage in the process of somatic embryogenesis. Successful conversion and germination of somatic embryos is a consequence of a proper maturity in respect of desiccation, accumulation of reserves and proteins for future conversion of embryos to seedlings. In alfalfa, this stage showed an increased level of storage proteins and free amino acids (Horbowicz et al. 1995; Lai and McKersie 1994).

It seems that the exogenous application of ABA during the development and maturation stages resulted desiccation tolerance, followed by post-maturation quiescence which prevented precocious germination and enhanced the conversion rate (Senaratna et al. 1995; Kuklin et al. 1994). In the case of *M. falcata*, exogenous ABA application is effective against precocious germination and it also favours successful development of single embryos to plantlets. The presence of GA3 in the medium enhanced this process further (Denchev et al. 1991).

The conversion of somatic embryos to plants is sometimes genotype dependent. In *M. truncatula* the percentage of conversion in genotype R 108 1 was 20 times higher than that in *cv*. Jemalong (Iantcheva et al. 2001). Even after osmotic pre-treatment to primary explants, genotype dominated the conversion process (Iantcheva et al. 2005).

5 Phenotype of Regenerated Plants via Somatic Embryogenesis: Somaclonal Variation

Regenerated plants from annual and perennial *Medicago* produced via somatic embryogenesis in most cases displayed normal and vigorous growth in the greenhouse, and morphologically resembled their donor plants with

flower and seed set (Matheson et al. 1990; Varga and Badea 1992; Arcioni et al. 1989; Nolan et al. 1989; Barbulova et al. 2002).

In *Medicago*, variation from tissue culture has, however, been observed. Hexaploid plants of *M. sativa* were obtained after tissue culture treatment from haploid (Latude-Data and Lucas 1983) or diploid (Reisch and Bingham 1981) donor plant material. Euploid and aneuploid alfalfa plantlets were regenerated via indirect somatic embryogenesis by Johnson et al. (1984). It is necessary to analyse regenerated plants in order to confirm their ploidy level and genome size. Larkin and Scowcroft (1981) proposed the general term "somaclonal variation" for the variation arising from tissue and cell culture. In *M. sativa*, somaclonal variation for qualitative genetic characters like disease resistance (Johnson et al. 1984; Latude-Data and Lucas 1983) and quantitative traits like forage yield (Johnson et al. 1984; Pfeifer and Bingham 1984) were previously reported. In Romania "Sigma" is the first cultivar from this forage crop created from in vitro regenerated somaclones via indirect somatic embryogenesis (Varga and Badea 1992). The same authors suggested the use of alfalfa somaclones in a breeding programme that could shorten the time for raising a new cultivar. In the paper of Arcioni et al. (1989) the authors' investigations on somaclonal variation do not provide novel phenotypes, absent in the donor cultivar. Among *Medicago* species, somaclonal variation is genotype specific and superior variants can be selected during the plant regeneration procedure. This issue needs further detailed studies, and methods such as DNA fingerprinting may be useful in this direction.

6 Conclusion

Somatic embryogenesis is the direct way to regenerate plants from single somatic cells, and opens up the possibility of understanding the process of cell cycle reprogramming from somatic to embryogenic type, cloning and characterization of genes involved in wounding, hormone activation, cell division, differentiation and developmental processes (Chugh and Khurana 2002). Considerable advances in the development of the somatic embryogenesis system in genera *Medicago* have been noted in the last 30 years. The development of a genome and proteome database of model annual *Medicago truncatula* species will serve as a genetically compatible model for alfalfa, which is tetraploid and perennial (Bell et al. 2001; Imin et al. 2004).

One of the important uses of somatic embryogenesis is to explore it as an approach to investigate the early events of zygotic embryogenesis in higher plants, because of the existing parallel events happening between the two processes (de Jong et al. 1993; Dodeman et al. 1997). The second important application of somatic embryogenesis is the mass propagation of commercially valuable genotypes—one of the most attractive uses of this

morphogenic pathway. Because of the huge number of somatic embryo structures, easy scale-up is possible. Single-cell origin also permits synchronized, homogeneous and stable plant material; thus, somatic embryogenesis is the preferred method of regeneration rather than organogenesis (Merkle et al. 1990). Another use of somatic embryogenesis is in the generation of transgenic plants. Gene transfer into embryogenic cells may help in conventional plant breeding and crop improvement programmes.

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