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Morphogenic Differentiation in *Medicago*

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ABSTRACT

Using somatic embryogenesis in *Medicago arborea* L. subsp. *arborea* and organogenesis in *Medicago strasseri*, we have achieved important advances in plant regeneration. The best results were obtained from *M. arborea* using petioles and anthers in the second phase of meiosis as explants. With *M. strasseri*, the best inducing explants were the petiole and leaf. The most efficient media were Murashige-Skoog (MS) medium with 2,4 dichlorophenoxyacetic acid (2,4-D) and kinetin. An attempt was made to optimise somatic embryogenesis by varying the composition of the culture medium. Embryogenesis was improved using proline but the collection of vigorous plants was poor. All (100%) of the somatic embryos obtained from anthers germinated and produced 63% green and 27% albino seedlings. The analysis of cytokinins in calli revealed clear differences in embryogenesis. The amount of total endogenous cytokinins: Z, [9R]Z, (diH)Z, (diH)[9R]Z, iP and [9R] iP, was greater in the non embryogenic than in embryogenic calli. The ratio between isopentyl derivatives and zeatin-like derivatives was lower in embryogenic than in non-embryogenic calli. Analysis of carbohydrates revealed that there were no significant differences in total sugars between embryogenic and non-embryogenic calli. The highest levels of reducing sugars were seen in embryogenic calli. During the development of somatic embryogenesis and organogenesis, very low starch levels were found in the calli. In embryogenic calli, the sucrose content was lower. Nitrogen levels decreased in the calli with an increase in culture time, mainly in embryogenic calli. Plant acclimatization was carried out over two months in glass pots containing sterile vermiculite and the plants were nourished every week with MS medium with half the concentration of salts. Then, they were transferred to sterile sand and after two weeks they were transplanted into flower pots. The humidity was gradually reduced over time. Of the somatic embryos induced, 28 ± 3% developed into plants with normal phenotypic characteristics.

Keywords: carbohydrates; cytokinins, embryogenic and non-embryogenic calli, *Medicago arborea*, *M. strasseri*, nitrogen compounds
Abbreviations: **ABA**, abscisic acid; **BA**, benzyladenine; **BAP**, 6-benzylaminopurine; **(2,4-D)**, 2,4 dichlorophenoxyacetic acid; **DH**, double haploid; **(diH)Z**, Dihydrozeatin; **EST**, α or β esterase; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **IDH**, isocitrate dehydrogenase, **IEDCs**, induced embryogenic determined cells; **iP**, N^6-(Δ^2-isopentenyl) adenine; **LAP**, leucine aminopeptidase; **MDH**, malate dihydrogenase; **MNR**, menadine reductase; **NAA**, α-naphthalene acetic acid; **PEDCs**, pre-embryogenic determined cells; **PEM**, proembryogenic mass; **PER**, peroxidase; **(diH)[9R]Z**, 9-β-D-ribofuranosyl-(diH)Z, **[9R]iP**, 9-β-D-ribofuranosyl-iP; **[9R]Z**, 9-β-D-ribofuranosyl-Z; **TDZ**, thidiazuron; **Z**, zeatin

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INTRODUCTION

As stated by Sanchez Ron (2003), "we are witnessing one of those periods in which, to say it one way, the earth shakes beneath our feet; a period in which we see that the future will be different from the present and that the present itself is changing rapidly precisely because of the instruments that are being created within this revolution". It is indeed true that we may envisage a future full of hitherto unimaginable possibilities, although they are also associated with the risks that may be generated by science and technology.

Humankind's unbridled activity is leading to the destruction of biodiversity. It is estimated that approximately half of the species forming the terrestrial flora are under threat of extinction (UICN 2004). The advances made in recent years in the generation of new biotechnological tools have allowed interesting alternatives to conservation programs and plant improvement (Cook 2000; Woodfield and Brummer 2000; Stein *et al*. 2001). Among the tools that have contributed to such developments is tissue culture (Martín *et al*. 2000a; Gallego *et al*. 2001; Rout *et al*. 2006; reviewed extensively in Teixeira da Silva 2006).

In plants, by means of the reproductive process – the

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union of two gametes of different sex that give rise to a zygote, which, through successive mitoses, will give rise to a new organism – different structures are formed: roots, stems, leaves etc. All these structures derive from a single cell and will therefore have the same genetic material. From this it may be deduced that in the formation of the different structures and organs of a plant certain factors must be involved that induce the selective expression of genes, whose result is the manifestation of different cellular characteristics. This is known as cellular differentiation, leading to the "biodiversity" of the plant kingdom.

It was logical to question whether the changes undergone by cells in the differentiation process were irreversible, such that the cells would be unable to dedifferentiate, or whether by contrast there existed the possibility of dedifferentiation. It was originally believed that the changes occurring in cells were permanent, but as early as 1878 Vöchting, working on the polarity of cuttings, demonstrated that the process was reversible. That author noted that the cells of the stem could give rise to both stems and roots and that it was the position of the cells in the plant that determined their function. Thus emerged the concept of cellular totipotency: most living cells of a plant, regardless of their degree of differentiation and their level of ploidy could, at a given moment and under appropriate conditions, originate a new plant (Hall 1998).

As an application of cellular totipotency, *in vitro* culture techniques were developed for plant organs, tissues, and cells (Segura 1993). These initially appeared as support techniques for basic physiological, biochemical, genetic and molecular studies. At the end of the sixties, the conception began to change. Investigators realised that that *in vitro* culture technology offered a broad range of possibilities in applied research, especially from the point of view of plant production and improvement. Currently, *in vitro* culture is considered a basic element in new technologies for plant improvement (Gallo-Meagher and Green 2002; Kumria *et al*. 2003).

When a piece of plant tissue is cultured in a sterile medium with appropriate nutrients and growth regulators, the division of many of its cells is stimulated. The fragment of plant with which *in vitro* culture is initiated (García-Luis *et al*. 1999; Stipp *et al*. 2001; Teng *et al*. 2002; Kumria *et al*. 2003) is called an explant. Often, these cells in culture proliferate indefinitely in a disorganized fashion, producing a mass of relatively undifferentiated cells called a callus (Bhalla and de Weerd 1999; Sakhanokho *et al*. 2001; Teng *et al*. 2002; Martin 2003). To a large extent, plant calli are formed from masses of vacuolised cells surrounded by small meristematic cells. From these cells, more callus mass may be produced or the cells may undergo a morphogenetic response, leading to the direct formation of organs: roots, buds, etc. i.e. organogenesis (Medina *et al*. 1998; Prakash *et al*. 1999; Schween and Schwenkel 2002; Singh *et al*. 2003), or else embryos may be formed, i.e. somatic embryogenesis (Hita *et al*. 1997; Gallego *et al*. 2001; Das *et al*. 2002; Pueschel *et al*. 2003; Linhares *et al*. 2006). When the callus is maintained, the cells loose their ability to differentiate, which can be recovered by manipulating the culture medium. The morphogenetic response consists of *de novo* differentiation, as a manifestation of cellular totipotency, when the cells are subjected to certain culture conditions. Most factors that affect the *in vitro* growth of plant organs, tissues and cells are similar to the factors that limit the *in vivo* growth of plants (Leifert *et al*. 1995). In recent years, such factors have been studied in detail and they include: 1) plant growth regulators (Lee *et al*. 2002; Singh *et al*. 2003; Tan and Furtek 2003; Preil 2003; Rout and Jain 2004; Quint and Gray 2006), 2) illumination (Medina *et al*. 1998; Cho *et al*. 1999; Medina *et al*. 1999; Luczkiewicz *et al*. 2002; Rout *et al*. 2006), 3) temperature (Rietveld *et al*. 2000; Von-Aderkas and Bonga 2000), 4) the mineral composition and pH of the medium (Morard and Henry 1998; Shibli *et al*. 1999; Pasqua *et al*. 2002), 5) gas exchange (Santamaria *et al*. 2000; Tisserat *et al*. 2002), and 6) the presence of harmful microorganisms (Nowak *et al*. 1997; Szendrak *et al*. 1997). Apart from these factors, for proper initiation it is also necessary to take into account the type and characteristics of the explant. In *in vitro* cultures, depending on the aim in mind, it is crucial to select the appropriate explant. Success in the collection of a given morphogenetic response partly depends on the choice of an appropriate explant. In fact, as long as the physical conditions and the culture medium are appropriate, any cell, tissues or organ of the plant can be used as explants (Bhau and Wakhlu 2001; Mithila *et al*. 2001; Singh *et al*. 2003). It is possible to start out with fragments of stem, root, or leaf, from meristems and from mature zygotic embryos i.e., of somatic tissue (Medina *et al*. 1998; Martin *et al*. 2000a; Gallego *et al*. 2001; Palacios *et al*. 2002; Chang *et al*. 2003), although it is also possible to initiate the culture of non-somatic cells or tissues, such as anthers, microspores, ovaries or ovules (Lionneton *et al*. 2001; Zheng *et al*. 2002; Kashyap *et al*. 2003; Lanas *et al*. 2006).

A key aspect in plant regeneration is the differentiation capacity of its cells, which may manifest their totipotency and give rise to organised structures that will in turn lead to complete plants (Benkirane *et al*. 2000; Tejavathi *et al*. 2000; Lee *et al*. 2002; Senaratna *et al*. 2002). All the above processes are structural manifestations of different physiological, biochemical and molecular events that reflect the expression of selective genes in cells (Dorion *et al*. 1999). The expression of these morphogenetic responses is determined by the interaction of many processes (Vergauwe *et al*. 1998; Dorion *et al*. 1999; Rey *et al*. 2000; Ananthakrishnan *et al*. 2003; Singh *et al*. 2003), among them, the genotype of the material cultured, the **pH** of the medium, temperature, light and gases.

Most cell cultures are developed at an initial optimum pH between 5.0 and 6.0 (Robert and Haigler 1994; Hasson and Poljakoff-Mayber 1995; Mauri and Manzanera 2003), measured before the addition of agar and autoclave sterilisation, that changes during the course of culture and may alter certain morphogenetic processes. The direction of cell expansion is also predominant at pH 6.5-7.0

Temperature is another important factor. Although nearly all tissues grow well at temperatures close to 25ºC, (Pérez-Francés *et al*. 1995; Cardoso and Oliveira 1996; Veramendi and Navarro 1996), the usual growth temperatures at which the species from which the explants have been obtained should be borne in mind: for example, explants from tropical species require much higher temperatures (Detrez *et al*. 1994).

Cultures of plant tissues are not photosynthetically efficient and are in general heterotrophic. Hence the importance of the **light** regime applied. In some cases, the optimum response is obtained under conditions of darkness (Westcott 1994; Liu *et al*. 2006); in others under conditions of light (Ekiz and Konzak 1997), and in many cases under photoperiod conditions (16 hours of light and 8 of darkness) (Cardoso and Oliveira 1996; Veramendi and Navarro 1996; Mauri and Manzanera 2003), or an alternation of light and darkness (induction medium in darkness and differentiation medium in the light (Zheng *et al*. 1996)). In some cases, low light treatment (5 μmol/m^2/s) greatly improves shoot organogenesis (Dong *et al*. 2006). The mechanism of light-regulated gene expression has been widely investigated. Thus, several *cis*-regulatory DNA elements and their corresponding binding transcription factors of light-regulated genes have been isolated and characterized (Terzaghi and Cashmore 1995; Inaba *et al*. 2000). Occasionally, cold treatment can be used to substitute light (Romano and Martins-Loução 1999).

Aeration is essential for the increase in biomass (Cassels and Roche 1993), and also affects the accumulation of metabolites. According to Tate and Payne (1991), a high concentration of O_2 versus that of CO_2 is always favourable for better callus development. Nevertheless, using cells from *Selinum candoli* grown on solid medium and covered with a layer of sterile mineral oil, which formed a barrier

between cells and the medium, thus preventing the free circulation of gases and creating an oxygen-poor reducing agent, Mathur (1991) demonstrated that a decrease in the production of calli occurred but that the number of somatic embryos obtained increased. In wheat cultures, it has also been demonstrated that low O_2 concentrations decrease the formation of somatic embryos to a considerable extent and that, additionally, the precocious germination of immature embryos decreases (Carman 1988). Another opinion is that of Kvaalen and von Arnold (1991), for whom the proliferation of embryonic tissues of *Picea abies* is stimulated by high partial pressures of both O_2 and CO_2. Cultures of *Cyclamen persicum* growing at a 40% oxygen partial pressure had a lower growth rate, markedly reduced cell viability, and showed a decrease in the pH of the medium 3.5. The poor cell proliferation was an effect of acidification of the medium rather than of the high O_2 partial pressure (Hohe *et al*. 1999).

In plant cells, the differentiation process does not involve a loss of genetic material or an irreversible change in the number of genes, but all the cells preserve the complete genetic patrimony, such that the molecular mechanism of differentiation is very probably based on a differential expression of genes (Kubota *et al*. 2000; Subramanian *et al*. 2002).

In tissue culture, the genotypic variability of species induces a large variety of responses. The extent of the variability and inheritability occurs more in plant regeneration than in callus induction (Gupta *et al*. 2006). Mutations are increasingly used to achieve improvements in plant propagation. *In vitro* mutation of chrysanthemum has been achieved using ethylmethanesulphonate (EMS) and imamture floral pedicels as explants (Latado *et al*. 2004). With *Solanum surattense*, streptomycin-resistant plantlets were induced showing encoged mutants from mutagenised (EMS and γ-rays) cotyledon explants (Rama-Swamy *et al*. 2005). Plant cell cultures can be used to select variant cell types in which herbicide resistance is expressed at the cellular level: for example, atrazine resistance (Venkataiah *et al*. 2005).

The regeneration and propagation of plants is thus considered essential to *in vitro* culture (Jayasankar *et al*. 2001; Amutha *et al*. 2003; Martín 2003; Pati *et al*. 2006). In this context, two important ways in which to carry out the *in vitro* regeneration of a plant (Vasil 1994) can be considered: organogenesis and somatic embryogenesis.

Organogenesis is the most common route for regeneration (Yu *et al*. 1997). It involves the inhibition of apical dominance and consists in using sections of a donor plant which, when cultured in the appropriate nutrient medium, will afford many sprouts (small seedlings) around the base of the initial apex. These sprouts can be placed in a new nutrient medium, where the process is repeated, or they can be transferred to a different medium, in which, by root differentiation, they will develop until they form whole plants (Palacios *et al*. 2002; Kadota and Niimi 2003; Singh *et al*. 2003). This process therefore leads to the differentiation of caulinar (caulogenesis) and/or radicular (rhizogenesis) meristems, which will give rise to stems and/or adventitious roots respectively (Decruse *et al*. 2003; Fratini and Ruíz 2003).

The *de novo* development of structures such as roots or stems begins with the transformations brought about in a small group of cells. These cells begin to divide until they produce a globular mass of meristematic cells (meristemoids) (Altamura *et al*. 1998; Idei and Kondo 1998; Biondi *et al*. 2001) able to respond to certain factors in the tissue and to give rise to a caulinar or radicular primordium.

Both rhizogenesis and caulogenesis may occur directly, on the explant, or indirectly, through the formation of a callus (Babaoglu and Yorgancilar 2000; Kadota and Niimi 2003; Teixeira da Silva 2003; Glocke *et al*. 2006). Both of the above processes represent the structural manifestation of different physiological, biochemical and molecular events that reflect the expression of selective genes in cells (Dorion *et al*. 1999). The expression of these morphogenetic responses is determined by the interaction of many factors (Rey *et al*. 2000; Ananthakrishnan *et al*. 2003; Singh *et al*. 2003), such as the genotype of the material cultured, the type of explant and its physiological status, the composition of the culture medium, environmental factors, and growth regulators.

In the control of morphogenesis, a crucial role is played by growth regulators, either endogenous or added exogenously (Gallego *et al*. 2001; Fiore *et al*. 2002; Quint and Gray 2006), although their mechanism(s) of action in this process has not been fully elucidated. The most widely accepted hypothesis states that organogenesis would be regulated by changes in the endogenous levels of auxins and cytokinins (Rey *et al*. 2000; Gentile *et al*. 2002; Kadota and Miimi 2003): the so-called "Skoog-Miller model". According to this model, caulogenesis (sprout differentiation) occurs if the auxin/cytokinin balance is in the favour of cytokinins while rhizogenesis occurs if the proportion of auxins is higher. However, the type of auxin used is crucial when obtaining a response. Thus, Choffe *et al*. (2000), using explants of *Echinacea purpura*, demonstrated that indolbutyric acid was effective in the induction or organogenesis; IAA was less effective, and NAA was completely ineffective. In *Lycopersicon cheesmanii*, IAA was effective in sprout production, while 2,4-D and NAA had an inhibitory effect on organogenesis (Dorion *et al*. 1999). The thidiazuron, substituted phenylurea, which is a potent bioregulator of morphogenesis *in vitro*, induced either organogenesis or somatic embryogenesis in *Cajanus cajan* L., depending on the concentration used in the culture medium (Singh *et al*. 2003).

In general, the gibberellins, ABA and ethylene do not appear to be essential in the induction of organogenesis, although there may always be exceptions (Charrière and Hahne 1998; Ananthakrishnan *et al*. 2003).

Somatic (or asexual) embryogenesis is considered to be the best method for the *in vitro* propagation of plants (Gallego *et al*. 2001; Vasic *et al*. 2001; Gallo-Meagher and Green 2002; Beena *et al*. 2003; Singh *et al*. 2003; Teixeira da Silva and Nhut 2003; Nhut *et al*. 2006). It is a process in which embryonic structures are formed from somatic cells with no need for gamete fusion for a zygote to be. In the literature, these embryonic structures receive different names: vegetative or adventitious embryos, somatic embryos or embryoids (Gray and Purohit 1991).

The formation of embryos from somatic cells was first described in the carrot by Steward *et al*. (1958) and since then somatic embryogenesis has been studied in many species, both angiosperms and gymnosperms, herbaceous plants and ligneous plants (Filonova *et al*. 2000; Gallego *et al*. 2001; Stipp *et al*. 2001; Wang *et al*. 2002; Singh *et al*. 2003).

It is possible to distinguish two types of somatic embryogenesis: direct and indirect (Gray and Purohit 1991; Buchanan *et al*. 2000; Maldonado *et al*. 2000).

Direct embryogenesis

In this, the embryo is generated directly from a cell or tissue without previous callus formation. It usually takes place from an explant maintained in a solid culture medium. The direct formation of somatic embryos from a given tissue seems to be associated with greater genetic and cytological uniformity than the indirect induction of embryogenesis (Gavrilenko *et al*. 2001; Choi *et al*. 2003; Martin 2003; Linhares *et al*. 2006).

The cells from which the embryos are originated were termed pre-embryogenic determined cells (PEDCs) by Sharp *et al*. (1980) because they only require the presence of growth regulators or favourable conditions for them to express their embryogenic condition.

Indirect embryogenesis

Here, embryos are obtained either from an undifferentiated tissue, the callus, in a solid nutrient medium or from unor-

ganised groups of cells growing in a liquid medium under conditions of shaking (Gallego *et al*. 2001; Sakhanokho *et al*. 2001; Suzuki *et al*. 2002; Choi *et al*. 2003).

The cells from which the embryos are formed are called induced embryogenic determined cells (IEDCs) and they produce embryos when they are induced to do so (Sharp *et al*. 1980). There are two types of cell that seem to be involved in embryogenesis: embryogenic cells and vacuolated cells.

The induction of somatic embryos starts at the surface of calli that have certain zones with a lobulate aspect and a compact texture, where growth seems to be more active (embryogenic masses), or at the periphery of the cellular mass (proembryogenic mass or PEM) in liquid cultures in suspension (Gray and Purohit 1991). Often, a mature somatic embryo may be the origin of the formation of new somatic embryos. This phenomenon takes place without prior callus formation, from epidermal cells of the somatic embryo (Vasic *et al*. 2001; Das *et al*. 2002; Martin 2003) and the process is known as secondary embryogenesis. It may occur in the absence of growth regulators (Fernández-Guijarro *et al*. 1995) and with different explants (Martinelli *et al*. 2001; Das *et al*. 2002; Martin 2003) but transformation into plants is in general lower than that obtained with normal somatic embryos (Vasic *et al*. 2001).

The *in vitro* development of a somatic embryo, regardless of whether its has developed directly or indirectly, involves several embryonic phases that, based on their morphology, have been called proembryonic (the phase in which a cell of the parenchyma is stimulated or induced), globular, cordiform or heart, torpedo and cotyledonary in the case of dicots and conifers, and globular, scutelar and coleoptilar in monocots (Gray and Purohit 1991; Xie and Hong 2001; Aly *et al*. 2002).

These phases are similar to those seen during the development of a zygotic embryo, although there are fundamental differences, such as the fact that in somatic embryos no fusion of gametes takes place and neither is the presence of reserve substances (gametophyte or endosperm) observed (Gray and Purohit 1991; Barry-Etienne *et al*. 2002).

Although the regulation of the first developmental processes in embryos derived from somatic cells may be somewhat different from that observed in embryos derived from gamete fusion, the morphological similarities between somatic and zygotic embryos, particularly at the end of development, reflect apparently similar morphological processes (Perán-Quesada *et al*. 1999; Mordhorst *et al*. 2002). The different embryonic phases that occur during the development of somatic embryos are similar to those taking place during the development of a zygotic embryo (Gray and Purohit 1991; Bandyopadhyay and Hamill 2000). However, there are also fundamental differences: somatic embryos do not show small storage tissues, whose function in zygotic embryos is nutrition, protection and the regulation of gas exchange (Gray and Purohit 1991; Barry-Etienne *et al*. 2002).

It is easy to find somatic embryos with certain structural anomalies, such as additional cotyledons, the fusion of hypocotyls, or poorly developed apical meristems (Nugent *et al*. 2002; Tan and Furtek 2003). Such anomalies interfere in the development of the embryo to the plant stage and they seem to be due to the culture conditions, nutrient imbalances, or growth factors (Guievin and Kirby 1997). They are not due to factors intrinsic to somatic embryos because immature zygotic embryos display similar irregularities when they are extracted from seeds and are developed *in vitro* (Gray and Purohit 1991).

One of the distinctive characteristics of somatic embryos is that they are bipolar structures, having both a caulinar apex and a root apex. This latter is anatomically closed, with no vascular connection with the maternal tissue that originated the embryo (Gray and Purohit 1991; Matsumoto *et al*. 1996; Linhares *et al*. 2006).

In the development of somatic embryos, four main phases can be distinguished (Segura 1993; Lindemann and Luckner 1997; Dupire *et al*. 1999; van Zyl *et al*. 2003):

The initial phase, or phase 0. In this, the grouping of small, round and dense cells occurs; these associate with vacuolar cells in response to the presence of auxin and will later give rise to PEMs. In these groupings, some cells will acquire the ability to give embryos; it is the hormone (auxin) that induces the formation of embryogenic cells. If these PEMs are kept in a medium with this hormone, they dissociate to form smaller masses, each of which will give rise to a new PEM.

The embryogenic cells from which embryos arise are characterised by generally being small and having a dense cytoplasm with a large nucleus, small vacuoles, many ribosomes and high concentrations of RNA and proteins, and abundant starch granules (Megia *et al*. 1993; Dutta-Gupta and Conger 1999). Additionally, these cells tend to be arranged at the periphery of the proembryonic masses (Marapara *et al*. 1999; Bozhkov *et al*. 2002). Their histochemistry and ultrastructure suggest intense metabolic activity and RNA synthesis (Williams and Maheswaran 1986). Another characteristic of embryogenic cells is the thickness of their cell wall. This thickening of the cell wall provides a certain degree of insulation that guarantees their expression of totipotency, such that inside the walls divisions are constructed by means of internal segmentation. In this way, discrete groups of cells – from two to eight – are formed, separated from the rest by a thick cell wall. The continuing divisions of those cell groups give rise to elongated structures with a bipoplar organization – the proembryos – which later develop to become embryoids. The number of proembryoids initiated is usually larger than the number of somatic embryos formed from them, since many of them degenerate or interrupt their development (Anbazhagan and Ganapathi 1999; Fernandez *et al*. 1999; Yu *et al*. 2000).

Together with the above embryogenic cells, there are other large, highly vacuolated cells (a large vacuole occupies most of their volume) lacking embryonic potential. Although they do not participate directly in the embryogenic process, it appears that they do synergise it (Chasan 1992); that is, their presence is necessary for embryogenesis to occur. Among these two types of cell (vacuolated and embryogenic), there is cytoplasmic continuity by means of plasmodesmata (Anbazhagan and Ganapathi 1999; Fernandez *et al*. 1999; Yu *et al*. 2000). Later, during the development of somatic embryos, this plasmodesmic continuity is lost (Kiyosue *et al*. 1992), which indicates an isolation of the tissue surrounding the embryogenic cells (Franz and Schell 1991).

Phase I: If PEMs are transferred to a medium without auxin, the cells begin to divide actively in certain zones of the periphery

Phase II: As a result of the activation of cell division at the periphery of the PEMs, globular embryos are formed.

Phase III: The embryos in the globular state detach from the proembryogenic masses and continue to develop, passing through the stages of heart, torpedo and cotyledonary, until they give rise to seedlings.

The different nutrient media used for *in vitro* culture are based on the hydroponic nutrient media used with plants *in vivo* (Leifert *et al*. 1995) – Knop medium (Knop 1865) and Hoagland medium (Hoagland and Amon 1938) – and the techniques usually used in microbiology.

Before 1960, most attempts to culture exscised plant tissues, calli or cells were carried out using similar mineral compositions to those used for *in vivo* cultures: macronutrients (N, S, P, K, Mg and Ca) and micronutrients (Fe, Mn, Zn, B, Cu and Mo) (Gautheret 1995). At that time, many investigators began to add carbohydrates (normally sucrose or glucose) and vitamins (inositol, thiamine, nicotinic acid, nicotine amide, pyridoxine, pantothenic acid, ascorbic acid, biotin, folic acid, choline chloride, *p*-amino benzoic acid, riboflavin, vitamin B12 and/or vitamin E) and amino acids (glycine, alanine, arginine, asparagine, aspartic acid cysteine, glutamine, glutamic acid, leucine, methionine, phe-

nylalanine, proline, serine, tyrosine and/or tryptophan (George *et al*. 1987, 1988).

Auxin regulates many physiological processes in plant development, among others embryogenesis and organogenesis (Quint and Gray 2006). In 1930, the identification of auxin as a natural regulator of growth and the isolation by Miller *et al*. (1955) of the first artificial cytokinin, known as kinetin, afforded a huge advance in the development of *in vitro* culture techniques. Some years later, in 1957 Skoog and Miller determined the concept of hormonal control over organ formation when they observed that the differentiation of roots and buds in tissue cultures of *Nicotiniana tabacum* occurred as a function of the proportion of auxin/cytokinins in the culture medium. During the sixties, experiments were begun with increasing concentrations of mineral nutrients in the culture media used previously (George and Sherrington 1984) and it was observed that in general these higher concentrations favoured the formation and growth of calli as long as suitable amounts of auxins and cytokinins were added to the culture medium.

Different combinations of nutrients have been used in culture media, for example: 1) Heller (1953); 2) Lin and Staba (1961); 3) Murashige and Skoog (MS) (1962); 4) White (1963); 5) Linsmaier and Skoog (LS) (1965); 6) Gamborg *et al*. (B5) (1968); 7) Nitsch and Nitsch (1969); 8) Nagata and Takebe (NT) (1971); 9) Schenck and Hildebrandt (1972); 10) Kao and Michayluk (1975); 11) Lichter 1981; 12) Lloyd and McCown (WPM) (1981) and 13) Muller, Missioner and Caboche (KL) (1983) and their variants.

In work on *in vitro* culture it is difficult to reach generalisations. Each medium may be appropriate for obtaining a given objective, depending on the type of explant used (Leifert *et al*. 1995). For example, Kao and Michayluck medium (1975), that of Lin and Staba (1961) or that of Nagata and Takebe (NT) (1971) are the media that have been most widely used to induce calli from protoplasts. Lin and Staba (1961) medium is the one that has most often provided the best results in the culture of protoplasts from *Rosacea* while that of Kao and Michayluck is the best one for Cruciferae, grasses and legumes (George *et al*. 1987).

The media most commonly used in work on *in vitro* culture are MS, B5 and Schenck and Hildebrand and favour callus growth and morphogenesis (Gray and Purohit 1991). Of these media, the macronutrients of MS medium (Murashige and Skoog 1962) have been used in approximately 25% of the culture media used for the micropropagation of plants (George *et al*. 1988; Leifert *et al*. 1991; Teixeira da Silva *et al*. 2005).

Currently, for the *in vitro* culture of ligneous plants WPM medium is sometimes used (Lloyd and McCown 1981; Rout *et al*. 1995). This medium is characterised by having a total ionic concentration that is approximately one half of that of MS medium and a low concentration of total nitrogen that is approximately one quarter that of MS medium.

In many cases it is necessary to add hormones or growth regulatory substances to basal media in order to achieve the induction and establishment of a morphogenetic response (Raj Bhansali *et al*. 1990; Zheng *et al*. 1996). Auxin alone (Lauzer *et al*. 2000; Gaj 2001; Martín 2003) or in combination with cytokinins (Gallego *et al*. 2001; Gallo-Meagher and Green 2002) are important inducers. Changes in osmotic pressure (Svobodová *et al*. 1999; Nakagawa *et al*. 2001) or in culture conditions (Nato *et al*. 2000; Blanckaert *et al*. 2002) also play an important activating role in the induction of embryogenesis.

MORPHOGENETIC DIFFERENTIATION: EXPLANT AND MEDIUM

In Mediterranean Europe, one of the biggest problems is the accelerated soil erosion and loss due to the abandonment of traditional crops. A good defence against this problem could be found in the planting of shrub species able to occupy – and productively so – some of these soils retired from agricultural activity, promotion of the diversity of the flora and fauna, and the implantation of shrubs that can be used directly by animals. Within policies aimed at improving soil quality with a view to increasing natural fertility and establishing a protective plant cover against erosion, legumes are promising candidates. Legumes are undemanding species because they are able to self-provide themselves with nitrogen and develop a broad root network in both the horizontal and vertical senses. These characteristics allow other species to benefit from the translocation of essential elements from the deeper horizons to the surface-most ones. This type of vegetation, apart from forage production, establishes a plant cover that may improve soil quality. As regards these aspects the genus *Medicago* offers many possibilities. However, with the exception of *M. arborea* and *M. truncatula*, few investigations have considered *in vitro* culture. *M. sativa* is one of the species that regenerates readily *in vitro* in comparison with other species of *Fagaceae*. Reports have also been made of regeneration systems for different diploid species: *M. littoralis* (Zagar *et al*. 1995), *M. lupulina* (Li and Demarly 1995), *M. murex* (Iiantcheva *et al*. 1999), *M. polymorpha* (Scarpa *et al*. 1993; Iantcheva *et al*. 1999), *M. truncatula* (Chabaud *et al*. 1996; Trieu and Harrisson 1996; Hoffman *et al*. 1997; Trinh *et al*. 1998; Iantcheva *et al*. 1999). Additionally, the somatic embryogenesis and regeneration of plants in other allogamous species that are generally tetraploid have been reported; examples are *M. coerulea* Less. and *M glutinosa* Mars. (Arcioni *et al*. 1982), *M. arborea* (Mariotti *et al*. 1984), *M. falcate* (Teoule 1983; Gilmour *et al*. 1987), *M.*

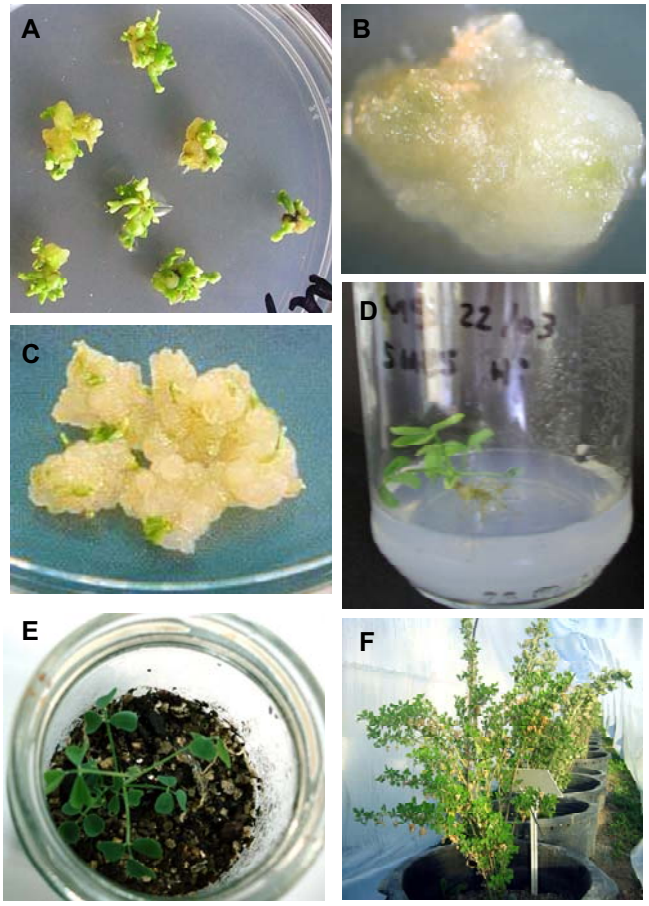

Plate I (**A**) Embryogenic calli obtained from *in vitro* culture of mature plants of *M. arborea*: WPM medium; (**B**) Soft whitish calli produced from the explants in MS media control; (**C**) Embryogenic calli obtained from *in vitro* culture of petioles from seedlings of *M. arborea*: H8 medium; (**D**) Seedlings of *M. arborea* in glass pots containing MS medium with half the concentration of salts but without sucrose and IBA; (**E**) Seedlings of *M. arborea* in glass pots with sterile sand; (**F**) Plants of *M. arborea*, with normal phenotypic characteristics, developed from somatic embryos.

varia Martyn (Gilmour *et al*. 1987) and *M. media* (Nagarajan *et al*. 1986; Brown *et al*. 1989).

The annual forage plant *M. truncatula* was proposed in 1992 as a model for crop legume species and has been widely adopted for genomic and genetic analyses (Gallardo *et al*. 2006).

Somatic hybridization experiments by means of protoplast fusion and the regeneration of plants have been reported and could represent and these techniques could represent a useful tool for solving the sexual impediments among *Medicago* species (Mariotti *et al*. 1984; Nenz *et al*. 1996).

In light of the above, our team selected two types of legume – shrubby, perennial and little studied – that might be good candidates as forage plants and in the recovery of degraded soils: *M. arborea*, which is able to produce significant amounts of dry matter under semi-arid climatic conditions (Martiniello *et al*. 1994), and *Medicago strasseri*, which is more tolerant to temperatures below 0ºC than *M. arborea* and whose growth rate is significantly higher (Alegre *et al*. 1993). Both species, which are tetraploids from the eastern Mediterranean, produce appreciable amounts of forage during the winter and under semi-arid conditions (González-Andrés and Ceresuela 1998). *M. citrina* could also be included within this group. This is a hexaplopid species endemic to the Mediterranean that grows better than *M. arborea* under saline conditions (Sibole *et al*. 1994), is tolerant to summer drough, and may be a good option for use as forage during the summer. The taxonomic relationships between the three species (*M. arborea*, *M. strasseri* and *M. citrina*) have been studied through the use of karyological, morphological, morphometric and molecular traits (Chebbi *et al*. 1995; González-Andrés *et al*. 1999). However, molecular characterisation is limited to a preliminary study using RAPD markers (Chebbi *et al*. 1995).

When we designed our study, it was first crucial to select a good cultivar and a good explant. Starting with *M. arborea*, we first attempted to select the optimal medium, following the different indications reported in the literature. For the *in vitro* culture of ligneous plants, WPM medium has often been used. This medium was very effective when mature plants of *M. arborea* were cultured (up to 85% of somatic embryos per callus; **Plate IA**), but totally negative results were seen when explants from young plants were used. In contrast to the above results, in most cases the use of young material as an explant (Gopi and Ponmurugan 2006) improves the capacity to induce embryogenesis: in mature tissues the response is much lower (Gray and Purohit 1991).

Using cotyledons, petioles, hypocotyls and leaves from young *M. arborea* plants as explants, we experimented with basal MS medium, varying the concentrations and types of hormone, as described in Gallego *et al*. (2001) and Martin *et al*. (2000). Treatments with growth regulators play a crucial role in embryogenic development but in general auxins and cytokinins are more effective in the induction of responses. With *M. arborea*, only 2,4-D and kinetin were able to induce embryogenesis. In an initial step, the explants were kept for two months in H8 medium (Gallego *et al*. 2001), consisting of basal MS supplemented with 2 mg l^{-1} of 2,4-D and 2 mg l^{-1} of kinetin. After this time, half of the explants were transferred to F0 medium, which consists of MS medium supplemented with 0.5 mg l^{-1} of 2,4-D but without kinetin. All cultures were carried out at 25ºC under conditions of 16 light and with an intensity of 20 µE $m^{-2}.s^{-1}$.

Despite the good results obtained using BA and TDZ in another type of plant (Mithila *et al*. 2002; Passey *et al*. 2002; Gopi and Ponmurugan 2006), in *M. arborea* no embryogenic response was obtained with TDZ, and BA was only effective in the induction of embryogenesis from petioles in the 2nd stage of culture, when BA was removed from

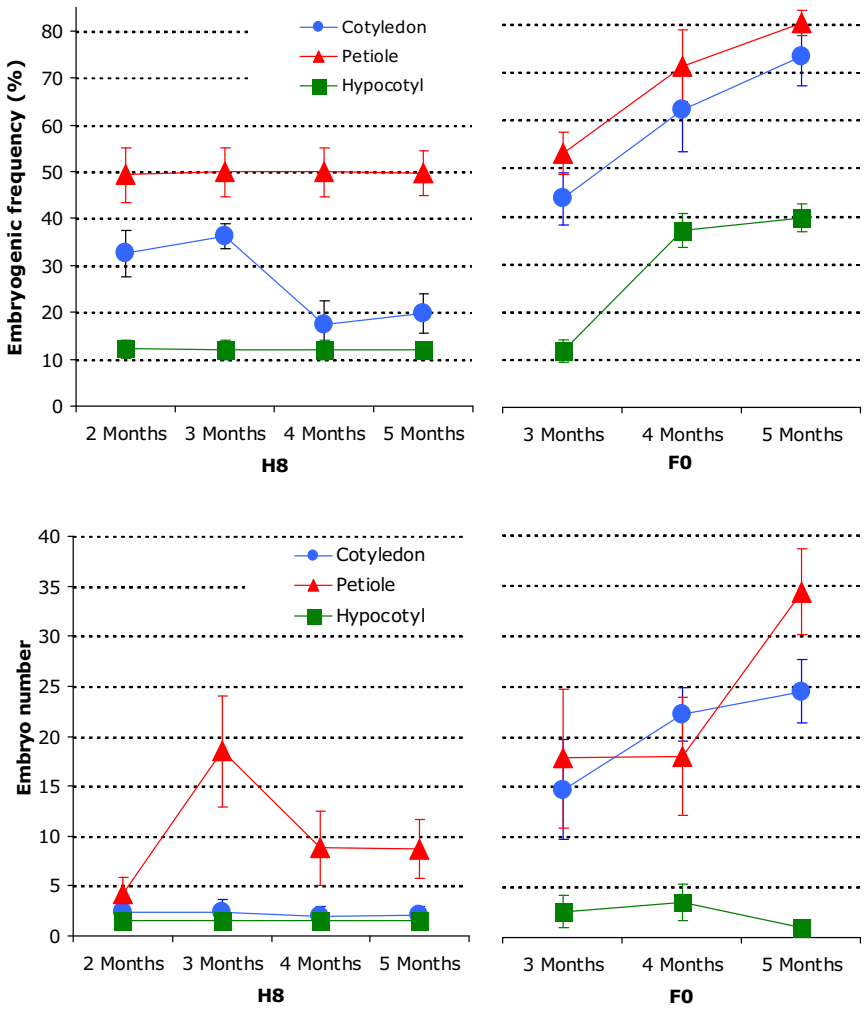

Fig. 1 Embryogenic frequency (%) and mean embryo number using explants of *Medicago arborea* L. cultured on H8 and F0 media.

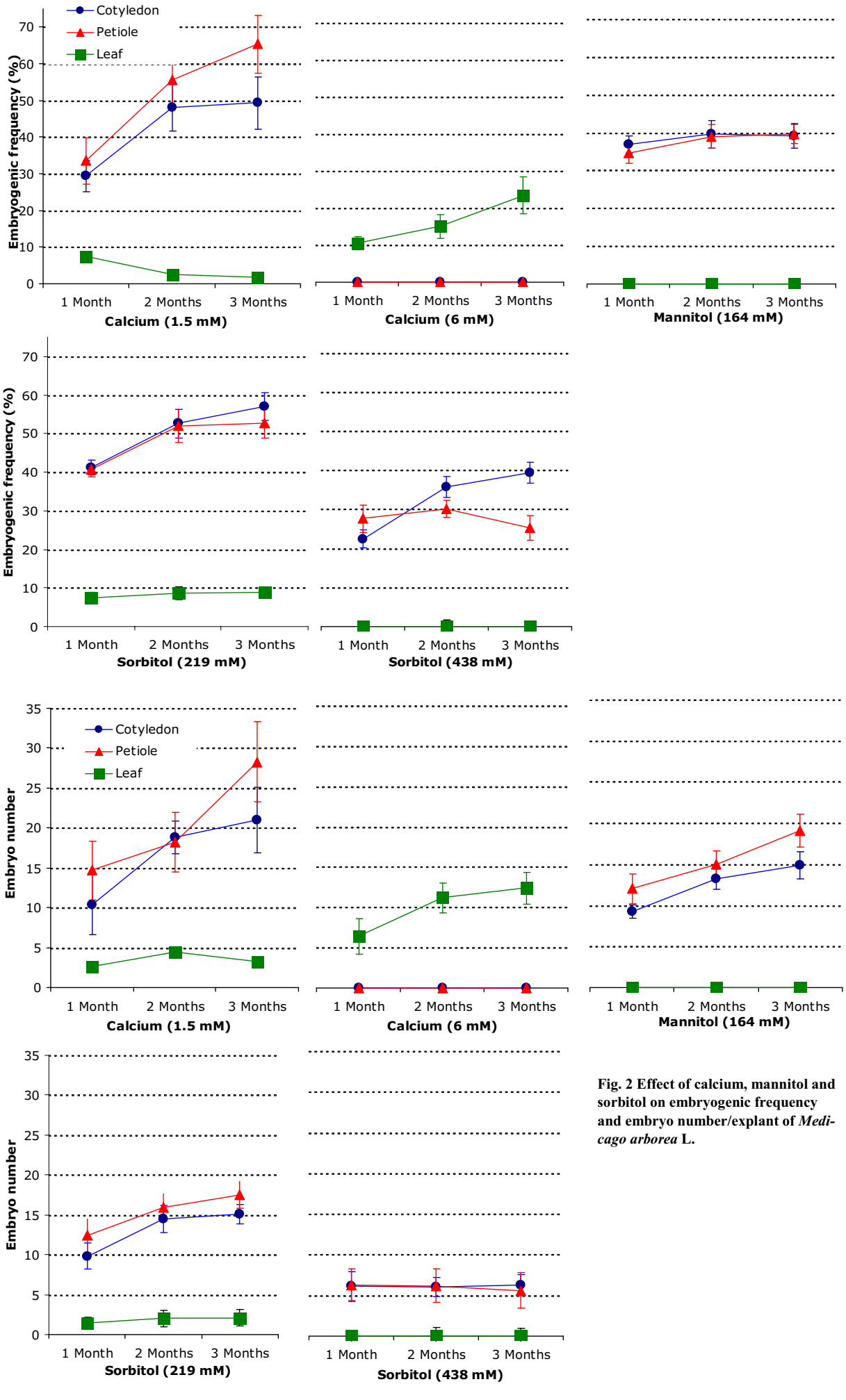

Fig. 2 Effect of calcium, mannitol and sorbitol on embryogenic frequency and embryo number/explant of *Medicago arborea* L.

the medium and the concentration of 2,4-D was reduced.

The morphology of the calli was also different depending on the hormones applied to the medium. MS media, with only 2,4-D, produced soft whitish calli from all the explants (**Plate IB**). The calli cultured with TDZ were light green, with dark green spots. The calli produced with BA had a uniform dark green colour. With kinetin, however, the embryogenic calli in the 1st step were whitish yellow, friable and slow-growing (**Plate IC**). The non-embryogenic calli were green, hard, and faster growing.

With H8 and F0, an important somatic response was obtained from cotyledons and petioles, as shown in **Fig. 1**. The petioles, used as explants, afforded the greatest embryogenic efficiency (embryogenic frequency × mean embryo number), above all in the second step of culture (F0 medium) (**Fig. 1**).

For the germination of somatic embryos, we used MS medium supplemented with indolbutyric acid (IBA) and half the concentration of sucrose. When the seedlings had acquired a developed root network and the first leaves they were transferred to glass pots containing MS medium with half the concentration of salts but without sucrose and IBA (**Plate ID**). One month later, the plants, with a length of approximately 10 cm, were transferred to glass pots containing sterile vermiculite and these were covered with parafilm to prevent drying. The plants remained under these conditions for two months, and were nourished with MS medium with half the concentration of salts, no sucrose and with IBA at 0.6%. During this time, the parafilm covers were progressively withdrawn, every 2 days to over three weeks, so that the plant could become adapted to the prevailing environmental conditions. They were then transferred to sterile sand (**Plate IE**) and after two weeks they were transplanted into flower pots to compete their development. Periodic watering was made with full MS medium diluted to 10% (v/v).

The somatic embryos that germinated were counted and their development was monitored until they became plants. Under normal culture conditions (Gallego *et al*. 2001) 80 ± 5% of the embryos germinated and 28 ± 3% developed into plants with normal phenotypic characteristics (**Plate IF**).

Although there has been little research into the effect of a given mineral nutrient or any given carbohydrate in a culture medium to obtain a response, it is important to bear in mind that knowledge in this field may be crucial to obtain good results. This idea was fundamental to achieve a medium that would produce better results in the embryogenic response (Hita *et al*. 2003).

Sometimes, the application of exogenous calcium to MS medium becomes necessary to obtain an *in vitro* morphogenetic response (Reynolds 2000) and it affects the process of somatic embryogenesis (Anil and Rao 2000). In the case of *M. arborea* (**Fig. 2**), a concentration of calcium of 1.5 mM reduced the embryogenic response with respect to control (medium H8, **Fig. 1**) and the embryos had very low viability. Thus, only 12 ± 3.7% germinated and of the seedlings obtained only 2 ± 0.3% were transformed into plants. Embryogenesis was completely inhibited at higher calcium concentrations (6 mM) (**Fig. 2**). Only when the leaves were used as explants did high calcium concentrations induce a greater number of somatic embryos than the control (H8 medium) and improve embryogenic efficiency (**Fig. 2**). High concentrations of calcium increased the hardness of the calli.

The type of carbohydrate and concentrations used in the culture media may vary the response *in vitro*, depending on the type of explant used (Leifert *et al*. 1995).

Sucrose exerts an important inducing role in somatic embryogenesis (Zhang *et al*. 2000; Karami *et al*. 2006). In general it is considered that the use of 3% sucrose in MS medium provides better results than 2% or 4%. However, there are many cases in which the use of higher concentrations of sucrose produces better results in the production of sprouts (Pati *et al*. 2006). Variations in the concentration of sucrose in H8 medium did not afford positive results. The highest concentrations (175.4 mM) applied to H8 medium totally inhibited embryogenesis, regardless of the explant used. With lower sucrose concentrations in H8 medium, embryogenesis was very reduced and the few somatic embryos obtained were larger and greener than those obtained in the control medium (**Plate IIA**).

The literature also contains reports that the addition of polyols, such as mannitol or sorbitol, to the culture medium improves the induction of somatic embryos (Li *et al*. 1995; Bronsema *et al*. 1997). With *M. arborea*, the addition of sorbitol to H8 medium elicited an embryogenic response that was more favourable than that obtained with mannitol, but embryogenic efficiency was always lower than that obtained with H8 medium (**Fig. 2**). The embryos obtained with these treatments were elongated and lacked vigour for their transformation into plants.

The presence of reduced nitrogen concentrations in the culture medium seems to stimulate somatic embryogenesis (Rienbothe *et al*. 1990; Gray and Purohit 1991). Thus, in carrot cultures the addition of NH_4Cl (10 mM) to an embryogenic medium containing KNO_3 (12-40 mM) produced a large number of embryos. NH_4^+ in small quantities, together with NO_3^- is necessary for the development of somatic embryos (Ammirato 1983; Srickland *et al*. 1987). In some cultures, such as in the case of *Digitalis lanata*, these nitrogen compounds cannot be completely replaced by the amino acids commonly used as a source of reduced nitrogen (glutamine, glutamic acid, alanine, arginine, asparagine or lysine) but, however, the addition of arginine together with NO_3^- produces elevated amounts of somatic embryos

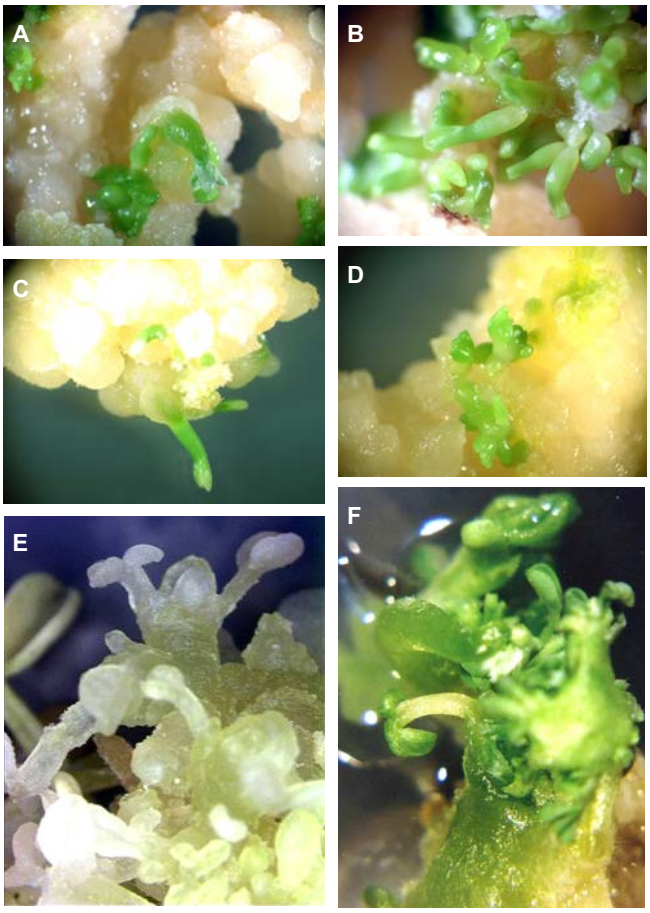

Plate II (**A**) Embryogenesis very reduced and somatic embryos larger and greener: H8 medium with half the concentration of sucrose; (**B**) Highest embryogenesis when petioles were used as explants: H8 medium with 2.5 mM proline; (**C**) Embryos induced in H8 medium with casein hydrolysate; (**D**) Somatic embryos small and less viable obtained with different culture media; (**E**) Albino seedlings obtained from anthers *in vitro* culture; (**F**) Organogenesis with explants from *M. strasseri*: H8 medium.

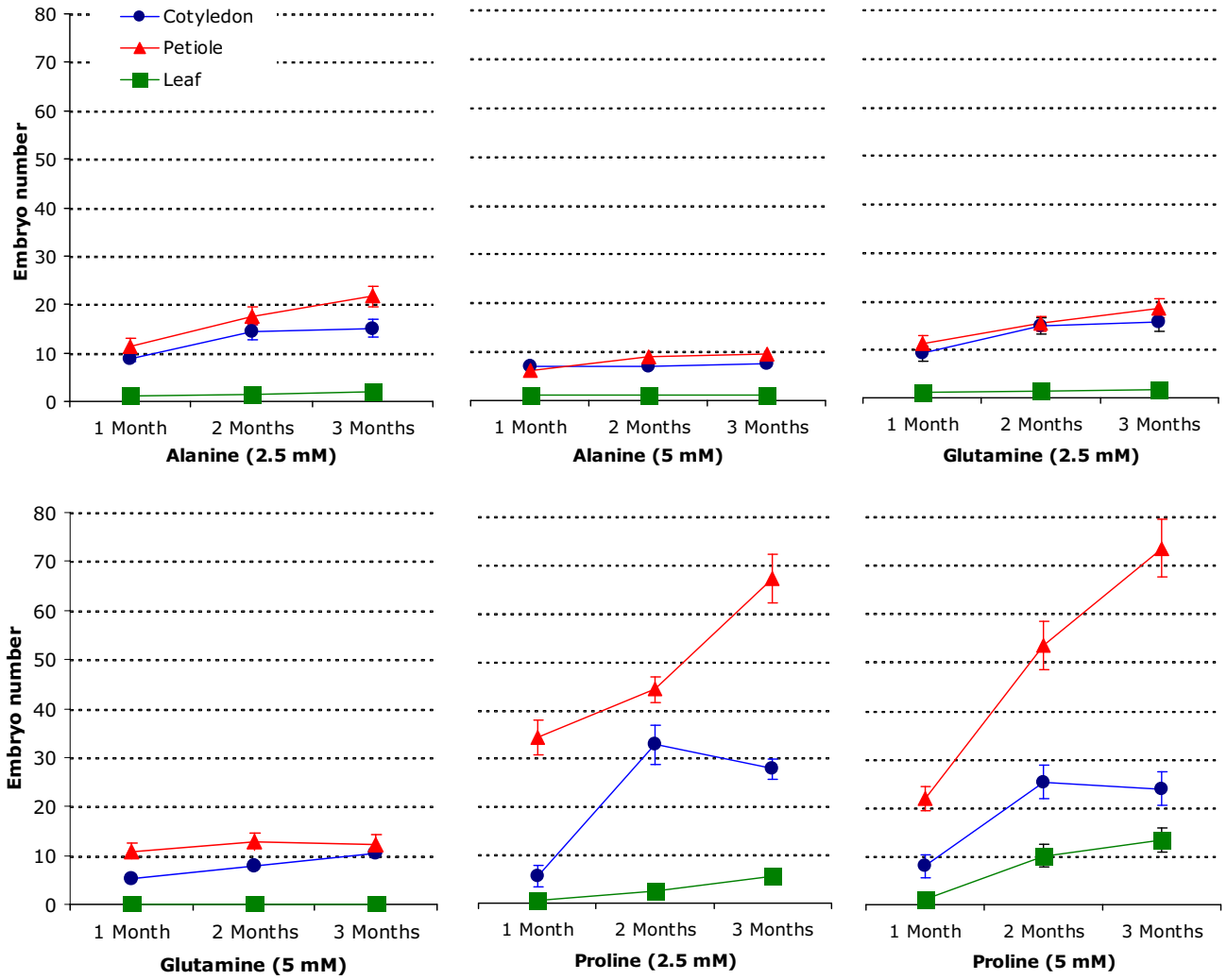

Fig. 3 Effect of alanine, mannitol and sorbitol on embryogenic frequency and embryo number/explant of *Medicago arborea* L.

(Rienbothe *et al*. 1990). The effect of the addition of amino acids to the medium varies considerably as regards somatic embryogenesis, depending on the type of amino acid and the species used as explant. Proline favours the embryogenic process (Shetty and Mckersie 1993). However, Loiseau *et al*. (1995) reported that the addition of glutamine, alanine, or proline does not elicit an increase in the number of embryos in pea hypocotyl cultures, but it does lead to a rapid proliferation and a better maturation of the embryos in embryogenic lines of *Abies alba* (Hristoforoglu *et al*. 1995). In general, the addition of different amino acids to the medium, such as serine, proline, asparagine and glutamine, decreases the germination frequency of the somatic embryos in *Eleusine coracan* Gaertn and hence the regeneration of plants from them (Eapen and George 1990). Glutamine as the sole source of nitrogen favours the maturation of somatic embryos in *Picea mariana* and *P. abies* (Khlifi and Tremblay 1995). Moreover, in conifers it is very beneficial for the induction of embryogenesis and maturation of embryos. In *Picea glauca* the type of amino acid influences the callus growth rate but not the total number of embryoids (Barret *et al*. 1997).

The importance of the nitrogen source in somatic embryogenesis is well documented. Accordingly, studies were conducted to address the effect that different nitrogen compounds may have on the results of embryogenesis obtained previously with *M. arborea* (Martín *et al*. 2000; Gallego *et al*. 2001).

According to the results obtained (**Fig. 3**), only proline exerts an important inducer effect (**Plate IIB**) producing a much better embryogenesis efficiency than that obtained previously by Gallego (2001). The use of a concentration of proline of 2.5 mM produced the highest frequency of embryogenesis when cotyledons and petioles were used as explants. When the concentration of proline was raised to 5 mM, a reduction in embryogenic callus production occurred with respect to that obtained in controls. Of special interest was the improvement in embryogenic frequency obtained with a proline concentration of 5 mM when leaves were used as explants; this contrasts with earlier results by Gallego with H8 and F0. The optimum proline concentration to achieve the maximum number of embryogenic calli (280 mg/l) was similar to that reported by Rao *et al*. (1995) to describe the improvement in the embryogenic production of *Sorghum bicolor*.

The positive effect of proline is also seen on observing the number of embryos per callus (**Fig. 3**). The optimum inducer concentration of proline, in the case of cotyledons, is 2.5 mM, but when petioles and leaves are used as explants a concentration of 5 mM is the most positive. The effect of proline, however, cannot be generalised to the rest of amino acids employed (**Fig. 3**), since their addition to the culture medium does not produce a similar negative response; in fact the response obtained when the control medium (H8) was used was reduced.

With the use of casein hydrolysate in the culture medium (**Fig. 4**) no outstanding results regarding embryogenesis were obtained with respect to the control. Only a concentration of 100 mg/l caused a slight positive effect and the use of higher concentrations (500 mg/l) reduced embryogenesis, which was completely inhibited when the concentration was increased to 1000 mg/l. These results conflict with those of Wu *et al*. (1996) and those of Wang *et al*. (1999), who demonstrated that casein hydrolysate added to the culture medium improved somatic embryogenesis in some plants, such as lily.

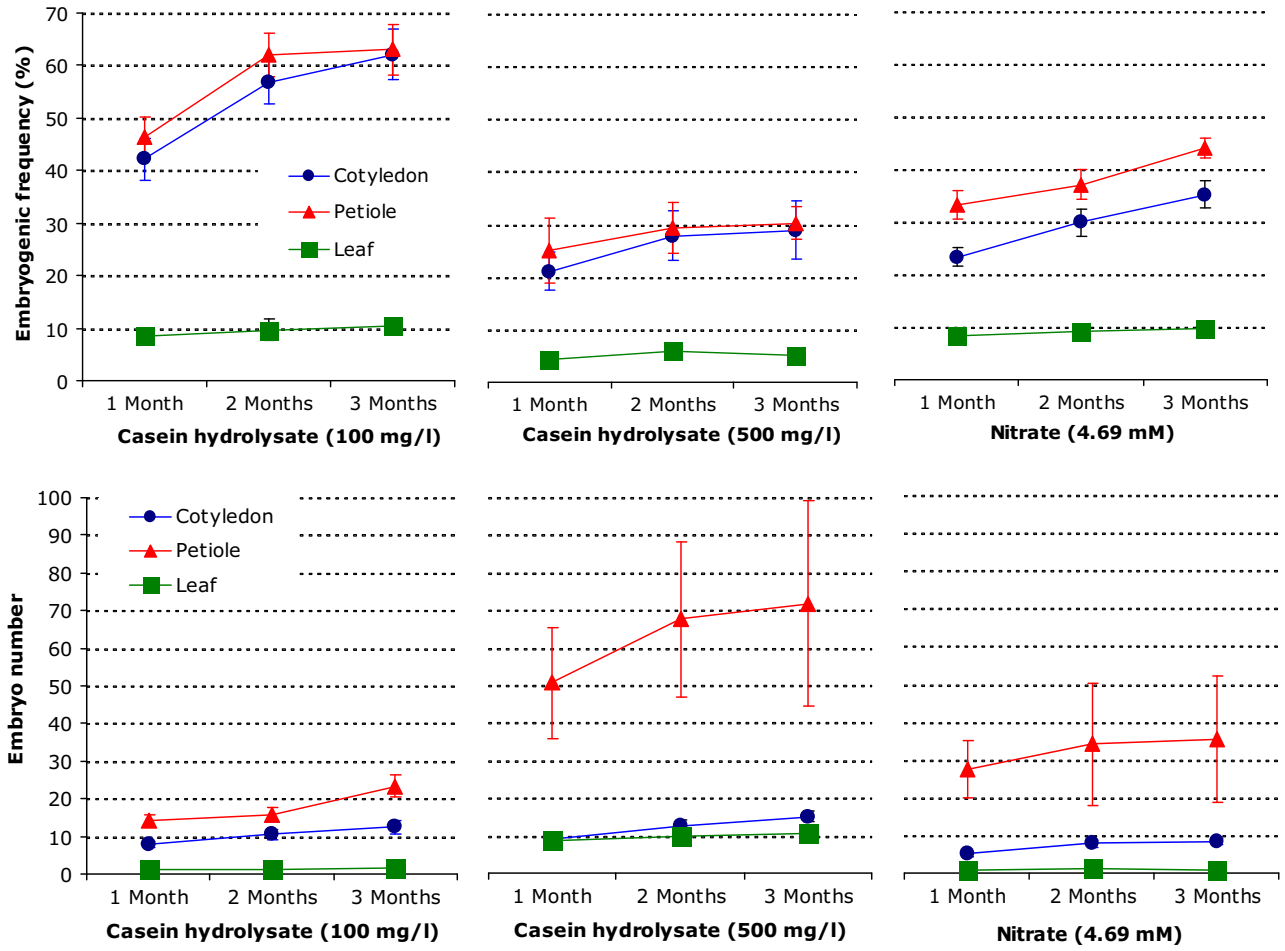

Fig. 4 Effect of casein hydrolysate and nitrate on embryogenic frequency and embryo number/explant of *Medicago arborea* L.

Alterations to the source of inorganic nitrogen of the medium may also change the embryogenic response (**Fig. 4**). A reduction by half of the nitrate concentration decreased efficiency. When it was reduced to one quarter, the production of somatic embryos was completely inhibited. This inhibitory effect was neutralised when casein hydrolysate was added to the medium with nitrate at a concentration of 100 mg/l. However, with this latter treatment, embryo induction continued to be lower than that obtained in the presence of proline.

The collection of a large number of embryos is not sufficient to consider that a good embryogenic response has been obtained. It is necessary to determine whether the different somatic embryos obtained are viable and whether they will give rise to vigorous plants able to acclimatise to the different environmental conditions.

The characteristics of the somatic embryos induced in the different media, depending on the nitrogen source are specified below.

Embryos induced in media with proline (**Plate IIB**) – both at 2.5 and 5.0 mM, were morphologically similar to those obtained in the control medium (H8); without proline, a higher proportion of embryos being produced in the cotyledonary phase together with greater synchronisation in their formation.

With casein hydrolysate, the embryos developed were longer and thinner than those obtained in the control medium (**Plate IIC**).

Finally, the embryos obtained with the rest of the media were small, were less viable and there was a greater production of embryos in the globular and torpedo phases (**Plate IID**).

One of the most important biotechnological initiatives aimed at plant production in recent years is the generation of dihaploid plants (Peng and Wolyn 1999), which on one hand has the advantage of shortening the improvement cycle, allowing the generation of genetic combinations that would otherwise be difficult to achieve, and, on the other, it offers the possibility of forming plants that are 100% homozygotic. That is, with the collection of dihaploid plants the production of new varieties can be accelerated.

Haploid is the general term used for sporophyte plants that have a gametic number of chromosomes (n). Thus, in a diploid sporophyte species (2n) the haploid may also be called monoploid (x) since it only has one set of chromosomes. In polyploidy species, haploids (n) have more than one set of chromosomes and are polyhaploid. Haploid plants coming from an autotetrapoid (4x) with four sets of chromosomes have been called dihaploid.

The differences between a dihaploid and a double haploid (DH) is that the latter is a haploid whose chromosome number has been duplicated and which has been formed from a monoploid or an allohaploid, such that it must be completely homozygotic. In contrast, a dihaploid is not homozygotic since it has two sets of chromosomes selected from 4 sets of the autotetraploid (Kasha and Maluszynski 2003).

There are two types of haploids: those deriving from the male gamete (androgenetic haploids) and those that derive from the female one (gynogenetic haploids).

The method most widely used nowadays for the production of double haploid plants is androgenesis (the induction of microspores to enter the process of cell division and differentiation) (Alché *et al*. 2000; Guo and Pulli 2000a, 2000b; von Aderkas and Bonga 2000; Liu *et al*. 2002; Germanà *et al*. 2006). This is because a more direct exposure of genetic traits occurs at haploid level. The regeneration of diploid plants from haploid microspores favours the production of fertile homozygotic individuals from a heterozygotic individual in a single generation, which reduces production time. To date, the results of these studies have given low response percentages, mainly due to a lack of

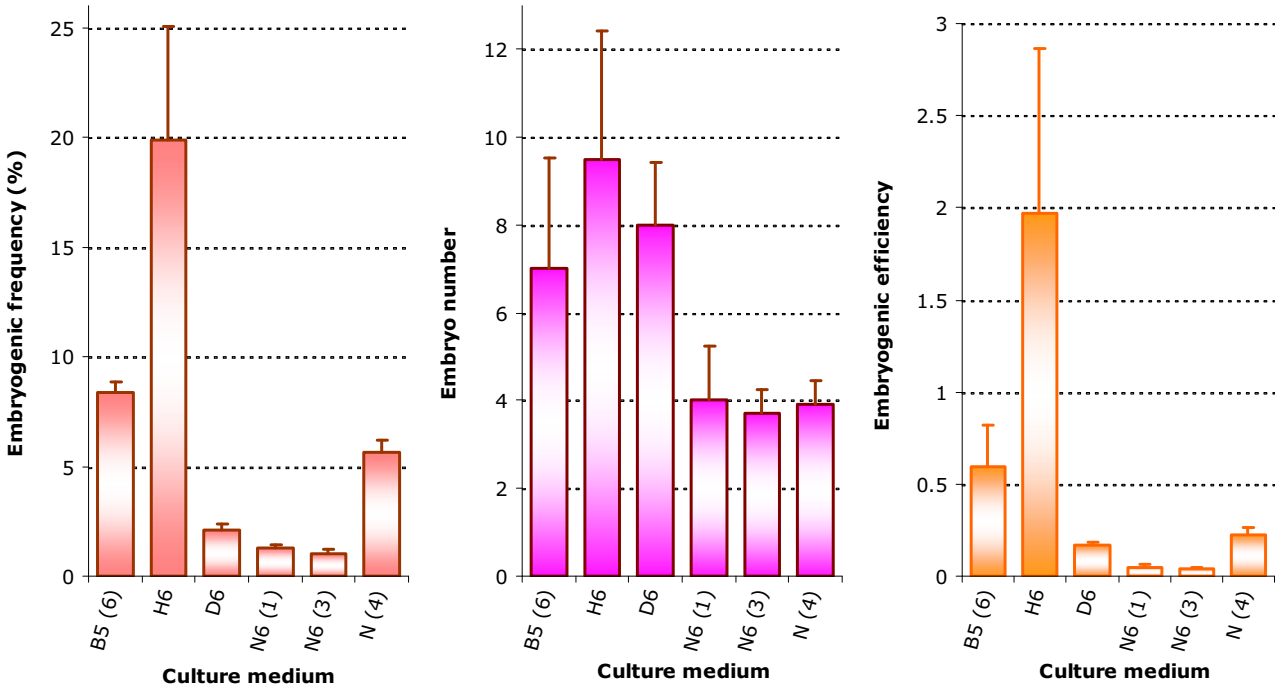

Fig. 5 Embryogenic frequency (%), mean embryo number and embryogenic efficiency (embryogenic frequency x mean embryo number) obtained from *in vitro* culture of 100 anthers of *Medicago arborea* L. Calli cultured for three months in the dark and two months under photoperiod conditions. Media abbreviations: B5 (6) (Gamborg *et al*. 1968); H6 (Murashige and Skoog 1962); D6 (Murashige and Skoog 1962); N6 (1) (Chu 1978); N6 (3) (Chu 1978); N (4) (Nitsch and Nitsch 1969).

knowledge about the molecular changes occurring during the development of pollen grains to induce androgenesis.

Our studies on haploidy were begun on the above basis (Lanas *et al*. 2006) and their aim was to improve the production and quality of the somatic embryos obtained by Gallego *et al*. (2001) from *M. arborea* seedlings.

First, it was necessary to select the moment of the inflorescences in which the anthers produced the best response. We thus collected flowers in the first stages of the development of the capituliform inflorescence; that is, when they contained pollen whose formation had not been completed, with the microspores in the second phase of meiosis. At that time the anthers were perfectly visible.

Through use of histological sections, it was observed that the anther tissue was in the mononuclear phase, with microspores in the 2nd phase of meiosis and tetrads were seen, confirming the existence of haploid cells.

Once the most suitable phase of the flower had been selected and anthers had been obtained under a lens in a laminar flow chamber, different culture media were used to determine the primary response (Lanas *et al*. 2006). Fifteen media were used, in which we varied the basal medium (of Gamborg, of Murashige and Skoog, of Chu, of Nitsch or of Lichter) and the type of auxins and the concentrations used. At three months of culture in the darkness and with two photoperiod conditions, of the 15 types of callus obtained differentiation only occurred in 6 of them (**Fig. 5**); the important embryogenic response obtained when anther culture was carried out in H6 medium was striking. Interestingly, this medium (H6) is very similar to the H9 medium used, with great efficiency, by Gallego *et al*. (2001) to collect somatic embryos of *M. arborea*. The difference between both media lies in the concentration of kinetin, which in anther cultures was 0.5 mg/l.

In general, as shown by Gurel *et al*. (2000) cold pretreatment increases embryogenic frequency in anther cultures. Our results, however, revealed that the response was not always improved. Of the different media used, only H6, with cold pretreatment, was able to induce embryogenesis and a much higher embryogenic efficiency – nearly twofold – than that obtained under normal conditions.

Despite this efficiency, obtained with cold pretreatment using H6 medium, this medium was used, without any pretreatment, for studies on the germination and regeneration of seedlings.

One of the particularities of haploidy is the production of albino plants (**Plate IIE**). To date, the production of albino seedlings has generally been high and the aim of different workers such as Bishnoi *et al*. (2000) has been to reduce the generation of albino plants and increase that of green plants In this initial study, we achieved a high percentage of green plants (more than 60%).

A striking observation on looking at the seedlings is that although those of the green and albino types were very similar the leaves showed differential characteristics that were the basis of performing analysis of fresh weight, dry weight, moisture content, starch and sucrose (**Fig. 6**). There was no difference in dry weight between either type of seedling and yet their fresh weight was very different owing to the high water content in the leaves of the albino seedlings. The sucrose and starch content is also very different (**Fig. 6**). Of special interest was the high level of sucrose in the albino plants; since this is an osmotically active compound it may be the cause of the high water content detected in this type of leaf, as reported by Talbott and Zeiger (1998).

The ion content of a seedling is extremely important for plant development. Each ion plays a crucial role in metabolism and its concentration may affect the vital and physiological functions of seedlings to an enormous extent, as reported by Di-Vaio *et al*. (2001). Upon analyzing the ions present in leaves (**Fig. 7**), marked differences were also observed, depending on the type of seedling. In general, with the exception of calcium the albino plants had lower concentrations of the ions studied, suggesting possible changes in metabolism which could be responsible for the observed albinism.

Comparative isoenzymatic analysis (Lanas *et al*. 2006) of the leaves of the albino and green plants also revealed interesting differences that merited analysis (Lanas *et al*. 2006). Six enzyme systems were chosen: leucine aminopeptidase (LAP), peroxidase (PER), isocitrate dehydrogenase (IDH) and α-B-esterase (EST) were monomeric, while malate dehydrogenase (MDH) and menadine reductase (MNR) were tetrameric.

A total of 29 alleles at 16 putative loci were identified in the *M. arborea* specimens analysed. For homozygosity assessment, of the *in vitro* plants obtained by androgenesis,

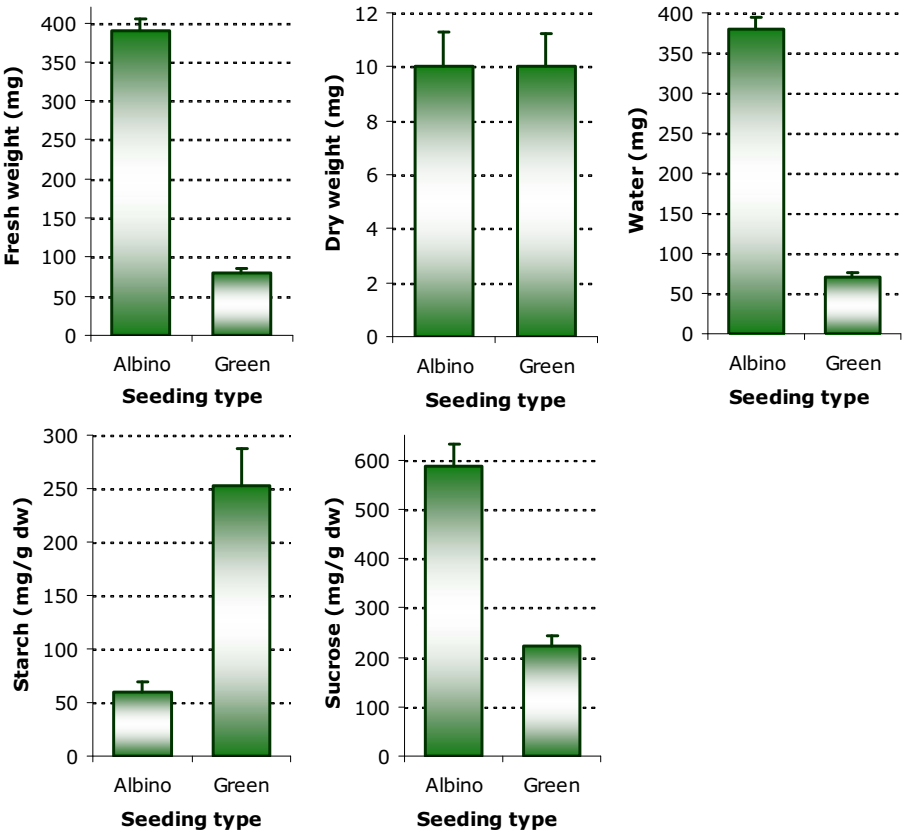

Fig. 6 Fresh weight, dry weight, water contents and starch and sucrose concentrations in the leaves of the albino and green seedlings grown on H6 medium.

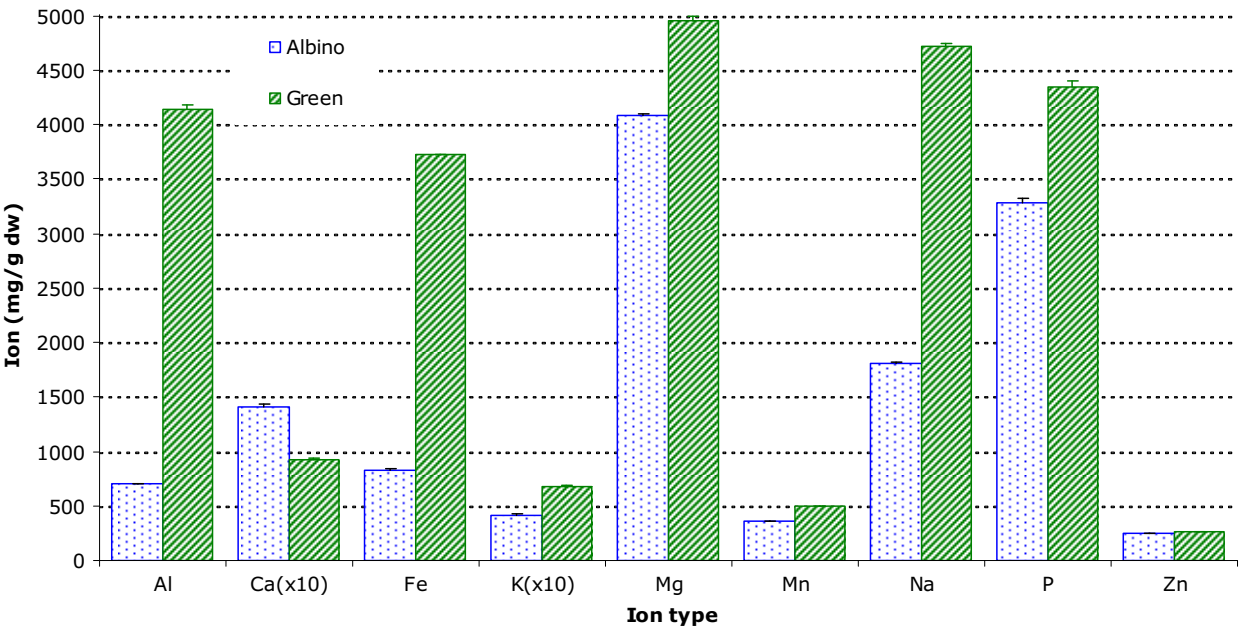

Fig. 7 Ion concentrations in the leaves of albino and green seedlings grown on H6 medium.

the segregation of 7 heterozygous isozyme markers (Per-B, Per-A, Per-C, Mnr-A, Lap-C, Est-C, Mdh-A) was analyzed between the *Medicago* "albino" and "green" plants (Lanas *et al*. 2006).

Although fragmentary, since they are based on only 11 individuals, our results suggest that the isozyme markers described here could provide a tool for the identification, control and characterisation of haploid individuals and for verification of parentage programs of haploid clone production. This conclusion is in agreement with previous reports about the same or similar problems in other species (Bouvier *et al*. 2002).

In our case the use of isozyme markers confirmed the homozygosity of all the "albino" individuals, and hence the existence can be inferred of a high percentage of haploid specimens within the total *in vitro* plants. This percentage reached 100% in the *in vitro* "albino" plants sampled.

The similarity, as ligneous perennial plants, between *M. arborea* and *M. strasseri* suggested that embryogenesis could be induced in the latter species by using the H8 and F0 media that had afforded such good results previously (Gallego *et al*. 2001). *M. strasseri* (Greuter *et al*. 1982) is a legume of great forage value owing to its resistance to the cold in winter, and in zones with a continental climate it could offer an alternative for animal feeding. It has been proposed that this species, implanted on a large-scale basis, could lead to huge savings in feed. *M. strasseri* is also a species considered of great importance as regards forestry repopulation in areas with lime soils, and owing to its contribution of nitrogen it could improve the growth of tree species.

Under photoperiod conditions, in which cotyledons,

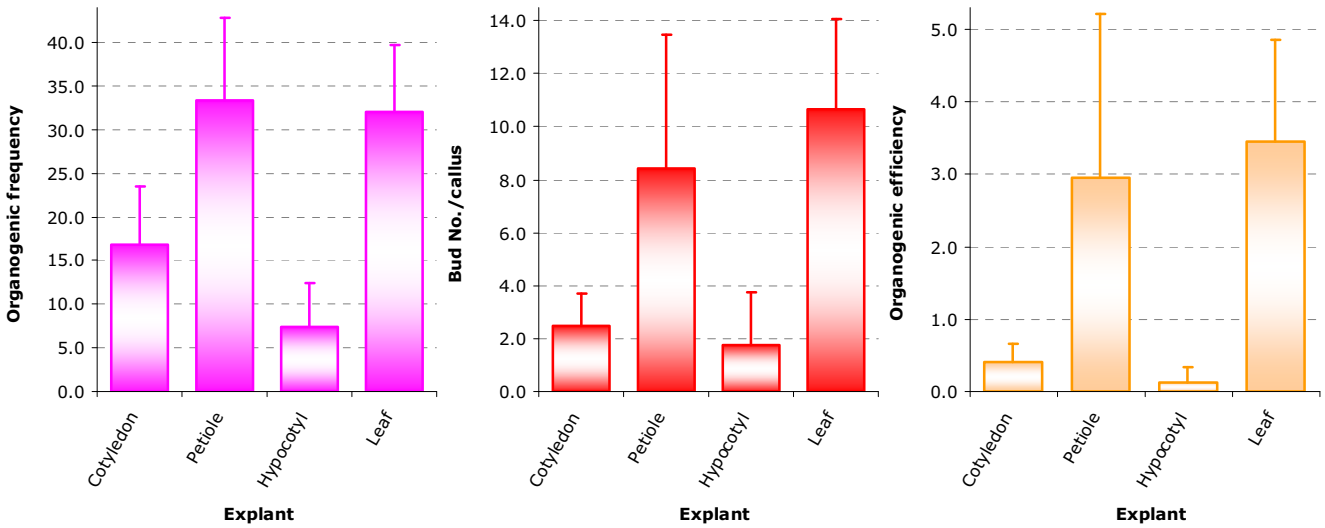

Fig. 8 Organogenic frequency (%), mean bud number and organogenic efficiency obtained from *in vitro* culture of several explants of *Medicago strasseri*. Culture medium: H8.

petioles, hypocotyls and leaves as explants and different culture media (with 2,4-D, TDZ, kinetin, BAP, glycerine, etc.) were used, only the media with kinetin and 2,4-D induced differentiation and a morphogenetic response. These observations contrast with those reported by authors such as Bama and Wakhlu (1995) and Kumar *et al*. (2001), who described good organogenic results with TDZ. Moreover, the medium supplemented with BAP also improved multiplication by organogenesis (Pati *et al*. 2006). H8 medium (which induced embryogenesis in *M. arborea*) afforded another type of response in this new species: i.e., organogenesis. The explants with a greater organogenic frequency, a higher number of shoots and a greater organogenic efficiency in this were the petiole and leaf, above all the latter (**Fig. 8**). Each medium may be suitable for achieving a specific aim, depending on the type of explant used (Leifert *et al*. 1995). What is known is that the *de novo* development of structures such as roots or stems is begun with the transformations brought about in a small group of cells. These cells then begin to divide until a globular mass of meristematic cells has formed (the meristemoid), able to respond to certain factors inside the tissue and give rise to a caulinar or radicular primordium.

Both rhizogenesis and caulogenesis may occur directly on the explant or indirectly through the formation of a callus. In the case of *M. strasseri*, the manifestation of organogenesis is the appearance of shoots on previously formed calli (**Plate IIF**). As suggested above, organogenesis is the structural manifestation of different physiological, biochemical and molecular events that reflect the expression of selective genes in cells (Brown and Thorpe 1986).

ENDOGENOUS CYTOKININS IN SOMATIC EMBRYOGENESIS

Endogenous cytokinins have been analysed (Pintos *et al*. 2002) during the induction and development of somatic embryos. The results have been important indicate of the behaviour of these hormones during the development of embryogenesis.

A high concentration of kinetin added to the H8 culture medium (2 mg/l) led to an increase in the endogenous content of total cytokinins (**Fig. 9**) with respect to the levels of total endogenous cytokinins found in the petioles from 4-week old seedlings (911.69/39.4 pmol/g dry weight, data not show).

After the second month of culture, a decrease in total endogenous cytokinins occurred as compared to the first

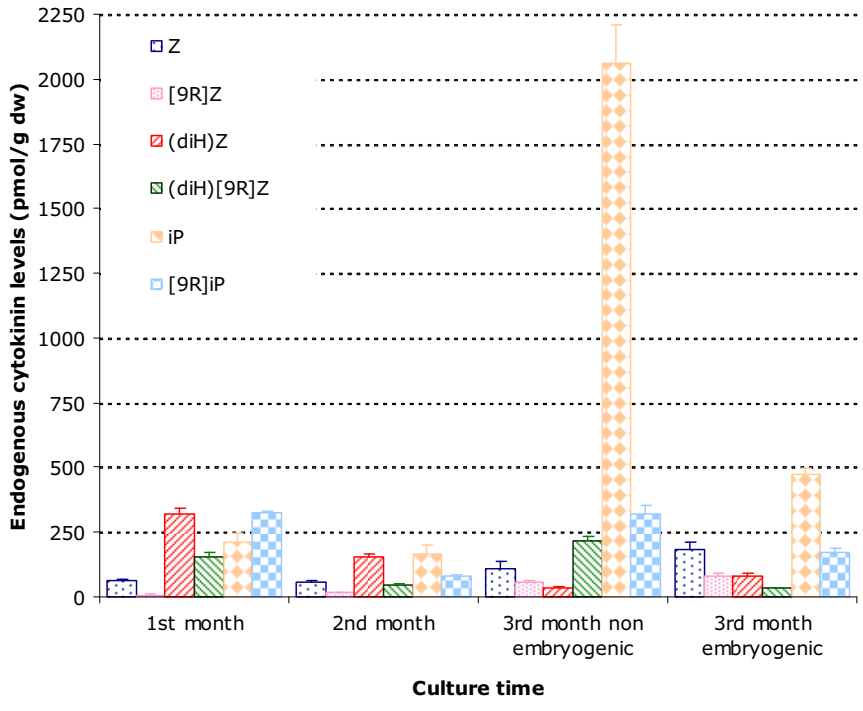

Fig. 9 Endogenous cytokinins in calli of *Medicago arborea* L. during 3 months of culture.

Table 1 Mean values ± S.E. of endogenous hormonal ratios (iP-type/total cytokinins and iP-type/Z-type cytokinins) and endogenous levels of iP+[9R]iP, found in embryogenic and non-embryogenic calli of *M. arborea* after 3 months of culture.

H8 medium	iP+[9R]iP(pmol/g)	iP-type/Total cytokinins (pmol)	iP-type/Z-type cytokinins (pmol)
Non-embryogenic	2379.58 ± 153.396	0.852624 ± 0.0091	5.785369 ± 0.4192
Embryogenic	645.05 ± 46.22	0.630097 ± 0.0093098	1.703417 ± 0.068

month (**Fig. 9**).

In the third month of culture, embryogenic and non-embryogenic calli were separated to carry out the analysis of endogenous cytokinins. In non-embryogenic calli, total endogenous cytokinins increased, and this increase was five-fold when 2 mg/l kinetin was used. For the induction media, the endogenous level of total cytokinins in the third month was greater in non-embryogenic than in embryogenic calli (**Fig. 9**).

The endogenous content of each of the cytokinins analysed (Z, (diH)Z, [9R]Z, (diH)[9R]Z, iP and [9R]iP) varied considerably in the different calli studied (**Fig. 9**). During the first 2 months of culture, similar values for isopentenyl forms (iP and [9R]iP) and dihydroderivatives [(diH)Z and (diH)[9R]Z) were found in both the first and second months of culture (**Fig. 9**). Furthermore, Z and [9R]Z showed the lowest values in both months. The levels of Z increased in the third month of culture, and this increase was more pronounced in the embryogenic than in the non-embryogenic calli from the induction media.

Upon analysing the relationships among the different combinations of endogenous cytokinins, significant differences were observed between embryogenic and non-embryogenic calli from the third month of culture (**Table 1**). The relationship between isopentenyl derivatives (iP and [9R]iP) and zeatin derivatives (Z + [9R]Z + (diH)Z + (diH)[9R]Z) and with respect to total cytokinins was always lower in embryogenic than in non-embryogenic calli (Martín *et al*. 2000).

In *M. arborea* calli, the levels of isopentenyl derivatives (iP-[9R]iP), and the iP-type/Z-type cytokinins and iP-type/total cytokinin ratios could be considered reliable indicators of the embryogenic response. High levels of isopentenyl derivatives with respect to other the endogenous cytokinins analysed do not appear to favour the development of the embryogenic response.

CARBOHYDRATE ANALYSES

Considering the calli obtained using different explants and media, of interest are the very different responses arising from small variations in the hormone composition of the media (Gallego *et al*. 2001). Only some calli, and only in a small fraction of the somatic cells of a callus, seem able to elicit an embryogenic or organogenic response. Accordingly, when we began our investigations we felt that a crucial step in the understanding of how somatic embryogenesis occurs would be to determine the metabolic differences that distinguish embryogenic calli from the non-embryogenic type. Analysis of this metabolism was begun with investigations of carbohydrates (Martin *et al*. 2000; Cuadrado *et al*. 2001). This study seemed to be essential since accor-ding to the literature the sucrose present in the medium plays an important role in callus formation (Frick 1991; Veramendi and Navarro 1996) and the glucose released through its hydrolysis initiates different metabolic processes that vary depending on the type of callus (Mangat *et al*. 1990; Vu *et al*. 1993).

The investigations were begun with *M. arborea*, later determining in *M. strasseri* whether the results were similar or different. Using three culture media (Gallego *et al*. 2001), two embryogenic (H8 and F0) and one non-embryogenic (basal MS), and 4 types of explant – cotyledons, petioles, hypocotyls and leaves – we began our investigation by studying total carbohydrates, starch, sucrose and reducing sugars.

In the phase of callus initiation, the calli showed a strong accumulation of starch; later, this decreased as the calli developed. Since amylase is an enzyme involved in the hydrolysis of starch we decided to study amylase activity in the different calli with a view to determining whether there might be any relationship between catabolism and amylase activity.

At the same time, since invertase is a key enzyme in sucrose hydrolysis, we considered that it must play a crucial role in the use of sucrose in the medium, hence providing the material and energy necessary for the processes of differentiation typical of embryogenesis or organogenesis to be brought about. We analysed both acid and alkaline invertase since their functions may differ (Masuda *et al*. 1988) at the different times of development of somatic embryos.

With all the above studies, which are basic but essential to gain insight into the cellular metabolism occurring during somatic embryogenesis or organogenesis, our aim was to determine whether there might be some difference or some fundamental phenomenon that only occurs during the induction and development of somatic embryos.

From the results obtained concerning total sugars two observations were salient: 1) the levels of these sugars were high, accounting for about 90% of the dry weight of the calli, with the exception of those obtained form leaves, and 2) there were no outstanding differences between the embryogenic and non-embryogenic calli.

Sugars are crucial for the growth and development of plants (Dimaxi-Theriou and Bosabalidis 1997). Of the different sugars investigated, reducing sugars play an impor-

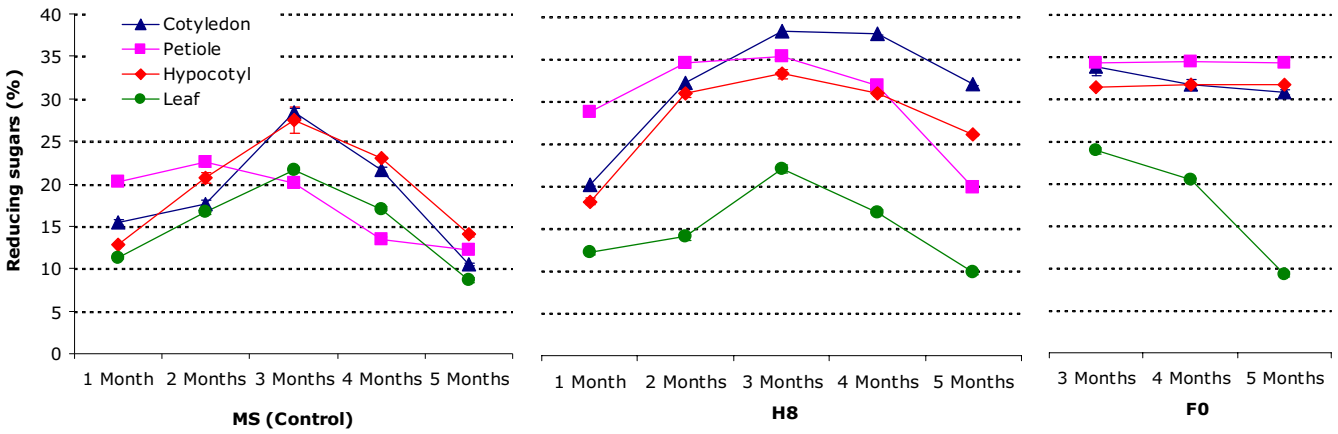

Fig. 10 Variation in the percentage of reducing sugars/total sugars in calli kept on different media, using several explants from *Medicago arborea* L.

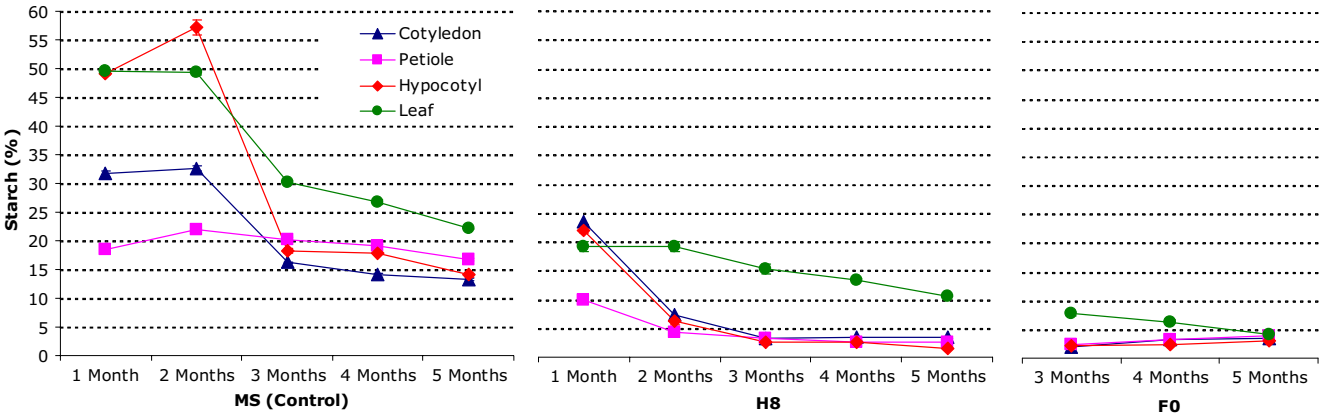

Fig. 11 Variation in the percentage of starch/total sugars in calli kept on different media, using several explants from *Medicago arborea* L.

tant role in callus formation and differentiation. The content of this type of sugar has been studied with different media and explants to see whether there were any important differences between the embryogenic and non-embryogenic calli. **Fig. 10** shows the percentages of reducing sugars with respect to the content in total sugars. The content of reducing sugars varied between 8.61% and 38.22%, depending on the explant used, the medium, and the time of culture. The highest percentages were seen when the calli were embryogenic (the calli obtained from petioles, cotyledons and hypocotyls in H8 and F0). In all cases, the highest percentage of sugars was obtained in the third month of culture.

A characteristic feature of *in vitro* culture is the rapid appearance of starch in callus cells at the start of culture (Branca *et al*. 1994) and also, in some cases such as bamboo, during the maturation phase of somatic embryos (Godbole *et al*. 2004). The results obtained on assessing starch levels are shown in the **Fig. 11**. In the calli developed in the MS control medium, a striking observation was the high levels of starch seen in the first two months of culture, especially when hypocotyls and leaves were used as explants. In general, a considerable decrease was observed in the starch content between the 2nd and 3rd month of culture. This decline continued very slowly as callus development progressed. Regarding the H8 medium (which induced embryogenesis), in the 1st month of culture the starch content of the calli was lower than that observed in the control calli. A striking observation with the H8 medium was a rapid decrease in the starch content of calli as from the 2nd month of culture. The levels of the polysaccharide remained very low from the 2nd to the 5th months of development. A clear difference was observed when leaves were used as explants: the starch content did not undergo the spectacular declines seen with the other types of explant, the level of the polysaccharide remaining high even in the 5th month of culture.

According to the results obtained with the MS and H8 media (**Fig. 11**), there were important differences not only in the starch content at the start of culture but also in the variation in its levels during the five months of development of the calli. In the first month of culture, the calli produced in MS medium (non-embryogenic) had a higher starch content than those formed in H8 medium (embryogenic). As the culture time progressed, a faster decrease was observed, accompanied by a very low starch content in the embryogenic calli (H8 medium). In light of the starch content, there seem to be differences between embryogenic and non-embryogenic calli: higher levels of starch in the non-embryogenic calli than in the embryogenic ones over the five months of culture. Transfer of the calli at two months of culture from H8 to F0 medium (**Fig. 11**) induced the development (except when leaves were used as explants) of a large number of somatic embryos per callus (Gallego *et al*. 2001). The determination of the starch content in the calli cultured in F0 medium revealed a decrease

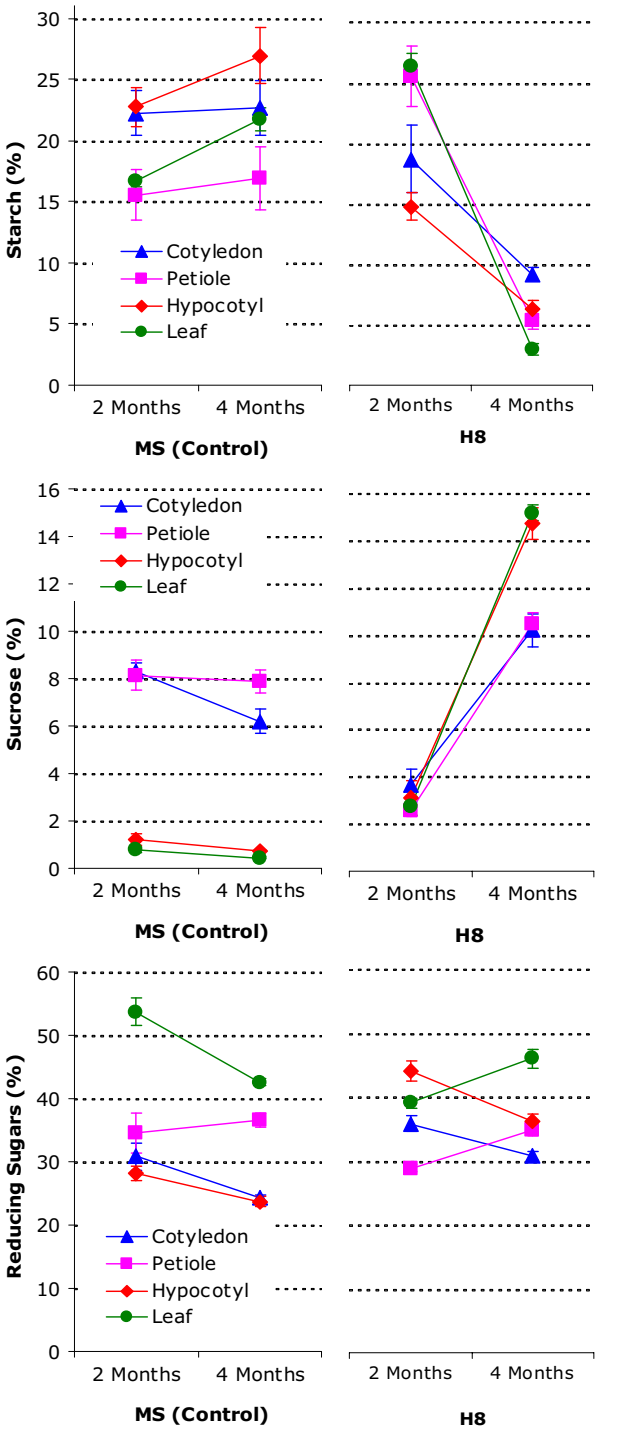

Fig. 12 Variation in percentage of reducing sugars/total sugars, starch/total sugars and sucrose/total sugars in calli kept on different media, using several explants from *Medicago strasseri*.

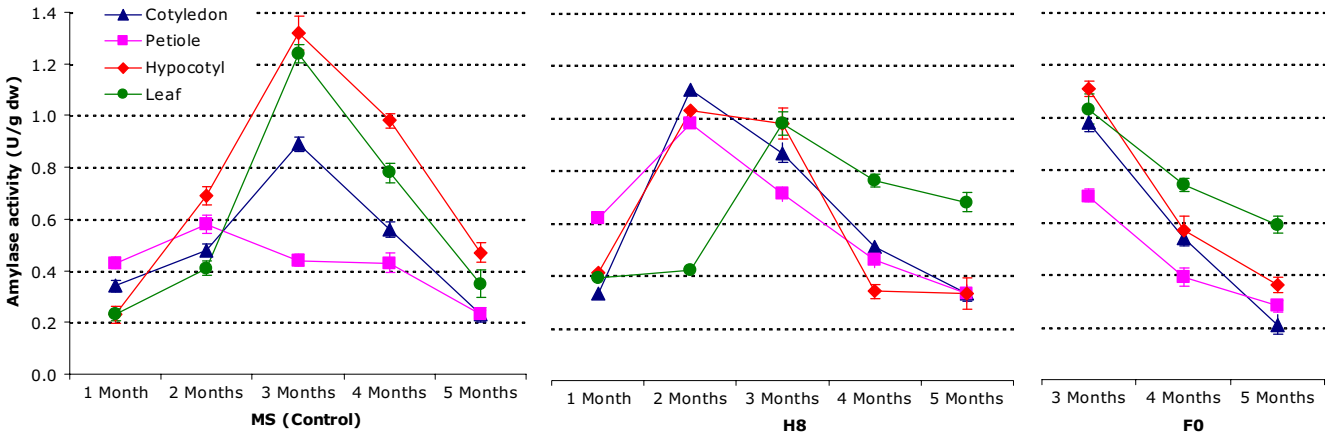

Fig. 13 Variation in amylase activity in calli kept on different media, using a range of explants from *Medicago arborea* L.

in the levels of the polysaccharide one month after the calli had been transferred from H8 medium. However, in contrast to what happened in the calli obtained with H8 medium, a small increase was observed in the starch levels as callus development progressed in F0 medium. The leaves, which are not able to induce embryogenesis in this medium, had calli whose behaviour was completely different as regards the starch content.

From the study carried out on the starch content in calli, two observations are important. First, at the start of callus development, the levels of starch were higher in the calli formed in the control medium (MS), which were never embryogenic. Second, during the development of somatic embryogenesis, starch levels were very low in the calli. The very slight increase detected in the calli obtained in F0 medium towards the end of development could be due to the accumulation of starch granules in the somatic embryos themselves as development progressed (Lai and McKersie 1994; Sreedhar and Bewley 1998).

Very similar results were obtained upon analysing the starch content in organogenic calli from *M. strasseri* (**Fig. 12**). A decrease was observed in the starch content before differentiation and the organogenic calli (H8 medium) contained the lowest starch contents. This strong accumulation of starch in the calli suggests later development and differentiation of the callus. Naidu and Kishoz (1995), working with tobacco, reported that the calli that formed organs had higher starch contents than those that did not. Many authors have also demonstrated that the accumulation of starch in callus cells is a prerequisite for the *in vitro* development of sprouts (Ho and Vasil 1983; Thorpe *et al*. 1986; Stamp 1987) and even for the development of somatic embryogenesis, although it cannot be considered a determinant factor of this latter (Keller *et al*. 1988).

Different authors (Mangat *et al*. 1990; Branca *et al*. 1994) have reported that at the beginning of the developmental processes *in vitro* starch is always accumulated and, according to Mangat *et al*. (1990), it is easy see why starch rapidly disappears during organogenesis or embryogenesis. Both processes require large amounts of energy, which can be provided by starch degradation. Degradation of the polysaccharide gives rise to the formation of glycolytic intermediates that, when catabolised oxidatively, produce large amounts of ATP, which can then be used for cellular metabolism.

Using *M. arborea*, amylase seemed to be involved in the starch hydrolysis in the calli because the maximum enzymatic activity was coincided with a marked drop in the starch content (**Fig. 13**). However, there were no clear differences in amylase activity between the embryogenic (H8 and F0) and non-embryogenic (MS) calli.

In *M. arborea*, sucrose is normally the main source of carbon for starch synthesis in seeds and it also plays an important role in starch formation in calli. With our analysis of sucrose levels in calli our aim was to determine whether, as happens with starch, there were any differences in the contents of this important disaccharide between embryogenic and non-embryogenic calli.

Considering the different media and explants, sucrose (**Fig. 14**) represented between 19% and 17.66% of total sugars. The highest percentages of the disaccharide were found in calli obtained in MS medium (non-embryogenic). The percentage of sucrose decreased in all calli as the culture time progressed. In general, in the embryogenic calli the percentages of sucrose were lower than in the non-embryogenic type. However, in the case of organogenic calli (**Fig. 12**), obtained in H8 medium with *M. strasseri*, the sucrose concentration increased in parallel with the increase in the starch concentration. These results suggest that some of the glucose released in starch hydrolysis would be used for the synthesis of sucrose, which could be stored as a temporary reserve in the vacuoles of cells so as not to alter the osmotic potential.

Plant invertases (β-D-fructofuranosidases) are highly

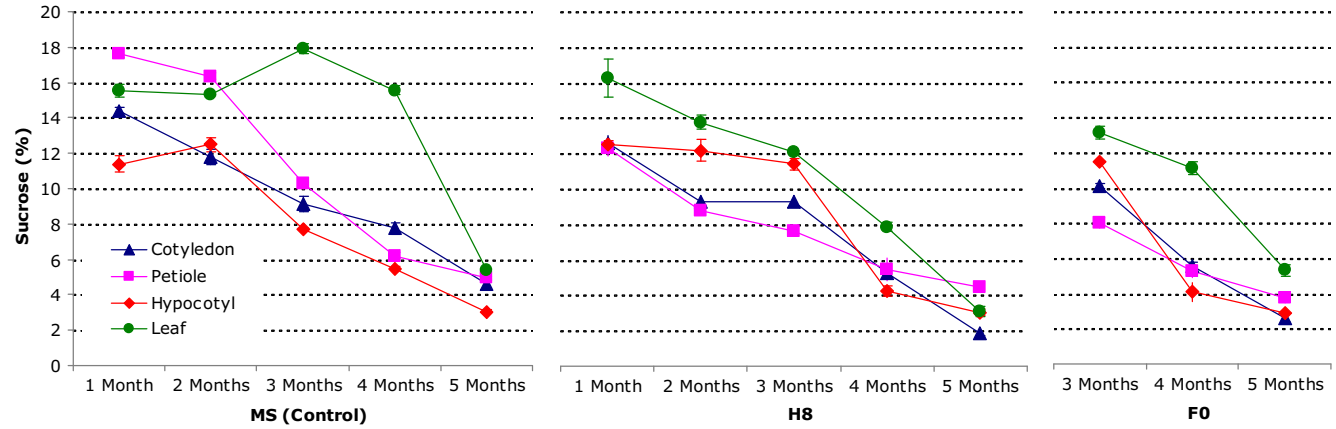

Fig. 14 Variation in the percentage of sucrose/total sugars in calli kept on different media, using several explants from *Medicago arborea* L.

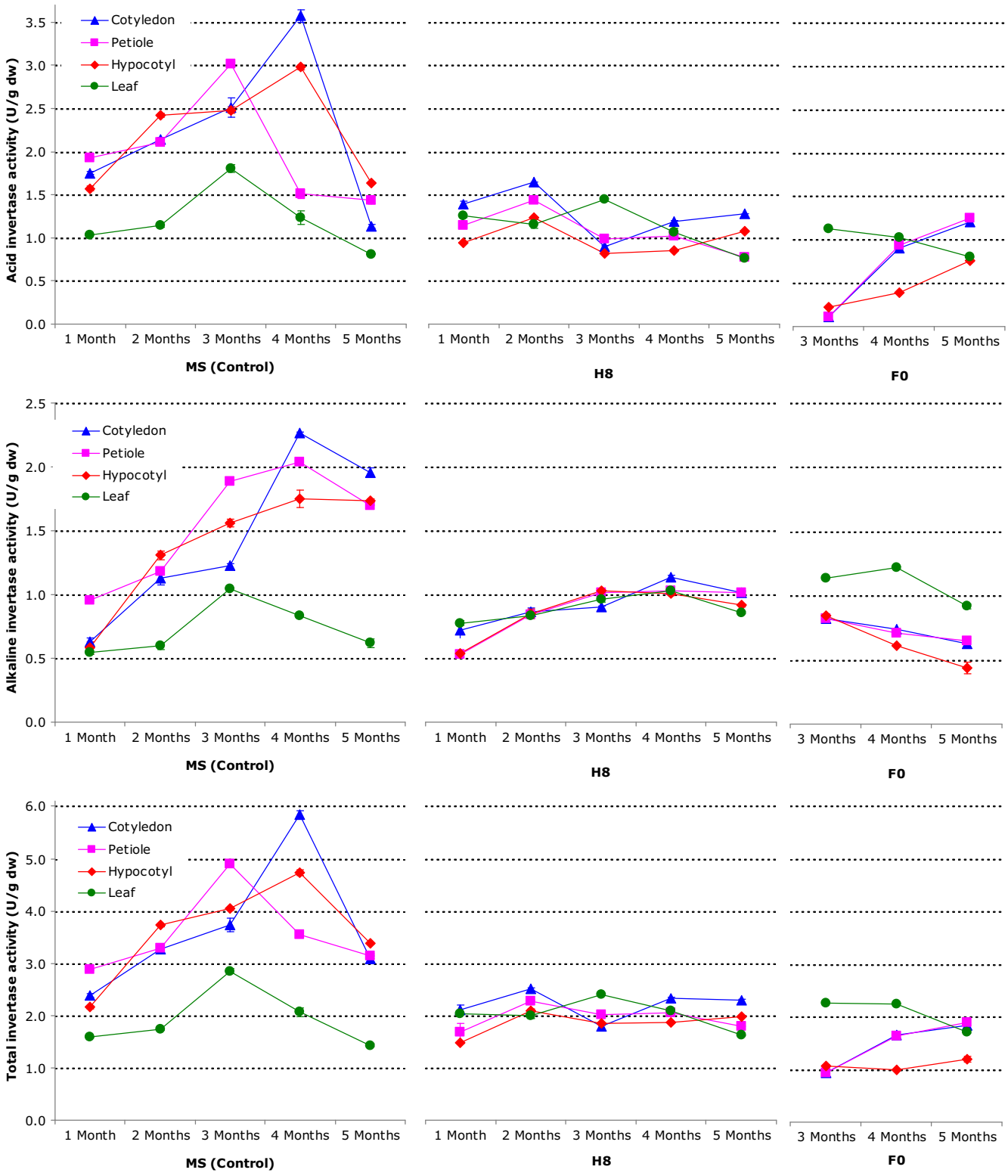

Fig. 15 Variation in invertase activity (acid, alkaline and total) in calli kept on different media, using several explants from *Medicago arborea* L.

polymorphic proteins involved in the degradation of sucrose (van den Ende and van Laere 1995; Tang *et al*. 1996; Koch 2004). They can be:

1) Soluble, located mainly in the cell cytoplasm;
2) Wall-bound and released at high salt concentrations, and
3) Extracellular. These remain in the cell wall after treatment with salt.

There are acid and alkaline invertases, defined on the basis of the pH required for their maximum activity. Both types of enzyme often appear in the same tissue (Kaur *et al*. 1992). In contrast to acid invertases, which are mainly found in immature tissues, alkaline invertase seems to be confined to mature storage tissues harbouring sucrose (van den Ende and van Laere 1995).

In the cytosol, sucrose hydrolysis is carried out by soluble, acid, and alkaline invertases (van de Ende and van Laere 1995) to provide the hexoses to be used as major substrates for the synthesis of structural and reserve polysaccharides (Venkataramana *et al*. 1991).

Considering *M. arborea*, total invertase activity was lower in the more embryogenic calli, such as those cultured in F0 medium (**Fig. 15**). The greatest part of this activity was due to soluble invertase. Wall-bound invertase showed maximum activity during the first 2 months of culture, thereafter decreasing. The highest activities were observed in non-embryogenic calli induced in MS medium.

In embryogenic calli, the activity of acid and alkaline invertase followed a similar trend that was different from

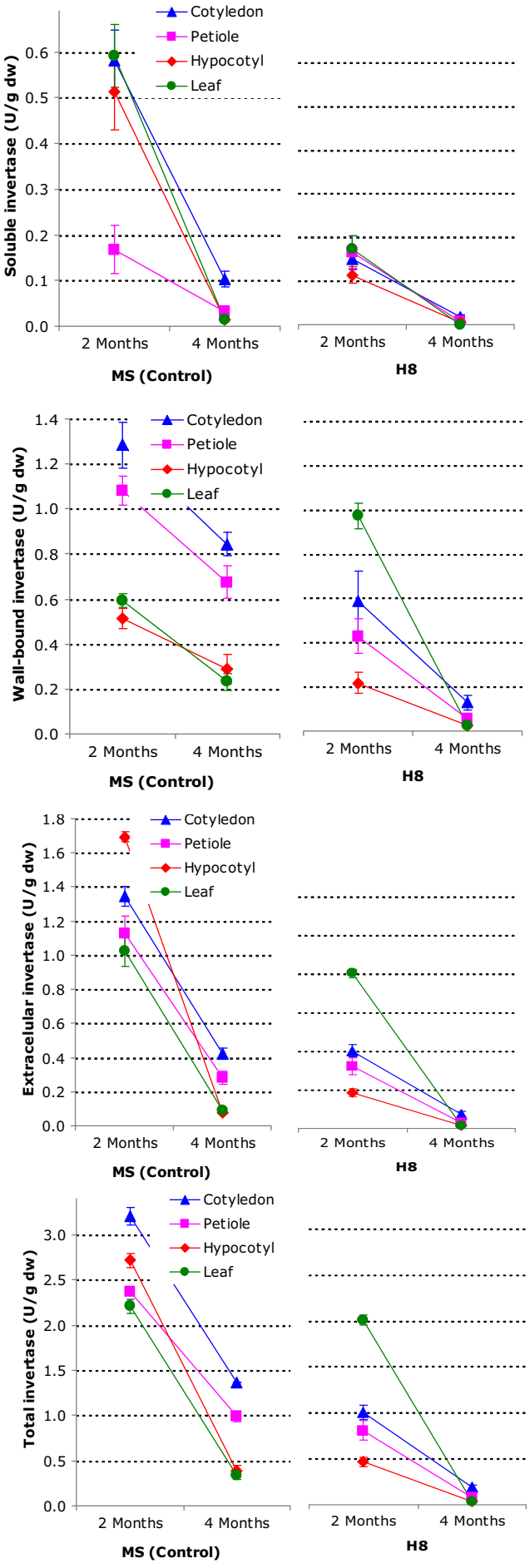

Fig. 16 Variation in alkaline invertase activity (soluble, wall-bound, extracellular and total) in calli kept on different media, using several explants from *Medicago strasseri*.

that observed in non-embryogenic calli: a lower percentage of acid invertase in the third month of culture (1st in F0 medium) and a later increase (**Fig. 15**). The percentage of alkaline invertase was higher in the third month or culture (1st in F0 medium) and thereafter decreased.

In the most embryogenic calli (**Fig. 15**), such as those obtained in F0 medium, lower levels of acid invertase were detected, together with higher alkaline invertase levels.

In the organogenic calli obtained with *M. strasseri*, study of alkaline invertase failed to give positive results since even though different buffers were used (25 mM Tris HCl pH = 8.0; 25 mM Tris HCl pH = 8.0 with 2% of Tri-ton X-100; 1 M sodium acetate buffer pH = 8.0) this type of activity was never detected in the calli. It is possible that in the culture time used in our studies alkaline invertase had still not been synthesised, since according to van den Ende and van Laere (1995) acid invertase is mainly located in imamture tissues, while neutral invertase is present in mature storage tissues. Some of the results concerning acid invertase in *M. strasseri* are shown in **Fig. 16**.

Regarding the overall set of data obtained on the assessment of total invertase (total acid invertase) in the calli, very high activity was observed in the non-organogenic calli from MS medium and lower activity in the organogenic calli from H8 medium. Most of the acid invertase activity detected was due to soluble invertase, followed by the wall-bound type.

ANALYSIS OF NITROGENOUS COMPOUNDS

The formation and maintenance of calli, in general, and of somatic embryos, in particular, require an active metabolism, reflected in the synthesis and degradation of different compounds.

The importance of carbohydrate metabolism in the development of somatic embryogenesis in *M. arborea* has been reported previously. Considering that nitrogen metabolism may also be crucial for unravelling the process of embryogenesis (Gutiérrez *et al*. 2006), we performed an analysis of total nitrogen and of total inorganic nitrogen components (ammonia, nitrate, nitrite) in embryogenic and non-embryogenic calli. The study was completed with an analysis of the activity of nitrate reductase, the enzyme responsible for the step from nitrate to nitrite. For this study we analyzed calli obtained in two culture media – H8 and F0 – from three explants of *M. arborea*: cotyledon, petiole and leaf.

Our investigations started with the analysis of total nitrogen (N) (**Fig. 17**) In the most embryogenic calli, induced from petioles, we detected the highest levels of nitrogen at the start of the culture. Nitrogen then decreased, which was not logical, taking into account that the source of nitrogen was not depleted because the culture medium was renewed monthly. We thus suspected that since the calli were already saturated it might be possible to elicit the exit of nitrogen from the medium. By taking samples of the culture medium during the fourth month of culture, when the greatest drop in nitrogen levels in the calli was observed, it was possible to confirm that in general nitrogen was released into the medium.

Another interesting observation was that the calli induced in F0 medium (more embryogenic) had lower nitrogen contents than those assessed after culture in H8.

Because nitrate is the form in which plants best take advantage of nitrogen, we analysed the content of nitrate in the calli, since according to Fernández *et al*. (1999) the induction of embryogenesis may be strongly related to the uptake of this compound. No clear differences were observed between the embryonic and non-embryonic calli upon evaluating nitrate levels (**Fig. 17**) and the same was the case of nitrite, ammonia (**Fig. 17**) and nitrate reductase activity.

When H8 medium was employed (**Fig. 17**), in the most embryogenic calli – obtained with petioles – the greatest reduction in nitrate was observed during the last two months of culture. This decrease may be due to the fact that nitrate

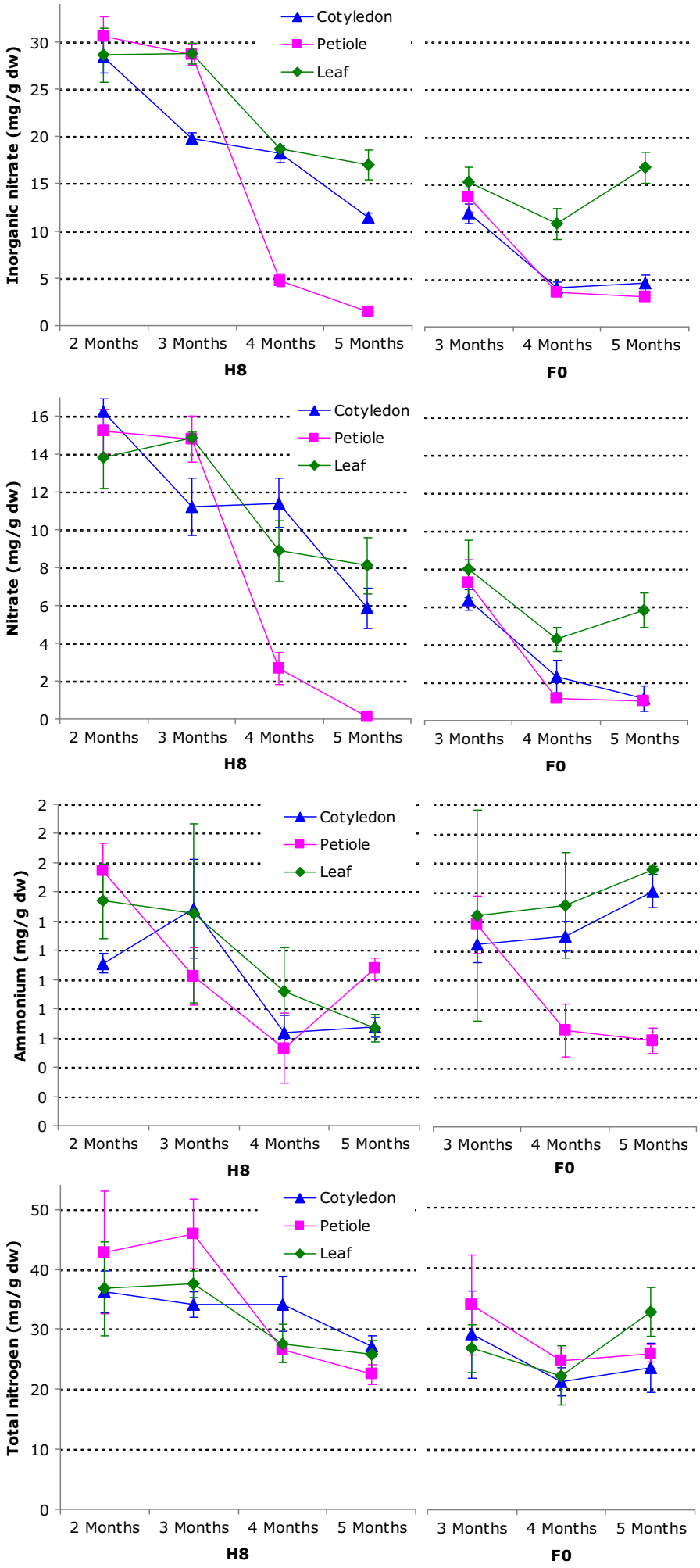

Fig. 17 Variation in total nitrogen, inorganic nitrogen, nitrate and ammonia in calli kept on different media, using several explants from *Medicago strasseri*.

metabolism is important for callus differentiation to occur, as pointed out by Cunha and Fernandes-Ferreira (1999).

With F0 medium (**Fig. 17**), we again observed a marked decline in nitrate contents as the culture time progressed. This decrease was more patent in the embryogenic calli obtained from cotyledons and petioles. It would thus appear that nitrate metabolism is more pronounced in the most embryogenic calli.

It was logical to surmise that the decreases in nitrate levels would coincide with increases in the content of nitrite. Nevertheless, these nitrogen compounds seem to be subject to rapid metabolism, thus preventing their accumulation up to toxic levels, as reported by Stohr and Mack (2001). In this case, we failed to observe any special type of behaviour that would differentiate the embryogenic calli, although the higher values observed in non-embryogenic calli were striking.

In fact, one important aspect in nitrogen metabolism during embryogenesis is the maintenance of suitable levels of ammonia in tissues in order to minimise the possible toxicity of this compound (**Fig. 17**). No differential characteristics between embryogenic and non-embryogenic calli were observed upon measuring ammonia levels. In the calli induced with F0 medium, an apparently active metabolism was only observed in the highly embryogenic calli obtained from petioles.

As reported above, of the inorganic nitrogen compounds analysed none exhibited any special kind of behaviour that might be characteristic of embryogenesis, despite the report by Montoro *et al*. (1995) stating that the induction of embryogenesis is characterised by an increase in nitrate uptake and a decrease in that of ammonia. Accordingly, if there are no differences in the behaviour of nitrate, nitrite and ammonia, it is possible that such differences would not be observed owing to the fact that these compounds are subject to a rapid conversion of nitrate to nitrite and from nitrite to ammonia, as reported by Mack and Schjoerring (2002), working with plants.

Regarding the total composition of inorganic nitrogen, differences were detected between embryogenic and non-embryogenic calli (**Fig. 17**). In the non-embryogenic calli (obtained from leaves in H8 and F0 media) high levels of inorganic nitrogen persisted. In the embryogenic calli, a decrease was observed in the content of this form of nitrogen, and an interesting observation was that in the calli obtained from petioles (the most embryogenic calli) a marked drop was observed between the 3rd and 4th months (the time at which the greatest embryo production was observed). It was also seen that in general the content in inorganic nitrogen in the calli obtained with F0 medium was lower than that of those induced with H8 medium. This supports the existence in embryogenic calli of a type of metabolism that may be directed towards the production of more nitrogen, apparently confirming the findings of Montoro (1995) in the sense of an increase in the protein synthesis as the development of somatic embryos progresses.

Nitrate reductase (NR) catalyses the key step in the assimilation of inorganic nitrogen. We thus believe that the activity of this enzyme could determine embryogenesis. From study of the activity of this enzyme, however, no striking observations would support this as characteristic of the induction and development of embryos.

Thus, even though nitrogen metabolism in calli may be an important factor in the collection of embryogenic responses there is much work to be done to fully elucidate which embryonic processes are specific to and determinant of embryogenesis.

CONCLUSIONS

The regeneration of *Medicago arborea* and *Medicago strasseri* plants has been achieved by somatic embryogenesis and organogenesis respectively, using young plants of these species as explants cultured in basal MS supplemented with 2 mg l^{-1} of 2,4-D and 2 mg l^{-1} of kinetin. The addition of proline to the medium increased the induction and germination of the somatic embryos but decreased seedling growth after the plants had been transferred to pots.

Different analyses have been performed to study possible markers of embryogenesis.

- An analysis and identification of endogenous cytokinins was made during the phase of induction and development of the somatic embryos. The amount of endogenous cytokinins was higher in the non-embryogenic calli. Study of the different cytokinins analysed allowed us to establish a relationship between the isopentyl derivatives (iP + iPa) and zeatin-like derivatives (Z+ DHZ + ZR + DHZR), this relationship being less marked in the embryogenic calli than in the non-embryogenic ones.
- A study was made of carbohydrates, nitrogenous compounds and enzymes involved in their metabolism, with the observation of clear differences between embryogenic and non-embryogenic calli.

The particular characteristics of these species, such as their ability to adapt to extreme conditions and their absence of a dormancy period, mean that they could be of special interest for the regeneration of marginal arid soils. They could also offer a viable alternative to Soya for the production of biomass and grain proteins in arid zones where *Medicago* species could be cultivated.

Our more immediate interest has focused on obtaining good forage plants. To do so, we shall initially study the genetic variability of different accessions of evergreen shrub species of the *Dendrotelis* section of the genus *Medicago* (*M. arborea*, *M. citrina* and *M. strasseri*). Additionally, we plan to carry out an analysis of the nutritional and anti-nutritional components of the accessions that show the greatest genetic diversity. Finally, the plants with the nutritional characteristics of greatest interest will be regenerated by *in vitro* culture.

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