TISSUE CULTURE OF *Trifolium polymorphum, T. carolinianum, Adesmia latifolia, A. bicolor* AND *Lotononis bainesii*

By

MARIA LAURA VIDOZ

A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2006

To my mother and to God, for their unconditional love.

ACKNOWLEDGMENTS

I would like to thank God for revealing His presence throughout my life, for His guidance during hard times and for His many blessings. I am thankful to my mother for her unconditional support and encouragement, for devoting so much time to my education, for giving me a deep love of nature, making it difficult to put into words how much she means to me.

I would like to thank my supervisory committee chair (Dr. Kenneth Quesenberry) for his advice and knowledge, and for introducing me to his wonderful family, all of which helped me professionally and personally. I am also grateful to the other members of my committee (Dr. Mimi Williams, Dr. Maria Gallo and Dr. David Wofford) for their critics and suggestions.

I thank Judy Dampier for her technical support, friendship and advice, making my stay in Gainesville a positive experience. I thank Loan Ngo for her technical assistance and sincere friendship. I also appreciate the technical support of Lindsay and Kailey Place.

I am thankful to Luis Mroginski and Dr. Hebe Rey, who introduced me to the tissue culture world and constantly encouraged me to grow in my career.

I would like to thank Lorena, Carlos, Gaby, Raquel, Jorge and Sonali for their company during these two years, and to all my friends in Argentina for their support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Thesis Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Master of Science

TISSUE CULTURE OF *Trifolium polymorphum, T. carolinianum, Adesmia latifolia, A. bicolor* AND *Lotononis bainesii*

By

Maria Laura Vidoz

December 2006

Chair: Kenneth H. Quesenberry
Cochair: Mary J. Williams
Major Department: Agronomy

Although they fix nitrogen, provide feed for livestock, improve soil properties and protect the soil from erosion, many forage legume species have been underutilized. Currently, breeding programs have an increased interest in several of the less-studied species that could improve pasture quality in subtropical regions of the world. *Trifolium polymorphum* Poir., *T. carolinianum* Michx., *Adesmia latifolia* (Spreng.) Vogel, *A. bicolor* (Poir.) DC. and *Lotononis bainesii* Baker are promising forages; however, they possess two major drawbacks: low seedling vigor and low dry matter production. The objective of this research was to develop *in vitro* plant regeneration protocols for these species that could assist breeding programs by potentially enabling the use of *in vitro* chromosome doubling techniques and genetic transformation.

Experiments using three types of basal medium (MS, L2 and B5) were conducted with *T. polymorphum* and *Adesmia* spp. In the latter, the effect of different plant growth regulators (TDZ, BAP and IBA) alone or in combination was evaluated. Cotyledon

culture on media supplemented with various plant growth regulators was assessed in *T. carolinianum*, *Adesmia spp*. and *L. bainesii*. Time required for shoot bud induction in *A. latifolia* was determined using immature leaflets as explants. Different leaf parts were evaluated for their morphogenetic potential in *A. latifolia* and *L. bainesii*.

In *T. polymorphum*, only callus formation was achieved using primarily B5 basal medium. With *T. carolinianum*, cotyledon culture on MS + 10 μM TDZ for 30 days with transfer to MS + 1 μM TDZ gave superior shoot bud organogenesis (20% of genotypes). Plant regeneration of *A. bicolor* was achieved through immature leaflet culture on MS or L2 + 4.5 μM TDZ for 60 days followed by transfer to MS devoid of plant growth regulators for 45 days. The highest frequency of plant regeneration in *A. latifolia* was obtained using immature rachises cultured on MS + 10 μM TDZ for 20 days and then transferred to MS without plant growth regulators for bud elongation and rooting. *L. bainesii* plant regeneration (in 90% of genotypes) was achieved by culturing immature leaflets on MS + 4.5 μM TDZ for 30 days and then transferring cultures to MS + 0.044 μM BAP + 0.049 μM IBA for bud elongation and rooting.

In conclusion, shoot organogenesis protocols were developed for four out of the five forage legume species evaluated, and plant regeneration was achieved with three of these species. Additional experiments to obtain rooting of *T. carolinianum* should be conducted. Studies to reduce the incidence of hyperhydricity in cultures of *Adesmia spp.* and to assess the influence of different plant growth regulators on shoot bud formation of *L. bainesii* are also needed.

CHAPTER 1
INTRODUCTION

In addition to providing feed for livestock, forages offer multiple benefits such as maintenance and improvement of soil characteristics, weed suppression, and protection of soil from erosion. When used as cover crops, forages may also assist in the recovery of degraded soils (Peters et al., 2003) by decreasing the loss of nutrients from leaching or erosion (Tilman et al., 2002). Forages that exhibit two or more of these characteristics are better accepted by farmers in agricultural systems (Peters et al., 2003).

The family Fabaceae comprises between 670 to 750 genera and 18,000 to 19,000 species, many of which constitute an important source of protein in human and animal diets, balancing the amino acids provided by cereals (Graham & Vance, 2003). According to Crews & Peoples (2004), legumes constitute a more environmentally friendly source of nitrogen than synthetic fertilizers due to their ability to fix nitrogen, reducing the risk of eutrophication and contamination of subterranean water.

However, numerous species of pasture legumes have thus far been underutilized (Graham & Vance, 2003), although many of them could be used to increase the genetic variability of their cultivated relatives or as a source of useful genes. For instance, legumes bred for drought and salinity tolerance would be useful in water-stressed areas of the world (Graham & Vance, 2003). In this scenario, forages that are naturally adapted to those conditions will be particularly valuable.

With approximately 240 species, the genus *Trifolium* L. is one of the most important genera in the Fabaceae family regarding the number of species it comprises

and their potential uses (Zohary & Heller, 1984). It is found in temperate to subtropical regions of Europe, the Americas, Asia and Africa (Lange & Schifino-Wittmann, 2000), but also occurs in the mountain and alpine zones in the tropics of West Africa and South America (Zohary & Heller, 1984). At least 25 species are useful as forages.

Trifolium polymorphum is endemic to subtropical South America and *T. carolinianum* is endemic to subtropical North America. Although common in native grasslands, neither species has been the subject of crop improvement research through plant breeding programs. *T. polymorphum* Poir. is a perennial subtropical species described in the United States, Argentina, Brazil, Chile, Paraguay, Peru and Uruguay (Quesenberry et al., 1997). It constitutes the only amphicarpic species in the genus and has been used in some cytogenetic and reproductive studies (Lange & Schifino-Wittmann, 2000). Agronomic evaluations conducted in New Zealand discovered one accession with 154 g kg^{-1} crude protein and 697 g kg^{-1} digestibility (Dodd & Orr, 1995). However, its marginal dry matter (DM) production and low seedling vigor is a major limitation for its use as a forage legume.

Trifolium carolinianum Michx. is an annual species native to the southeastern US. It produces seed and nodulates profusely, but like *T. polymorphum*, it has low seedling vigor and low DM production. Genetic improvement through traditional breeding and transformation can overcome these restrictions. Transformation, however, usually requires an efficient protocol for plant regeneration to be in place. Although *in vitro* regeneration protocols are available for several *Trifolium* species (Ding et al., 2003), there are none for *T. polymorphum* or *T. carolinianum*. Additionally, induction of polyploidy can also be used to increase seedling vigor and overall plant size, as was

shown previously in the genus *Trifolium* (Taylor et al., 1976; Furuya et al., 2001). Once again, the availability of an *in vitro* protocol would permit the use of additional techniques to duplicate chromosomes in *T. polymorphum* as has been demonstrated in bahiagrass (*Paspalum notatum* Flügge) using colchicine, trifluralin and oryzalin (Quesenberry et al., 2003). Moreover, the use of an *in vitro* protocol to induce chromosome duplication could prevent the recovery of chimeric plants that often are obtained when vegetative meristems are treated with chromosome doubling agents. In an *in vitro* system single cells are doubled and, consequently, plant regeneration from these single cells, as in the case of adventitious bud formation, renders a plant whose cells uniformly contain duplicated chromosomes.

Adesmia DC. (Fabaceae) is the only genus of the South American tribe Adesmieae (Benth.) Hutch and it comprises approximately 240 species of herbs and shrubs (Ulibarri & Burkart, 2000). Many *Adesmia* species, such as *A. bicolor* (Poir.) DC., *A. latifolia* (Spreng.) Vogel and *A. punctata* (Poir.) DC., constitute promising forage materials because of their satisfactory winter growth, high crude protein values and good *in vitro* organic matter digestibility. These species have been reported to be useful for soil cover and erosion control (Coelho & Battistin, 1998; Tedesco et al., 2000; Tedesco et al., 2001). In addition, *A. bicolor* showed tolerance to low phosphorous fertility and possesses valuable morphological characteristics when compared to other legumes (Dodd & Orr, 1995). In spite of these desirable characteristics, there are very few reports related to the biology of the genus (Coelho & Battistin, 1998; Dodd & Orr, 1995; Tedesco et al., 2000; Tedesco et al., 2001), and no reports regarding their use in tissue culture.

The genus *Lotononis* belongs to the tribe Crotalarieae and comprises approximately 150 species, from herbs to small shrubs (Jaftha et al., 2002). These species are distributed from Southern Africa to the Mediterranean region and India, and are found under a range of climates and geographical situations. *L. bainesii* Baker is a perennial herb whose forage value has been demonstrated in Australia (Jaftha et al., 2002). Although it has been previously reported to be a cleistogamous species, Real et al. (2004) have conducted molecular studies that suggest that it requires pollinators to set seed and some genotypes are self-incompatible. There is only one report on tissue culture of *L. bainessi*, where a low frequency of plant regeneration was obtained (Bovo et al., 1986).

Limited quantities of seeds may constitute a problem for release of new cultivars (Frame, 2004) but tissue culture may offer a solution, shortening the period of time for the availability of propagules. In addition, in cross pollinated species such as *L. bainesii*, *A. latifolia* and *A. bicolor*, each seed is potentially a different genotype. Therefore, it is not possible to propagate a plant exhibiting exceptional characteristics through seeds since the progeny may segregate for the trait of interest. However, micropropagation offers a solution for the mass propagation of an individual plant.

CHAPTER 2
LITERATURE REVIEW

All plant cells have the potential to be totitpotent, i.e., to be able to dedifferentiate, divide and regenerate into whole plants (Loidl, 2004). This was the idea that Gottlieb Haberlandt had in mind when he first attempted plant tissue culture in the early 20th century (Caponetti et al., 2004). Although he failed in his venture to regenerate plants from isolated tissues, his work attracted the attention of the scientific world and, consequently, abundant research was developed on the topic.

Tissue Culture Concepts as Applied to the Fabaceae Family

Tissue culture is usually defined as a heterogeneous group of techniques in which explants (protoplasts, cells, tissues or organs) are aseptically placed onto a culture medium of defined chemical composition, and incubated under controlled conditions (Mroginski et al., 2004b). There are three types of plant regeneration systems that are used most frequently: micropropagation, organogenesis and somatic embryogenesis. Micropropagation consists of the *in vitro* propagation of selected genotypes through improved axillary shoot production from explants with pre-existing meristems (Kane, 2004). In contrast, the other two regeneration schemes are based on the use of non-meristematic tissues as explants: organogenesis is the *de novo* formation of organs (shoots, roots or flowers), and somatic embryogenesis is the production of embryos without a previous fusion of gametes (Radice, 2004).

Members of the Fabaceae family have traditionally been regarded as recalcitrant to *in vitro* regeneration, particularly in the case of cultivated grain legumes (Griga, 1999;

Veltcheva et al., 2005; Mundhara & Rashid, 2006). Veltcheva et al. (2005) suggest that recalcitrance in grain legumes could be caused by the narrow genetic base of the cultivated varieties that have undergone inbreeding and selection for long periods of time. In addition, they suggest that in forage species the outbreeding and lower genotype selection may account for easier identification of responsive genotypes.

Some of the factors that affect *in vitro* response of a given species are genotype, explant, composition of the culture medium and conditions under which explants are incubated (Radice, 2004). The genotype of the donor plant is one of the most critical factors since it influences *in vitro* responses, from the establishment of the explant to the regeneration of whole plants, as well as *ex vitro,* during the acclimatization of regenerated plants. The importance of the genotype on *in vitro* plant regeneration of cultivated peanut (*Arachis hypogaea* L.) has been demonstrated by Chengalrayan et al. (1998), who assessed 16 genotypes for responsiveness *in vitro* using a protocol to induce somatic embryogenesis. These authors found differences in the frequency of response at each stage of the process and suggested that genotype could be the primary factor influencing conversion of somatic embryos to plantlets. A similar experiment was carried out in soybean (*Glycine max* (L.) Merrill), in which 17 breeding lines were evaluated for their response and ability to regenerate plants through somatic embryogenesis (Tomlin et al., 2002). Among these lines, a significant difference in the percentage of responsive explants, number and quality of somatic embryos were observed. Other legume species in which differences among genotypes were reported, particularly regarding somatic embryogenesis are *Medicago sativa* L. and *T. pratense* L. (Lakshmanan & Taji, 2000).

For *T. pratense*, Quesenberry and Smith (1993) increased genotype regeneration frequency from less than 5% to almost 70%, after five cycles of recurrent selection.

The explant type used for culture establishment depends on the objectives that are pursued since it determines responsiveness of the plant material *in vitro* (Lakshmanan & Taji, 2000). The aspects that should be considered in explant selection are: explant tissue (leaves, petals, anthers, roots, meristems, cotyledons, epicotyls, hypocotyls), explant size, explantation time, topophysis and polyphenol oxidation (Kane, 2004). It is widely accepted that immature zygotic embryos and young seedlings are the most responsive explants to induce somatic embryogenesis in legume species. This is because areas where cells show active division are more responsive to the embryogenic stimulus (Griga, 1999; Mundhara & Rashid, 2006). However, a range of explants have been used with success to induce somatic embryogenesis in Fabaceae family. These have included mature seeds, shoot apices, seedlings, hypocotyls, cotyledons, leaves, petioles, internodes, roots, endosperms, cell suspensions and protoplasts (Lakshmanan & Taji, 2000). For the induction of organogenesis in legume species, a similar variety of explants has been used. As an example, in *Arachis*, several explants have been capable of regenerating plants: fully expanded leaves (Dunbar & Pittman, 1992), leaflets from young seedlings (Akasaka et al., 2000), epicotyls, petioles (Cheng et al., 1992), cotyledons, embryo-axes, mature whole seeds (Radhakrishnan et al., 2000), protoplasts (Li et al., 1993), mature zygotic embryo-derived leaflets (Chengalrayan et al., 2001) and shoot apices (Radhakrishnan et al., 1999).

Culture medium composition is determined by the type and concentration of inorganic salts (macro- and micro-nutrients), organic compounds (sugar, vitamins,

activated charcoal, etc.), plant growth regulators (mainly auxins and cytokinins), gelling agents or other support system and the gaseous atmosphere inside the culture vessel (Radice, 2004). The most widely used basal medium for legume regeneration is MS medium developed by Murashige and Skoog (1962) for callus cultures of tobacco (Murashige & Skoog, 1962). This basal medium, which has a high salt concentration, has been used to achieve plant regeneration in several legume genera, such as *Arachis* (Rey & Mroginski, 2006), *Astragalus* (Luo et al., 1999), *Cajanus* (Singh et al., 2003), *Cassia* (Agrawal & Sardar, 2006), *Cicer* (Chakraborti et al., *Dalbergia* (Singh & Chand, 2003), *Glycine* (Tomlin et al., 2002), 2006), *Lathyrus* (Barik et al., 2005), *Lotus* (Akashi et at., 2003), *Phaseolus* (Delgado-Sanchez et al., 2006), *Pisum* (Loiseau et al., 1998), *Trifolium* (Ding et al., 2003) and *Vigna* (Saini & Jaiwal, 2002). However, other basal media have been specifically developed for certain legume species, such as *G. max* (Gamborg et al., 1968) and *T. pratense* (Collins & Phillips, 1982). Several plant growth regulators have been used with success in plant regeneration protocols for legume species, but the type of response and effectiveness of the compounds are highly dependent on the species and even on genotypes within a species. In general, auxins are used to induce somatic embryogenesis, whereas cytokinins are used to induce organogenesis. Nevertheless, there are some exceptions such as in *T. repens* L., *Medicago sativa* and *Phaseolus* spp., in which it was possible to achieve somatic embryogenesis by using cytokinins instead of auxins (Lakshmanan & Taji, 2000).

In addition to media composition factors, incubation conditions under which explants are incubated must be controlled. These include temperature, light quality and intensity, photoperiod, humidity and hygiene (Mroginski et al., 2004b). In general, the

temperature for incubation of cultures is between 23-29 ºC, depending on optimal growth requirement of the species (Radice, 2004). In some cases, when the process to be induced is somatic embryogenesis, cultures are incubated in the dark, since light is not required for this developmental pathway. In contrast, when organogenesis is to be induced, cultures are usually kept under light conditions with a specific photoperiod. In addition, light quality (spectral quality) and quantity (photon flux) are reported to have an important role in morphogenetic processes *in vitro* and on the subsequent growth of the regenerated structures (Lian et al., 2002).

Applications of Tissue Culture for the Fabaceae Family

Since its origin in the early 20th century, tissue culture procedures have been used for a variety of purposes, such as basic studies of particular physiological processes because the use of tissues instead of whole plants usually simplifies the study of the phenomenon (Mroginski et al., 2004b). Another use of tissue culture is the production of plants free from certain specific pathogens, generally viruses, through meristem or shoot tip culture alone or combined with thermo/chemotherapy. However, the most important application of these techniques from an economic point of view is related to micropropagation. This method is particularly important in horticulture, since it generally maintains genetic stability (Kane, 2004), and allows propagation of periclinal chimeras. This kind of chimera may be important in ornamental species and cannot be propagated through organogenesis or somatic embryogenesis. Tissue culture may also be used for the production of interspecific hybrids where zygotic embryos abort early in their development and have to be rescued, or in the case of plants with a rudimentary embryo (Mroginski et al., 2004b). The production of dihaploid, homozygous plants is also possible through anther or ovule culture, which reduces the time required to achieve

homozygosis in breeding programs. Other applications of tissue culture include the induction of somaclonal variation, production of secondary metabolites using cell culture, generation of somatic hybrids through protoplast fusion, and plant regeneration after transformation protocols. From the species preservation point of view, tissue culture constitutes a valuable technique for medium and long-term germplasm conservation (*in vitro* and cryoconservation), as well as plant material exchange since pathogen-free plants are used for this purpose (Mroginski et al., 2004b).

Production of Plants Free from Certain Specific Pathogens

Since plants were first domesticated, diseases and pests have threatened crop productivity. Some diseases caused by fungi and bacteria may be controlled if certain practices are used during the cultivation of the crop. However, in the case of viruses, the control is usually more difficult and in many cases the only indication of the presence of a virus is a reduction in crop yields. Viral diseases are transmitted rapidly particularly when the crop is vegetatively propagated (Kartha, 1984).

Meristem culture is one of the tools to eliminate viruses from plant material, provided that the rate of virus multiplication and movement in the plant is lower than the rate at which the meristematic region elongates. This is often the case since vascular tissues do not reach the meristem. In the Fabaceae family, meristem culture has been applied successfully to the rescue of interspecific hybrids between *A. hypogaea* and *A. stenosperma* Krapov & W.C. Gregory, and *A. hypogaea* and *A. otavioi,* which showed symptoms of peanut stripe virus (Radhakrishnan et al., 1999). Meristem culture with or without thermo/chemotherapy has also been used to eliminate peanut mottle virus, peanut stripe virus and tomato spotted wilt virus from interspecific hybrids of *Arachis* that were maintained vegetatively in the germplasm collection at the Southern Regional Plant

Introduction Station (Griffin, GA) (Dunbar et al., 1993a). In contrast, shoot tip culture was not an effective procedure to regenerate plants free of the peanut mottle viruses. Prasada Rao et al. (1995) excised seed axes from peanut stripe virus infected seed and cultured them on a medium containing ribavirin to obtain peanut plants free of the virus. Meristem culture has also been successfully applied to other legume genera, such as *Trifolium* and *Phaseolus*, for the production of plants free of common viruses (Phillips and Collins, 1979; Veltcheva et al., 2005).

Micropropagation

Micropropagation, the true-to-type propagation of a genotype through tissue culture techniques, is a useful tool in breeding programs. Among other advantages, it enables the production of uniform plants from a selected genotype at a high multiplication rate (Olmos et al., 2004). The stages for micropropagation from shoot explants are: a) donor plant selection and preparation, b) axillary shoot proliferation, c) pretransplant or rooting, and d) transfer to the natural environment (Kane, 2004).

Cultivated peanut has been reported to have limited reproductive efficiency, which is a drawback when large populations are required for breeding purposes (Radhakrishnan et al., 2000). Micropropagation may be used to overcome this situation, provided that an efficient *in vitro* protocol is available. For this species, Radhakrishnan et al. (2000) developed a high frequency micropropagation protocol from embryo axes and plant regeneration from other juvenile explants. Successful micropropagation protocols have also been developed for other species such as *Vigna mungo* (L.) Hepper, a grain legume important in South Asia and Australia, where plants were regenerated from shoot tips, embryo axes and cotyledonary nodes (Saini & Jaiwal, 2002).

Production of Dihaploid, Homozygous Plants

The production of haploid plants following anther, pollen or ovule culture is desired in breeding programs because it would result in a reduction in the number of cycles to achieve complete homozygosity after the duplication in the number of chromosomes of the regenerated haploid plants. Several attempts have been made in the Fabaceae family but with little success. For instance, in the genus *Arachis*, Bajaj et al. (1980) reported androgenesis but no plant regeneration from pollen cultures of *A. hypogaea* and *A. glabrata* Benth. Bajaj et al. (1981) reported plant regeneration from anther cultures of *A. hypogaea* and *A. villosa* Benth., with a chromosome number varying from haploid to octaploid. In soybean, Rodrigues et al. (2004) studied the origin of embryo-like structures from anther cultures using molecular techniques. They found both homozygous and heterozygous structures, suggesting that embryogenesis and androgenesis occurred simultaneously. Anther culture in *Phaseolus* resulted in callus formation with cell ploidy levels ranging from haploid to polyploidy (Veltcheva et al., 2005).

Generation of Interspecific Hybrids: Embryo Rescue and Protoplast Fusion

In many cases, wild species offer traits of interest that could be useful in breeding programs if incorporated into their cultivated relatives. Nevertheless, there may be interspecific barriers that need to be overcome in order to be able to transfer the trait of interest. One of the possibilities to circumvent this would be to use tissue culture techniques to rescue the embryo before it aborts, followed by micropropagation of the hybrid if it has low fertility.

Arachis villosulicarpa Hoehne, a wild relative of cultivated peanut (*A. hypogaea*), is rich in oil and is resistant to *Cercospora arachidicola* and *Cercosporidium personatum*

(Mansur et al., 1993). However, hybrids between this species and *A. hypogaea* could not be obtained due to cross compatibility barriers that caused abnormal endosperm development (Pittman et al., 1984). Somatic hybridization may be used to overcome this incompatibility, provided that an *in vitro* protocol is available. For this purpose, Mansur et al. (1993) developed a protocol for plant regeneration from cotyledons, leaves and cell suspensions of *A. villosulicarpa*.

In the genus *Trifolium*, a few interspecific hybrids have been produced with the aid of tissue culture techniques, such as hybrids between red clover (*T. pratense*) and *T. sarosiense* Hazsl. where aseptic embryo rescue was used before *in situ* abortion (Phillips et al., 1982). Przywara et at. (1996) regenerated hybrid plants from crosses between *T. repens* and *T. nigrescens* Viv. using *in vitro* pollination followed by embryo rescue. If hybrid embryos did not grow, they were transferred onto MS medium supplemented with growth regulators and achieved plant regeneration through organogenesis.

To apply somatic hybridization for the recovery of interspecific hybrids, at least one of the parents should be able to regenerate plants from protoplasts, but both should be able to undergo protoplast culture. In addition, it should be possible to select the somatic hybrids (Myers et al., 1989). There are reports on protoplast culture, which in most cases resulted in plant regeneration, in several species of the genus *Trifolium* such as *T. fragiferum* L. (Rybcznski, 1997), *T. pratense* (Myers et al., 1989; Radionenko et al., 1994), *T. repens* (Webb et al., 1987), *T. resupinatum* L. (Oelck et al., 1982) and *T. rubens* L. (Grosser & Collins, 1984). Plant regeneration from protoplasts has also been obtained in other legume genera such as *Astragalus* (Hou & Jia, 2004).

Other grain legume genera where embryo rescue techniques have been applied to obtain interspecific hybrids are *Cicer* and *Phaseolus*. In order to determine the phase when embryo rescue should occur in wide hybrids, Clarke et al. (2006) used selfed chickpea (*Cicer arietinum* L.) and selfed wild relatives to study the stage of embryo development at which abortion occurs. Several protocols have been developed to regenerate plants through embryo rescue after crosses between cultivated and wild *Phaseolus* species; however, no somatic hybridization has been reported for the genus (Veltcheva et al., 2005).

Plant Regeneration after Transformation Protocols

The application of molecular approaches to plant breeding usually requires an efficient *in vitro* system to regenerate plants from transformed single cells in order to obtain nonchimeric plants (Gill & Ozias-Akins, 1999). *In vitro* protocols amenable to molecular breeding have been developed for a wide range of legume genera: *Arachis* (Ozias-Akins & Gill, 2001; Vidoz et al., 2006; Rey & Mroginski, 2006); *Astragalus* (Luo et al., 1999), *Cajanus* (Singh et al., 2003), *Cassia* (Agrawal & Sardar, 2006), *Cicer* (Chakraborti et al., 2006); *Dalbergia* (Singh & Chand, 2003); *Glycine* (Tomlin, 2002), *Lathyrus* (Barik et al., 2005), *Lotus* (Lombari et al., 2003), *Macroptilium* (Ezura et al., 2000), *Phaseolus* (Delgado-Sanchez et al., 2006), *Trifolium* (Ding et al., 2003) and *Vigna* (Saini & Jaiwal, 2002). Some examples of legume species in which tissue culture has assisted transformation protocols are *A. hypogaea* (Ozias-Akins & Gill, 2001), *Lotus japonicus* (Regel) K. Larsen (Lombari et al., 2003), *G. max* (Olhoft et al., 2003), *T. repens*, *T. pratense* and *T. subterraneum* L. (Ding et al., 2003).

Medium and Long-term Germplasm Conservation and Plant Material Exchange

As civilization advances, the centers of diversity of many important plants for food and forage are threatened. This situation implies the loss of valuable genes contained in the wild relatives of the cultivated species that could be used in breeding programs. For this reason, germplasm is kept in storage facilities, mainly as seeds, which require considerable land and labor to be renewed. For many species belonging to the genus *Arachis*, seed viability decreases abruptly after 2-3 years of storage. However, some protocols have been developed to recover plants from seeds that would not germinate by themselves through the *in vitro* culture of embryonic axes (Dunbar et al., 1993b; Morris, et al., 1995).

Cryopreservation constitutes an alternative to the laborious and time consuming storage of seeds. Not only does it allow for long-term storage, but it also ensures genetic stability, requires little space and is low maintenance (Gagliardi et al., 2003). The ultra-low temperatures of liquid nitrogen cause interruption of all biochemical reactions protecting the plant material from physiological and genetic changes (Yamada et al., 1991). In addition, plants are kept free from pathogens when propagated from plants that have been indexed for the presence of specific microorganisms. Protocols for cryopreservation have been developed for several species: *A. burchellii* Krapov. & W.C. Greg., *A. hypogaea*, *A. retusa* Krapov. et al., (Gagliardi et al., 2003), *A. macedoi* Krapov. & W.C. Greg., *A. pietrarellii* Krapov. & W.C. Greg., *A. prostrata* Benth. *A. villosulicarpa* (Gagliardi et al., 2002) and *T. repens* (Yamada et al., 1991) among others. Medium-term conservation of germplasm can also be done by maintaining plants under *in vitro* conditions, which has similar advantages as cryopreservation: little space, low maintenance, and protection from pathogens. Moreover, plants kept *in vitro* are a ready

source of material in case the production of a large number of plants is required (Bhojwani, 1981).

Although there are many reports on tissue culture of legume species, there are no *in vitro* protocols for *T. polymorphum*, *T. carolinianum*, *A. bicolor* or *A. latifolia*, four promising forage species. For *L. bainesii* there is only one report of plant regeneration from cotyledons (Bovo et al., 1986). Therefore, the main objective of this research was to develop protocols for plant regeneration of these species that may then be used to improve their forage potential. Medium basal salts, plant growth regulators, explant type and time of exposure were the main factors evaluated.

CHAPTER 3
MATERIALS AND METHODS

Procedures common to all experiments are described here. The modifications made for specific experiments are described in corresponding chapters.

Plant Material

Experiments were carried out with four species representing three different genera of the Leguminosae family: *A. bicolor*, *A. latifolia*, *L. bainesii*, *T. carolinianum*, and *T. polymorphum* (Table 3-1). Seeds were scarified using concentrated sulphuric acid (98%) for 5 minutes (*Adesmia spp*., *L. bainesii*), 7 minutes (*T. polymorphum*) or 10 minutes (*T. carolinianum*) and then were rinsed for 10 minutes in running tap water. Subsequently, seeds were surface disinfected by immersion in a solution of sodium hypochlorite containing 0.571 % W/V available chlorine for 5 minutes and rinsed with distilled sterile water three times. Disinfected seeds were placed on half-strength Murashige and Skoog (1962) (MS) basal medium, with 15 g L^{-1} sucrose and 0.7 g L^{-1} agar (Sigma®[1] A-1296) in 100 mm diameter x 15 mm deep petri dishes.

Culture Conditions

Basal medium consisted of either MS, L2 (Collins & Phillips, 1982) or B5 (Gamborg et al., 1968). Media were prepared using MS basal salt mixture (Sigma M5524) and MS vitamins (Sigma® M7150) mixed in proportions corresponding to Murashige and Skoog (1962), B5 basal salt mixture (Sigma® G5768) and B5 vitamins

[1]Use of brand name is for identification purposes only and does not imply exclusion of other similar brand products.

(RPI® G37010) were used in quantities specified in Gamborg et al. (1968) or L2 concentrated stock solutions mixed in the adequate proportions according to Collins & Phillips (1982). Concentrated stock solutions of plant growth regulators were prepared by dissolving the correct quantity of the product {[TDZ= thidiazuron, 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (Sigma® P6186)], [BAP= benzylaminopurine (Sigma® B3408)], [KIN= kinetin, 6-furfurylaminopurine (Sigma® K0753)], [PIC= picloram, 4-amino-3,5,6-trichloropicolinic acid (Sigma® P5575)], [2,4-D= 2,4-dichlorophenoxyacetic acid (Sigma® D6679)], [IBA= indolebutyric acid (Sigma® I5386)] in distilled water and kept frozen at -14ºC.The pH of the media was adjusted to 5.8 with the addition of drops of 1 N KOH or 1N HCl before the addition of agar (Sigma® A-1296). Culture medium was sterilized by autoclaving for 20 minutes at 0.103 Mpa.

For the first steps of all experiments, 100 mm diameter x 15 mm deep petri dishes were used and explants were placed with the abaxial side down on 20 mL of culture medium. After the induction, cultures were transferred onto MS medium with or without the addition of 0.044 μM BAP + 0.049 μM IBA to achieve bud elongation and rooting of shoots. To induce further growth of shoots, cultures were subsequently transferred to magenta boxes (76.2 mm x 76.2 mm x 101.6 mm) (Magenta Corporation®) containing 50 mL of MS devoid of plant growth regulators. Cultures were kept in a growth chamber at 26 ± 2ºC with a 16-hour photoperiod and 85 μmol m^{-2} s^{-1} provided by cool fluorescent lights.

Regenerated plants were removed from magenta boxes and rinsed under running tap water to completely remove the culture medium and were then placed in plug trays containing vermiculite and covered with a humidity dome. Trays were placed in a growth

chamber at 22 ± 2ºC with a 14-hour photoperiod. Plants were watered daily to keep a high humidity level during the first two weeks and a solution containing 2 g/L of Captan® [4-cyclohexene-1,2-dicarboximide, N-(trichloromethyl) thio] was applied twice during this period. During the third week, covers were removed gradually for longer periods of time and plants were finally transferred to the greenhouse.

Evaluation and Experimental Design

Cultures were evaluated every 30 days for most experiments, in order to determine the number of explants that died, remained irresponsive, produced callus or buds. Whenever buds were regenerated, the number of buds per explant was also recorded. Each treatment was applied to 10 explants and experiments were repeated 2-3 times. Experiments were treated as completely random factorial experiments or completely randomized designs.

Table 3-1. Plant material used as a source of explants in the experiments.

Species	Accession No. *	Origin
A. bicolor	U5	Lavalleja (Uruguay)
	U6	Rocha (Uruguay)
	U7	Paysandu (Uruguay)
	U8	Tacuarembo (Uruguay)
	U10	Paso de los Toros/Tacuarembo (Uruguay)
	U11	Durazno (Uruguay)
	U12	Canelones (Uruguay)
	U13	Rocha (Uruguay)
	U14	Castillos/Rocha (Uruguay)
A. latifolia	U17	Valle Eden/Tacuarembo (Uruguay)
	U18	Mina de Corrales/Rivera (Uruguay)
	U19	Velásquez/Rocha (Uruguay)
T. polymorphum	U1	Estancia El Rincón/Florida (Uruguay)
	U2	Uruguay
	U3	Uruguay
	CPI 87102	
		Pilar (Paraguay)
		Rio Grande (Brazil)
		Uruguay
T. carolinianum	PI 516273	Gainesville, Florida, USA
L. bainesii	Cv INIA Glencoe	Uruguay

* U preceding a number is a locally assigned University of Florida number, CPI is Commonwealth Plant Introduction number from Australia and PI is USDA/NPGS Plant Introduction number

CHAPTER 4
TISSUE CULTURE OF *Trifolium polymorphum* AND *T. carolinianum*

Introduction

The genus *Trifolium* originated in the Mediterranean region, but subsequently spread to the Americas, Asia and Africa as well (Zohary & Heller, 1984). It consists of 8 sections and approximately 240 species, 25 of which are used as forage (Lange & Schifino-Wittman, 2000). *T. polymorphum* is a highly palatable forage legume native to eastern Argentina, Uruguay, Paraguay, central Chile and southern Brazil (Speroni & Izaguirre, 2003). It is an amphicarpic species that produces aerial and subterranean flowers on the same individual. It has been reported that the latter produce seeds more profusely, acting as a seed bank, whereas seed production from aerial flowers may be affected by grazing and insect attacks. The above-ground flowers have a morphology that seems to stimulate insect pollination; however, the species does not appear to exhibit self-incompatibility and self-pollination can occur, even before anthesis (Speroni & Izaguirre, 2003). Other workers report that the above-ground flowers are almost exclusively cross-pollinated (Daniel Real personal communication, 2006). *T. carolinianum* is an annual species and one of only three clovers native to the southeastern US. It is self-pollinated and re-seeds abundantly, but due to small plant size, has only limited forage potential.

Plant regeneration has been achieved in several species of the genus *Trifolium* including *T. repens*, *T.pratense*, *T. subterraneum*, *T. michelianum* Savi, *T. isthmocarpum* Brot. (Ding, et al., 2003), *T. nigrescens* (Konieczny, 1995), and *T. rubens* (Grosser & Collins, 1984) via either organogenesis or somatic embryogenesis. Many of these

protocols have used explants consisting of immature zygotic embryos (Maheswaran & Williams, 1984) or seedling derived explants such as hypocotyls and radicles (Heath et al., 1993), cotyledons (Konieczny, 1999), or leaves (Radionenko et al., 1994). However, these explants may not be appropriate for the propagation of selected genotypes in cross pollinated species in which seeds coming from the same plant may exhibit different genotypes. In this case, explants obtained from vegetative tissues in fully developed plants are more suitable for the propagation of selected individuals. *Trifolium* plant regeneration from non-meristematic tissues has been achieved using leaves (Rybcznski, 1997) and petioles (Quesenberry & Smith, 1993).

T. polymorphum and *T. carolinianum* exhibit low seedling vigor and low DM production that limit their forage use. Therefore, *in vitro* chromosome duplication may be used to increase plant vigor. In addition, chimeral plants could be avoided provided that plant regeneration is obtained from single cells that were previously doubled by treatment with chromosome doubling agents such as colchicine, trifluralin or oryzalin (Quesenberry et al., 2003). Consequently, the objective of these experiments was to develop a plant regeneration protocol that could be used for *in vitro* research to produce polyploid plants of *T. polymorphum* and *T. carolinianum* with increased seedling vigor and DM production.

Materials and methods

Trifolium polymorphum

Seeds from several accessions (Table 3-1) were germinated as described in chapter 3. Plants were maintained aseptically in magenta boxes containing MS medium devoid of growth regulators and were transferred approximately monthly. Petioles from immature fully expanded leaves were excised and cut on sterilized filter paper into pieces of 5 to 6-

mm length, which were used as explants. Five pieces were placed per Petri dish, onto one of the three basal media prepared as indicated in chapter 3 with the addition of 4.5 μM TDZ. The three treatments were applied to all germinated genotypes and experiments were repeated twice. Incubation conditions were the same as described in chapter 3 and data was recorded after 30 days of culture. The experiment was statistically analyzed as a factorial arrangement in a completely randomized design using PROC GLM from PC SAS (SAS Institute, 2003). Tukey's HSD Multiple Range Test at $p \leq 0.05$ level was used to compare the means of the basal media for each genotype.

Trifolium carolinianum

Seeds were scarified and germinated as indicated in chapter 3. Cotyledons from 45 1-week old seedlings were excised and cut longitudinally along the midrib into two pieces so that four cotyledon explants were obtained per genotype. Each explant was placed onto MS alone or MS with 10 μM TDZ, 10 μM BAP or 10 μM KIN. Five pieces were placed per petri dish and the identity of the genotypes was maintained. Seedlings without cotyledons were placed onto MS medium and kept *in vitro* for further experiments. After 30 days of culture, regenerated buds were transferred onto L2 medium supplemented with 1 μM TDZ. Incubation conditions were the same as described in chapter 3.

Results and Discussion

Trifolium polymorphum

After 30 days of culture, the only response obtained was the formation of friable, light brown callus that died after subculturing onto the fresh culture medium. The statistical analyses revealed an effect of basal media, genotypes and basal medium x

genotype interaction (Table 4-1). Basal medium B5 was significantly better than the others for the induction of callus formation in 9 of the 15 genotypes. In this experiment, TDZ was used as the growth regulator since it has a high cytokinin activity (Huetteman & Preece, 1993) and was reported to effectively induce organogenesis in a number of species of the genus *Trifolium* and *Medicago* (Ding et al., 2003). In addition, pieces of petioles were chosen as explants, since this would allow for the propagation and manipulation of selected genotypes. Petioles were successfully used as explants in *T. pratense* (Quesenberry & Smith, 1993) and *T. rubens* (McGee et al., 1989).

The failure in the regeneration of shoot buds could be due to the plant growth regulator used in the experiment, which may not be adequate to trigger the organogenic process or might have been present in a concentration toxic for this species. It is also possible that other auxins such as PIC or 2,4-D could be more effective. However, it would be necessary to evaluate a larger number of genotypes since there were significant differences among them.

Seedlings grew vigorously *in vitro* after slow germination, which started a week after the scarification and continued for over a month. Nevertheless, after approximately two months in culture, they started to decay producing few leaves even with frequent transfers to fresh medium. This factor limited the number of replications and experiments that could be carried out with this species. In order to determine if the presence of plant growth regulators in the germination medium promoted multiple shoot formation, seeds from different genotypes were scarified as in the first experiment and placed onto culture media containing either 10 μM TDZ or 1 μM BAP. Germination was not enhanced but multiple shoots were produced in some cases (data not shown). Nevertheless, the rate of

growth was very slow, and seedlings became chlorotic approximately a month after germination started.

Trifolium carolinianum

After 30 days of culture, it was observed that all explants placed on the basal medium devoid of plant growth regulators had died. Among the plant growth regulators that were evaluated in the experiment, KIN was not effective for callus induction or bud formation in any of the 45 genotypes and BAP only resulted in shoot bud formation in one genotype and callus formation in one other. TDZ effectively induced shoot bud organogenesis in 20% of the genotypes and callus formation in another 20% of the explants. Moreover, the mean number of buds was considerably low (2.1) suggesting that the species may be recalcitrant to tissue culture (Table 4-2). In all cases, calli were small, friable and light brown colored, but were already dead at the time the data was recorded.

Thirty days after transfer, the mean number of buds across genotypes increased markedly from 2.1 to 18.7 and buds began to elongate although growth was slow (Figure 4-1). To achieve bud elongation, cultures were transferred to L2 medium because it has a lower salt concentration than MS medium. In addition, the concentration of TDZ was reduced from 10 to 1 μM because it has been reported that TDZ may inhibit bud elongation (Huetteman & Preece, 1993).

Almost all responses were with TDZ, indicating that this plant growth regulator would be preferred for induction of organogenesis in *T. carolinianum.* In a previous report, TDZ proved effective for shoot bud induction in several *Trifolium* species (Ding et al., 2003). However, most of the reports on plant regeneration through organogenesis

in this genus mention the use of other plant growth regulators such as BAP (Heath et al., 1993; Konieczny, 1999) and 2-isopentenyladenine (Konieczny, 2000).

Trifolium carolinianum is an annual, self pollinated species, and therefore it has high homogeneity and homozygosis is expected within populations and plants. For this reason, in order to develop a tissue culture protocol, cotyledonary explants were used. Considering that seeds from a single plant and even a population should exhibit similar genotypes, this constitutes a suitable explant that is available in large quantities and can be easily disinfected and handled. This is not the case in cross pollinated species because the progeny of an individual plant may not reflect the trait of interest in the mother plant, which generally limits the use of seed-derived explants.

Interestingly, there were unexpected differences in response among genotypes in that explants from some genotypes were completely irresponsive while others produced up to 35 buds. This might indicate that the population that was used in the study is not highly homogeneous, probably due to the occurrence of some cross pollination. Another possibility could be that the difference in the responsiveness of the genotypes was caused by the treatment received by the plant material. For example, if some seeds had a thinner coat, the scarification process with sulphuric acid could have affected the cotyledons and their ability to regenerate *in vitro*. Another possible cause of variation could be size of the cotyledons among seedlings, which may explain the difference in the number of buds in the responsive genotypes.

Although it does not explain the variability observed in the experiment, it is possible that the unresponsive genotypes were more sensitive to the high concentration of salts in the culture medium. In this case, L2 basal medium should also be used for the

induction of organogenesis in this species. This would be in contrast to other work on *Trifolium* spp which showed that the use of MS as the basal medium allowed the development of an efficient protocol for *T. repens*, *T.pratense*, *T. subterraneum*, *T. michelianum* and *T. isthmocarpum* (Ding et al., 2003).

Conclusions

In *T. polymorphum*, a significant interaction between genotypes and basal media was observed for callus production. There were also significant differences among genotypes and culture media. However, the culture media tested to achieve plant regeneration through organogenesis were not effective and yielded only callus. Among the three basal media tested, a higher percentage of callus formation was observed with B5. The lack of shoot organogenesis may have been caused by the type or level of plant growth regulators, and therefore, other cytokinins or auxins should be tested. Moreover, additional experiments are required in order to determine the best culture conditions to maintain germplasm actively growing *in vitro*.

In contrast, *T. carolinianum* shoot bud formation was achieved in 20% of the genotypes when the culture medium contained 10 μM TDZ. This might suggest that in this species a potent cytokinin is required in order to induce this morphogenetic process. Callus formation was observed in another 20% of the genotypes, and the rest remained non-responsive and died. This difference was not expected considering that it is a self pollinated species, and populations are expected to be highly homogeneous. Shoot bud elongation occurred in MS supplemented with 1 μM TDZ but simultaneously, the number of adventitious buds continued to increase. Additional experiments are being

conducted in order to achieve rooting of the regenerated shoots and an adequate growth of the plantlets that would likely result in a higher acclimatization rate *ex vitro*.

Table 4-1. Mean percentage of explants producing callus from petiole pieces of *T. polymorphum* in three basal media after 30 days of culture.

	Basal medium		
Genotype	MS	L2	B5
CPI.1	0 [a*]	0 [a]	20 [a]
CPI.2	90 [b]	20 [a]	100 [b]
CPI.3	0 [a]	10 [a]	100 [b]
CPI.4	0 [a]	0 [a]	80 [b]
CPI.5	0 [a]	0 [a]	0 [a]
CPI.6	0 [a]	0 [a]	80 [b]
CPI.7	0 [a]	0 [a]	0 [a]
CPI.8	0 [a]	0 [a]	0 [a]
CPI.9	0 [a]	0 [a]	30 [b]
CPI.10	0 [a]	0 [a]	100 [b]
CPI.11	0 [a]	0 [a]	90 [b]
Urug.1	0 [a]	0 [a]	90 [b]
Parag.1	0 [a]	0 [a]	90 [b]
Parag.2	0 [a]	0 [a]	60 [b]
U 02.1	0 [a]	0 [a]	100 [b]

*Within rows, different letters indicate significant differences according to Tukey's HSD Multiple Range test at $p \leq 0.05$ level.

Table 4-2. Number of buds per explant in the responsive genotypes of *T. carolinianum* cultured on medium supplemented with 10 μM TDZ after 30 and 60 days of culture.

Genotype	30 days of culture	60 days of culture
8	4	35
9	1	8
10	3	21
11	2	14
23	2	22
24	3	28
32	1	5
41	1	13
43	2	22

Figure 4-1. Shoot bud organogenesis through cotyledon culture of *T. carolinianum*, 15 days after transfer to L2 + 1 µM TDZ (bar: 20 mm).

CHAPTER 5
PLANT REGENERATION OF *Adesmia latifolia* AND *A. bicolor*

Introduction

The tribe Adesmieae belongs to the Fabaceae family and consists of only one genus, *Adesmia* DC., and approximately 240 species endemic to South America (Ulibarri & Burkart, 2000). Most of these species of herbs and shrubs grow in the Andes mountains and semi desert zones in the Patagonia. Some species, such as *A. latifolia*, *A. bicolor* and *A. punctata* are considered promising forages because of their ability to grow during the winter season, high crude protein value and high *in vitro* organic matter digestibility (Tedesco et al., 2000). In addition, due to the stoloniferous morphology of plants, they may be used for soil cover and erosion control (Coelho & Battistin, 1998).

Adesmia bicolor and *A. latifolia* are cross pollinated species although some self pollination may occur (Tedesco et al., 2000). As a consequence, selected genotypes cannot be propagated through seeds because their progeny could segregate for the trait of interest. Even though these species could be multiplied vegetatively using stolons, tissue culture could provide a more rapid and efficient method of propagation. In addition, an *in vitro* protocol for plant regeneration from single cells, such as organogenesis and somatic embryogenesis, could be used to double the chromosome number in an attempt to increase plant productivity. Moreover, the development of a tissue culture system to propagate and maintain plants *in vitro* would prove useful for germplasm exchange (Mroginski et al., 2004b) since plants are protected from pathogens and do not require a quarantine period. Another contribution of tissue culture techniques to plant breeding is

the *in vitro* selection for biotic and abiotic stresses, which has been applied in alfalfa for resistance to specific fungal diseases (Dita et al., 2006).

It has been suggested that in forage legume populations there is greater heterogeneity in comparison to grain legume populations, due to the lower selection pressure of high seed producing genotypes (Veltcheva et al., 2005). This condition would result in an easier identification of *in vitro* responsive genotypes. However, the development of an efficient plant regeneration protocol implies the regulation of the many factors that influence *in vitro* responses and not only the genotype. These other factors include selection of an adequate explant, based on the objectives of the culture, composition of the basal medium and plant growth regulators used to induce the morphogenetic response, and conditions of incubation (Radice, 2004).

Tissue culture protocols have been developed for some forage legumes such as *T. pratense*, *T. repens* (Ding et al., 2003), *A. pintoi* Krapov. & W.C. Greg. (Rey & Mroginski, 2006), *A. glabrata* (Vidoz et al., 2006), *Lotus corniculatus* L. (Akashi et al, 2003), *Astragalus melilotoides* Pall. (Hou & Jia, 2004) and *Macroptilium atropurpureum* (DC.) Urb. (Ezura et al., 2000). Nevertheless, currently there are no reports on tissue culture in the genus *Adesmia*. The objective of these experiments was to develop for the first time, an *in vitro* plant regeneration system that could assist *A. bicolor* and *A. latifolia* breeding programs.

Materials and Methods

Plant Material

Seeds of *A. bicolor* and *A. latifolia* were scarified, sterilized and germinated as described in chapter 3. Plants were maintained aseptically in magenta boxes on MS medium without the addition of plant growth regulators. Unless indicated, explants

consisted of leaflets from immature leaves, at approximately 50% of expansion, that were excised from those *in vitro* grown plants.

Basal Media Experiment

Seven genotypes of *A. bicolor* and 16 of *A. latifolia* were used as explant sources. Leaflets were placed onto the three basal media indicated in chapter 3, with the addition of 4.5 μM TDZ. Each treatment was applied to 10 leaflets of each genotype and experiments were repeated three times. After 60 days of culture, buds were transferred to magenta boxes containing MS devoid of growth regulators for 45 days, where buds elongated and rooted. Incubation and acclimatization of regenerated plants was performed as described in chapter 3.

Response variables analyzed were: percent organogenesis (%= number of explants that produced buds/total number of explants*100), mean number of buds per explant (Σ number of buds per explant/ number of explants that produced buds) and regeneration index (index= % shoot bud formation * mean number of buds per explant / 100). This index was used in order to evaluate the influence of the basal medium on the percentage of shoot bud formation and mean number of buds per explant simultaneously. A higher index value indicates that a genotype is capable of producing a higher total number of buds. These response variables were also used in subsequent experiments.

This experiment was statistically analyzed as a factorial arrangement in a completely randomized design using PROC GLM from PC SAS (SAS Institute, 2003). Tukey's HSD Multiple Range Test at $p \leq 0.05$ was used to compare the means of the basal media for each genotype.

Factorial Experiments with TDZ and BAP

Among the responsive genotypes in the basal media experiment, *A. latifolia* U18.6 and U18.8 were chosen as explant sources for subsequent experiments, because their micropropagation rate (2.74 and 3.12 fold increase per month, respectively) provided adequate amounts of tissue. Explants were placed onto MS supplemented with 0, 1, 10, 30 or 60 μM TDZ alone or with the addition of either 0, 0.1 or 1 μM IBA, such that 15 treatments were used. These same concentrations and combinations were assayed replacing TDZ by BAP. Each treatment was applied to 10 leaflets of each genotype and experiments were repeated twice. After a month, cultures were transferred onto MS + 0.044 μM BAP + 0.049 μM IBA for 30 days to achieve bud elongation and then to magenta boxes containing MS without growth regulators for the same period of time. Incubation and acclimatization of regenerated plants was carried out as described in chapter 3.

Since both genotypes responded similarly, data were analyzed and presented as a combination of the two; however, results for each genotype are presented in the Appendix (Tables A-1 to A-9). Data from subsequent experiments were also pooled for genotypes. In addition to the variables mentioned above, shoot number, length of the longest shoot and acclimatization rate (% survival= number of plants that survived after a month / number of plants transferred to *ex vitro* conditions *100) were also determined. Statistical analyses were performed as a factorial arrangement in a completely randomized design using PROC GLM from PC SAS (SAS Institute, 2003). Tukey's HSD Multiple Range Test at $p \leq 0.05$ was used to compare the means of treatments. Regression analysis was performed using Microsoft Office Excel (Microsoft, 2003).

Induction Time Experiments

The same explants and sources from the previous experiment were used to evaluate the influence of exposure time to TDZ. Leaflets were placed onto MS + 10 μM TDZ for 0, 1, 4, 7, 14, 20 or 30 days and then transferred onto MS devoid of plant growth regulators. Each treatment was applied to 10 leaflets of each genotype and experiments were repeated twice. Forty-five days after the initiation of the experiment, all cultures were transferred onto fresh medium for 30 days, and then into magenta boxes containing the same culture medium. Incubation conditions were the same as described in chapter 3.

The experiment was analyzed as a completely randomized design using PROC GLM from PC SAS (SAS Institute, 2003). Tukey's HSD Multiple Range Test at $p \leq 0.05$ was used to compare treatment means. Regression analysis was performed using Microsoft Office Excel (Microsoft, 2003). Additional data for each genotype are presented in Appendix (Tables A-10 to A-12).

Type of Explant Experiment

The same two genotypes of *A. latifolia* were used as explant sources. Leaflets were excised and placed onto sterile filter paper where they were divided into petiole, rachis and leaflets. Petioles and rachises were subsequently divided into 5-6 mm pieces. All petiole and rachises pieces obtained from each leaf and eight leaflets were placed in each petri dish containing MS + 10 μM TDZ. This procedure was repeated 10 times for mature, fully expanded and immature, actively expanding leaves from both genotypes. Thirty days after the initiation of the experiment, explants were transferred onto MS medium without growth regulators to achieve bud elongation. The conditions under which cultures were incubated were as those indicated in chapter 3.

Statistical analysis was performed as a factorial arrangement in a completely randomized design using PROC GLM from PC SAS (SAS Institute, 2003). Tukey's HSD Multiple Range Test at $p \leq 0.05$ was used to compare the means of treatments. Additional data for each genotype separately is presented in Appendix (Table A-13).

Results and Discussion

Effect of Basal Medium on Shoot Organogenesis

After 60 days of culture, shoot bud formation was observed in 18 of the 23 genotypes evaluated (Table 5-1). Four of the non-responsive genotypes (U5.3, U5.5, U12.5 and U13.1) corresponded to *A. bicolor* and one (U19.3) to *A. latifolia*. Both genotype and basal medium had highly significant effects on the percentage of shoot bud formation. The interaction between these factors was also highly significant.

The basal medium MS resulted in a higher number of responsive genotypes and was superior to L2 in two of them. The average percentage of shoot bud formation across all genotypes was not significantly different between MS and L2 (22 and 16.6%, respectively); however, both were superior to B5 (5.5%). In general, higher percentages were obtained in those genotypes derived from accession U18 of *A. latifolia*, whereas those corresponding to *A. bicolor* were the least responsive.

For the variable mean number of buds per explant, only genotype was a significant source of variation. This was also evident when the average number of buds per explant across genotypes is considered. This may indicate that the basal medium may have had a major influence during the period in which explants dedifferentiated or acquired competence to respond to the plant growth regulator stimulus, but once induction had occurred, it had a low influence on the number of buds arising per explant. In all cases,

the mean number of shoot buds arising per explant was rather low and never higher than five, although up to 20 buds per individual explant were formed in some cases.

The regeneration index proved to be useful in this study involving numerous genotypes since in some cases a high number of buds per explant were produced, but a low percentage of explants was responsive. Even though the identification of highly responsive genotypes was not the main purpose of this experiment, this index proved useful for the selection of genotypes that could be used in further experiments to adjust the *in vitro* plant regeneration protocol. Averaging regeneration index data over genotypes, the means for MS and L2 were significantly higher than for B5. However, the index masked the effect of genotype x medium interaction observed on percentage, since the statistical analysis revealed only genotype and basal medium effects.

The results of this experiment suggested that MS medium produced higher proliferation of shoot buds in *A. latifolia* and *A. bicolor* than the other basal media. Although MS was originally developed for callus culture of tobacco (Murashige and Skoog, 1962) and B5 and L2 were developed for legume species (Gamborg et al., 1968; Collins & Phillips, 1982), MS basal medium has been successfully used for tissue culture of several legume genera including forages like *Trifolium* and *Medicago* (Ding et al., 2003), grain legumes like *Cicer* (Chakraborti et al., 2006) and *Vigna* (Saini & Jaiwal, 2002) and trees such as *Dalbergia* and *Cassia* (Singh & Chand, 2003; Agrawal & Sardar, 2006). Moreover, shoot bud induction was possible using TDZ as the plant growth regulator, which is in agreement with reports on other legume species as *A. hypogagea* (Akasaka et al, 2000), *A. correntina* (Burkart) Krapov. & W. C. Greg. (Mroginski et al.,

2004a), *Cajanus cajan* (L.) Millsp. (Singh et al., 2003) and *Vigna radiata* (L.) R. Wilczek (Mundhara & Rashid, 2006).

Regarding the acclimatization *ex vitro* of regenerated plants, the overall performance of plants obtained in MS treatment was markedly higher (72.4% survival *ex vitro*) than those of plants obtained in L2 (38.7%) or B5 (45.6%) (Figure 5-1 E). This higher survival rate of plants regenerated on MS may be due to reduced problems associated with hyperhydricity. One of the factors that lead to this condition characterized by morphological and physiological abnormalities is water availability (Hazarika, 2006). The higher salt concentration of MS basal medium may result in lower water availability for plant material, so that the incidence of hyperhydricity is lower in MS than in L2 or B5 medium. Low survival rates could also be due to other disorders common to plants exhibiting heterotrophic growth *in vitro*, such as a low photosynthetic rate, abnormalities in structure and functioning of stomata, and poor mesophyll and vascular system development (Hazarika, 2006).

Influence of TDZ on Organogenesis

After 15 days of culture, shoot buds were observed in all plant growth regulator combinations in both genotype 18.6 and 18.8 of *A. latifolia.* Similarly, TDZ has been successfully used to induce shoot formation in other legume genera; however, in some species such as *Vigna radiata* (Mundhara & Rashid, 2006), *A. hypogaea* (Gill & Ozias-Akins, 1999), *Trifolium* spp (Ding et al., 2003) and *C. cajan* (Singh et al., 2003), explants consisted of seedling-derived parts which are usually much more responsive than non-juvenile tissues.

In most of these *Adesmia* explants, callus formation was rare and, in some cases, buds seemed to arise almost directly from the surface of the explant. This is a desirable

characteristic since prolonged maintenance in tissue culture may result in somaclonal variation (Ozias-Akins & Gill, 2001). For the percentage of explants exhibiting bud organogenesis and mean number of buds per explant, there was a highly significant effect for TDZ but not for IBA concentration or its interaction with TDZ concentration. Since IBA effect was not significant, the average across IBA concentrations is presented. Only the treatment without TDZ was significantly differed from the rest for the percentage of shoot bud formation. A separate analysis was performed by excluding the 0 TDZ treatment in order to detect differences among TDZ concentrations and acquire a more accurate understanding of the response (Tables 5-2 and 5-3). Similarly a regression analysis was carried out without considering the 0 TDZ treatment and the simplest model that best explained culture responses was chosen in each case. When the 0 TDZ treatment was dropped, numerically the percentage adventitious bud formation increased with increasing TDZ concentration at 30 days of culture, but this increase was not significant (Table 5-2). By 60 days of culture, there was a significant quadratic effect of TDZ concentration (R^2=0.96) (Table 5-3 and Figure 5-2). Regardless of TDZ concentration the percentage of responsive explants increased from 30 to 60 days of culture which indicates that some explants required a longer period of time to become competent and differentiate buds (Appendix Tables A-1 and A-3).

The same statistical procedures and considerations described above were applied to the other response variables. Even when the 0 TDZ treatment was dropped from the analysis, TDZ concentration did not affect mean number of buds per explant at either 30 or 60 days (Tables 5-2 and 5-3). In some cases, the number of buds per explant of *A. latifolia* U18.8 actually decreased at 60 days of culture at higher concentrations of TDZ

(Appendix Table A-3) due to the death of some buds. These bud deaths were probably due to some phytotoxic effect of this potent plant growth regulator, as reported in peanut (Kanyand et al., 1994).

The same regeneration index as in the basal media experiment was applied here (Tables 5-2 and 5-3). This index showed a quadratic response with increasing TDZ concentrations at 30 days of culture (R^2= 1) (Figure 5-3.), but by 60 days of culture the effect of TDZ concentration had disappeared (Table 5-3). Shoot number (Table 5-3 and Figure 5-4) and shoot length (Table 5-3 and Figure 5-5) showed a linear decrease with increasing concentrations of TDZ (R^2= 0.87 and 0.79, respectively) (Figures 5-1 A-D). The reduction in shoot elongation is a common response reported in several species when TDZ is added to the culture medium (Huetteman & Preece, 1993). Shoot length is an important factor for the success in *ex vitro* acclimatization since longer shoots may have more reserves to produce roots which promote higher survival rates (Table 5-3). This was evident in both *A. latifolia* genotypes (Appendix Tables A-2, A-4 and A-5) where *ex vitro* survival decreased linearly with increasing concentrations of TDZ (R^2=0.88) (Figure 5-6). In addition, there was a higher incidence of hyperhydricity in plants regenerated in medium containing 30 and 60 µM TDZ, probably caused by a hormonal imbalance in the culture medium (Hazarika, 2006).

Influence of BAP on Organogenesis

For most variables considered in this study, the effect of BAP concentration was significant whereas the effect of IBA and the interaction between these factors were not. Consequently, the mean across IBA concentrations was obtained and values were treated as in the previous experiment regarding Tukey's test and regression analysis. After 30 days of culture, there was a quadratic effect of BAP concentration (R^2=0.24) (Figure 5-7)

although the percentage of explants that had produced buds was low and some explants remained non-responsive (Table 5-4). By 60 days of culture, the response to BAP concentration remained quadratic (R^2=0.57), but the percentage of adventitious bud formation increased in at all concentrations (Table 5-5 and Figure 5-9). This response is similar to what has been reported for *A. hypogaea,* where the addition of BAP to the culture medium required a longer period to induce shoot organogenesis than when was TDZ was added to the medium (Kanyand et al., 1994). Bud formation occurred in explants of most treatments at 60 days after initiation of cultures (Table 5-5 and Appendix Tables A-6 and A-8). Additionally, an increase in the percentage of bud formation occurred in those that had responded earlier. This pattern was also observed in the number of buds per explant. Similar to percentage of bud organogenesis, regeneration index response to increasing BAP concentration after 30 days of culture was quadratic (R^2=0.25) (Figure 5-8) being highest for explants placed onto 10 µM BAP. After 60 days of culture, the regeneration index response was still quadratic (R^2=0.48) (Figure 5-11), but the index values were significantly lower for explants cultured onto 1 µM BAP than for those placed onto 10-60 µM BAP (Tables 5-4 and 5-5).

For mean number of buds per explant, there were no differences among treatments after 30 days of culture, but after 60 days, the response to BAP concentrations was quadratic (R^2=0.56) (Tables 5-4 and 5-5, Figure 5-10). At 90 days of culture, shoot length also exhibited a quadratic response to BAP concentration (R^2=0.50) (Figure 5-12), reaching a maximum at 10-30 µM BAP and then decreased (Table 5-5, Appendix Tables A-7 and A-9). This reduction in length at high levels of the plant growth regulator may

have resulted from the induction of numerous shoot primordia that competed among each other and/or to the higher number of hyperhydric shoots.

The addition of BAP to basal medium has proved useful to induce organogenesis in some legume genera such as *Desmodium* (Rey & Mroginski, 1997), *Aeschynomene* (Rey & Mroginski, 1996) and *Trifolium* (Heath et al., 1993). Interestingly, BAP was less effective than TDZ for shoot bud induction in *A. latifolia*. This is in agreement with a report in *A. hypogaea*, in which several cytokinins were tested but TDZ proved to be the most efficient (Akasaka et al., 2000).

Induction Time for Adventitious Bud Formation

In order to determine the minimum time of exposure to TDZ required for shoot bud induction in *A. latifolia*, explants were maintained in a culture medium supplemented with this plant growth regulator for increasing periods of time. It was observed that one and four days of culture were enough to induce organogenesis in *A. latifolia* U18.8 and U18.6, although with a very low percentage of bud formation (Appendix Tables A-10 and A-11).

In general, the percentage of bud organogenesis increased as explants remained for longer periods of time in contact with TDZ. There were no significant differences between the 1- and 4-day treatments or among 7- to 30-day treatments (Table 5-6). An increase in the percentage of responsive explants was also observed from 30 to 60 days after the initiation of cultures. This suggests that, as in the case of four to seven days of exposure to TDZ, a period of over 30 days may be required for buds to arise even though cells have been induced to follow this developmental pathway. In most cases the mean number of buds per explant increased from 30 to 60 days after the initiation of cultures. Exceptions to this were the case when some buds became stunted and died. However,

there were no significant differences in mean bud number when explants were placed between on TDZ 4 and 30 days (Table 5-6). Regarding the regeneration index, there were significant differences between 1 or 4 days on TDZ and 7 to 30 days on the medium with this plant growth regulator after 30 days of culture. Thirty days later, the highest index value was observed for the longest treatment, but it was not significantly different from 7 to 20 days of culture on TDZ. Several studies showed that in order to regenerate normal shoots in *A. hypogaea* , explants should not remain in a medium supplemented with TDZ for more than 7 days at 45.4 µM TDZ or 21 days at 4.54 µM TDZ (Akasaka et al., 2000). In *A. latifolia* there were no significant differences in shoot length after culture of explants on TDZ for 1 to 30 days (data not shown). Regression analysis for all response variables showed a quadratic increase for length of time on TDZ supplemented medium (R^2 from 0.80 to 0.95) (Figures 5-13 to 5-15 and Appendix Figures A-1 to A-3).

Another experiment was conducted with shorter intervals between treatments to determine more precisely the number of days required for buds to arise. The percentage of shoot bud formation was highest for 10 days of culture on TDZ containing medium; however, it was not significantly different from percentages obtained after 5 to 9 days of culture on TDZ (Table 5-7). There were no differences among treatments regarding the mean number of buds per explant, and the regeneration index showed a significant difference only between 10 days of culture on TDZ and 1 or 2 days of exposure to this plant growth regulator but not with longer exposures (Table 5-7). In addition, the percentages of response were lower than in the first experiment (Appendix Table A-12). Regression analysis showed a linear increase of percentage of shoot bud formation and regeneration index with increasing exposure to TDZ (R^2= 0.92 and 0.93, respectively)

(Figures 5-16 and 5-17), but this is probably because treatments corresponded to the first phase of the quadratic model observed in the previous experiment.

Effect of Explant Type on Shoot Organogenesis

Results from an exploratory experiment with several genotypes of *A. latifolia* revealed that leaflets were more responsive than petioles, suggesting that the part of the leaf used as explant had a major influence on the *in vitro* responsiveness. The percentage of bud formation was significantly higher when rachises were used as explants (Table 5-8). Leaflet explants produced a significantly higher percentage of organogenesis than petioles, which in general remained non-responsive. In the best case, only 16% organogenesis was achieved in contrast to the 90% responsiveness that was obtained using rachises from immature leaflets (Appendix Table A-13). These response patterns were observed in both immature and mature leaves. Mean number of buds per explant and bud formation index also showed the higher responsiveness of rachises over the other two types of explants. For these response variables, there were no significant differences between leaflet and petiole explants (Table 5-8).

The higher frequency of shoot bud formation from rachises may be due to the presence of intercalary meristems in the rachises because they are the last segment of the leaf to undergo the maturation process. Nevertheless, this does not explain that this explant, when excised from mature leaves, gave almost as high responses as when harvested from actively expanding ones. The only disadvantage of using rachises would be the lower amount of plant material in cases in which few or small plants are available, since each leaf provides between 10 to 20 leaflets, but each rachis from immature leaves gives no more than two explants.

Regarding explant age, there were no significant differences between immature and mature leaves although explants from expanding leaves were more responsive than those from fully expanded, mature ones. This may be due to the meristematic activity of young leaves with less differentiated cells that could readily respond to an external stimulus, i.e. TDZ. In *A. hypogaea*, leaf developmental stage is a primary factor in somatic embryo induction, as unfolded leaflets usually lose embryogenic potential (Baker & Wetzstein, 1998). Nevertheless, shoot bud formation was possible in a number of wild *Arachis* species (Dunbar & Pittman, 1992). In contrast to what might be expected, cotyledons of *A. bicolor* and *A. latifolia* were completely irresponsive *in vitro* when cultured onto PIC, 2,4-D or TDZ (data not shown).

Conclusions

It was possible to achieve plant regeneration from *A. latifolia* and *A. bicolor* through immature leaflet culture. Shoot bud organogenesis was successfully induced in several genotypes of both species using MS, L2 or B5 as the basal medium. Even though there were no marked differences in bud formation frequency between MS and L2, the former one promoted a higher *ex vitro* survival of regenerated plants and therefore was used in subsequent experiments. Interestingly, *A. bicolor* proved to be much less responsive than *A. latifolia* and all regenerated plants failed to acclimatize *ex vitro.*

The plant growth regulator TDZ was more efficient than BAP for shoot bud induction, not only in the frequency of bud formation, but also in the time required for buds to arise from explants. In general, the percentage of organogenesis increased with higher levels of TDZ, but this was associated with a reduction in bud elongation. Shoot length was a critical factor in *ex vitro* acclimatization of regenerated plants and as a consequence, plants originated in TDZ concentrations of 1 and 10 μM showed higher

survival rates. Despite the lower organogenesis frequency of explants cultured on BAP, this plant growth regulator did not affect shoot length as drastically as TDZ, and in general the most effective concentrations were 10 and 30 μM BAP.

Shoot bud organogenesis was observed after exposures to TDZ as short as one day, although the percentage of bud formation increased with prolonged culture in a medium containing this plant growth regulator up to 10 days. Exposure for 30 days to 10 μM TDZ allowed a higher frequency of organogenesis and did not show a negative effect in shoot elongation, which was observed in the previous experiment at higher levels of the plant growth regulator.

A major influence of explant type on shoot bud formation of *A. latifolia* was revealed in a follow-up experiment. A higher frequency of shoot bud formation and mean number of buds per explant was obtained when rachises were used as explants. The age of the source leaf was not as important as the part of the leaf placed into culture. Nevertheless, an increase in bud formation was achieved when using explants from immature, expanding leaves instead of mature, fully expanded ones.

A suggested protocol for *A. latifolia* plant regeneration is as follows:

1. Use immature rachises as explant source (immature leaflets may be used with some reduction in efficiency if large amounts of explant tissue are desired)

2. Culture on MS + 10 μM TDZ for 20 days

3. Transfer to MS with no plant growth regulators for bud elongation and rooting

Table 5-1. Effect of three basal media on percentage of adventitious bud formation (%), mean number of buds (No.) and regeneration index (Index†) in *A. bicolor* (U5.2-U13.3) and *A. latifolia* (U17.1-U19.6) after 60 days of culture.

Genotype	Basal Medium								
	MS			L2			B5		
	%	No.	Index†	%	No.	Index	%	No.	Index
U5.2	3.3 [a*]	1.7	0.2 [a]	3.0 [a]	1.0	0.1 [a]	0 [a]	0	0 [a]
U5.3	0 [a]	0	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U5.5	0 [a]	0	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U12.2	0 [a]	0	0 [a]	3.3 [a]	0.7	0.1 [a]	0 [a]	0	0 [a]
U12.5	0 [a]	0	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U13.1	0 [a]	0	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U13.3	6.7 [a]	0.7	0.1 [a]	3.3 [a]	0.3	0 [a]	3.3 [a]	0.3	0 [a]
U17.1	50.0 [b]	1.9	1.1 [a]	6.7 [a]	1.7	0.2 [a]	3.3 [a]	0.3	0 [a]
U17.2	53.3 [b]	3.7	1.9 [a]	26.7 [ab]	2.8	1.1 [a]	16.7 [a]	1.1	0.3 [a]
U17.3	3.3 [a]	0.3	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U18.1	63.8 [ab]	3.6	1.8[ab]	80.0 [b]	3.4	2.9 [b]	36.7 [a]	2.9	1.0 [a]
U18.2	78.6 [b]	2.3	1.9 [b]	37.2 [a]	3.7	1.5[ab]	13.3 [a]	0.8	0.2 [a]
U18.3	17.4 [a]	2.2	0.4 [a]	24.4 [a]	1.9	0.5 [a]	3.3 [a]	1	0.1 [a]
U18.4	23.3 [a]	0.7	0.5 [a]	16.7 [a]	1.1	0.6 [a]	0 [a]	0	0 [a]
U18.6	56.7 [b]	1.6	0.9 [a]	46.7	1.2	0.8 [a]	6.7 [a]	0.3	0.1 [a]
U18.8	46.7 [b]	2.6	1.1[ab]	80.0 [b]	3.9	2.6 [b]	13.3 [a]	4.1	0.8 [a]
U18.9	53.3 [b]	2.1	1.3 [a]	23.3 [ab]	1.6	0.4 [a]	16.4 [a]	0.7	0.2 [a]
U19.1	3.3 [a]	0.3	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U19.2	3.3 [a]	0.3	0 [a]	3.3 [a]	1.7	0.2 [a]	0 [a]	0	0 [a]
U19.3	0 [a]	0	0 [a]	0 [a]	0	0 [a]	0 [a]	0	0 [a]
U19.4	5.6 [a]	0.5	0.1 [a]	4.2 [a]	0.3	0 [a]	0 [a]	0	0 [a]
U19.5	13.3 [a]	0.6	0.2 [a]	0 [a]	0	0 [a]	3.3 [a]	0.3	0 [a]
U19.6	23.3 [a]	3.9	2.7 [b]	23.3 [a]	1.8	1.2[ab]	9.1 [a]	1.6	0.4 [a]
Mean	22.0 [B]	1.3[A]	0.6[B]	16.6[B]	1.2[A]	0.5[B]	5.5[A]	0.6[A]	0.1[A]

* Within rows, means for a given variable followed by different lower case letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05 level. Means over genotypes followed by different upper case letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05 level. † Index= % shoot bud formation x mean number of buds / 100

Table 5-2. Effect of TDZ concentration on percentage of adventitious bud formation (ABF), mean number of buds per explant, and regeneration index (Index†) in *A. latifolia* after 30 days of culture.

TDZ (µM)	ABF	Buds	Index†
	---%---	---No.---	
0	0	0	0
1	52.5[a]*	1.7[a]	0.9[a]
10	57.5[a]	1.9[a]	1.1[a]
30	67.5[a]	2.0[a]	1.4[ab]
60	71.7[a]	2.3[a]	1.6[b]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. †Index= % shoot bud formation x mean number of buds / 100

Table 5-3. Effect of TDZ concentration on percentage of adventitious bud formation (ABF), mean number of buds per explant, and regeneration index (Index†) after 60 days of culture; number of shoots and shoot length after 90 days of culture; and percentage of *ex vitro* survival in *A. latifolia*.

TDZ (µM)	ABF	Buds	Index†	Shoots	Length	Survival
	--%--	---No.---		---No.---	---mm---	-----%-----
0	0	0	0	0	0	0
1	65.8[a*]	2.7[a]	1.7[a]	10.4[c]	31.3[c]	76.5[b]
10	79.2[ab]	2.8[a]	2.3[a]	7.8[bc]	18.2[b]	60.3[b]
30	87.5[b]	2.5[a]	2.2[a]	4.5[ab]	5.3[ab]	15.0[a]
60	85.8[b]	2.7[a]	2.4[a]	3.3[a]	2.6[a]	4.2[a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table 5-4. Effect of BAP concentration on percentage of adventitious bud formation (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* after 30 days of culture.

BAP (μM)	ABF	Buds	Index†
	---%---	---No.---	
0	0	0	0
1	2.5[a*]	0.3 [a]	0 [a]
10	18.3[b]	0.8 [a]	0.2 [b]
30	1.7[a]	0.2 [a]	0 [a]
60	0.8[a]	0.1 [a]	0 [a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table 5-5. Effect of BAP concentration on percentage of adventitious bud formation (ABF), mean number of buds per explant and regeneration index (Index†) after 60 days of culture; and shoot length after 90 days of culture in *A. latifolia*.

BAP (μM)	ABF	Buds	Index†	Length
	--%--	---No.---		---mm---
0	0	0	0	0
1	13.3[a*]	1.4 [a]	0.2 [a]	15.0 [a]
10	65.8 [b]	3.2 [b]	2.2 [b]	41.7 [b]
30	57.5 [b]	2.9 [b]	1.7 [b]	30.0 [ab]
60	50.0 [b]	2.9 [b]	1.6 [b]	17.5 [a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table 5-6. Effect of different times of exposure to TDZ on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* after 30 and 60 days of culture, respectively.

	30 days of culture			60 days of culture		
Days in TDZ	ABF	Buds	Index†	ABF	Buds	Index
	---%---	---No.---		---%---	---No.---	
0	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]
1	2.5 [a]	0.3 [a]	0 [a]	2.5 [a]	0.3 [ab]	0 [a]
4	12.5 [a]	0.8 [ab]	0.2 [a]	30.0 [a]	2.1 [bc]	0.6 [ab]
7	57.5 [b]	2.0 [bc]	1.2 [b]	77.5 [b]	2.0 [bc]	1.5 [abc]
14	70.0 [b]	1.7 [bc]	1.2 [b]	77.5 [b]	2.5 [c]	2.0 [bc]
20	72.5 [b]	2.6 [c]	1.9 [b]	87.5 [b]	2.3 [c]	2.0 [bc]
30	80.0 [b]	1.8 [bc]	1.4 [b]	90.0 [b]	2.8 [c]	2.5 [c]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table 5-7. Effect of short exposure to TDZ on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* after 30 days of culture.

Days in TDZ	ABF	Buds	Index†
	---%---	---No.---	
0	0 [a]	0 [a]	0 [a]
1	0 [a]	0 [a]	0 [a]
2	2.5 [a]	0.3 [a]	0 [a]
3	5.0 [a]	0.5 [a]	0.1 [ab]
4	5.0 [a]	1.0 [a]	0.1 [ab]
5	7.5 [ab]	0.8 [a]	0.1 [ab]
6	12.5 [ab]	0.8 [a]	0.2 [ab]
7	17.5 [ab]	1.1 [a]	0.2 [ab]
8	20.0 [ab]	1.4 [a]	0.3 [ab]
9	27.5 [ab]	1.4 [a]	0.4 [ab]
10	35.0 [b]	0.9 [a]	0.4 [b]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table 5-8. Effect of explant type on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* after 30 days of culture.

		Type of explants	Immature leaves	Mature leaves	Mean
ABF	---%---	Petioles	9.1	8.0	8.6 [a]
		Rachis	80.0	65.0	72.5 [c]
		Leaflets	38.1	20.6	29.4 [b]
Buds	--No.--	Petioles	0.8	0.4	0.6 [a]
		Rachis	2.3	2.7	2.5 [b]
		Leaflets	1.2	1.0	1.1 [a]
Index†		Petioles	0.2	0.1	0.15 [a]
		Rachis	2.3	2.7	2.5 [b]
		Leaflets	0.6	0.3	0.5 [a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.05$. † Index= % shoot bud formation x mean number of buds / 100

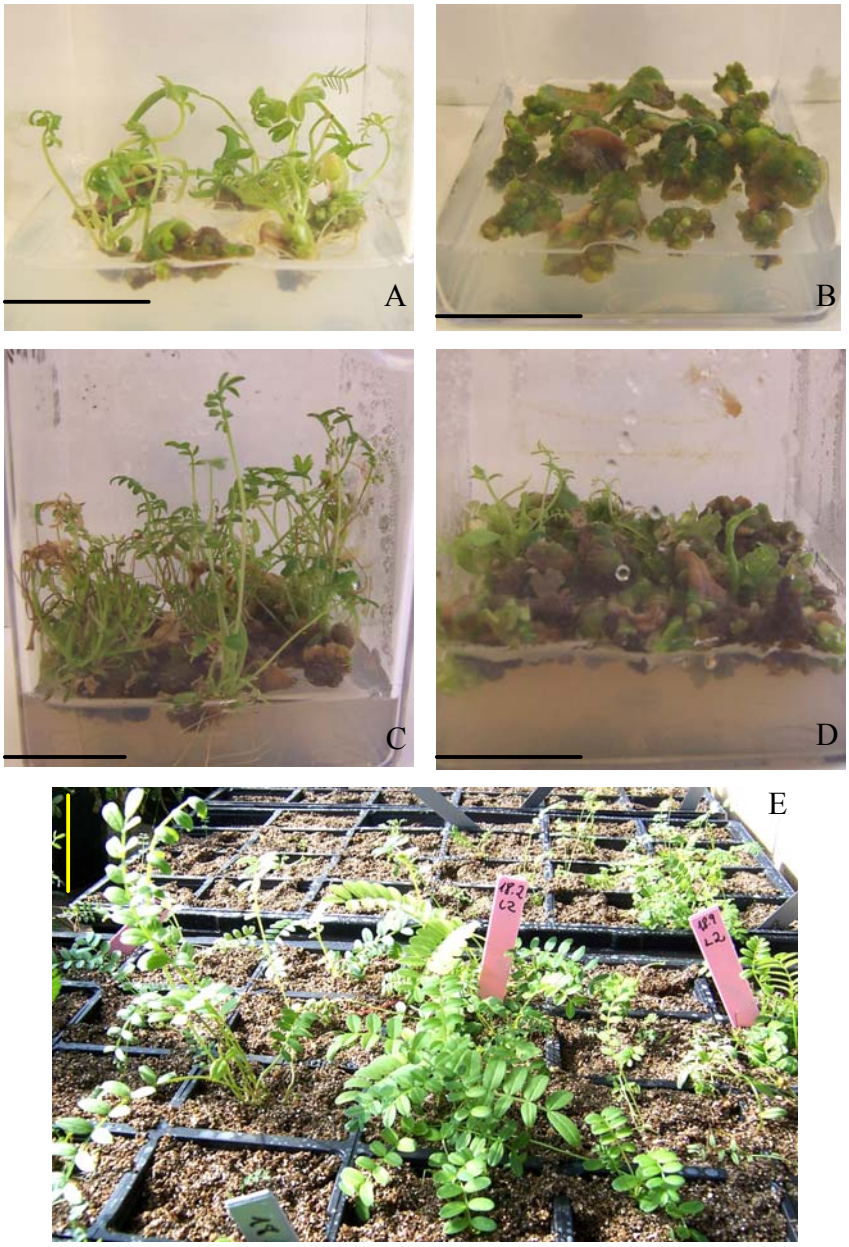

Figure 5-1. Organogenesis in *A. latifolia*. A), B) Shoot bud formation 60 days after initiation of cultures and C), D) shoot elongation 90 days after initiation of cultures; A), C) in MS + 1 µM TDZ and B), D) in MS + 60 µM TDZ (bar: 20 mm). E) Successful acclimatization of regenerated plants, 45 days after transfer to *ex vitro* conditions (Bar: 50 mm).

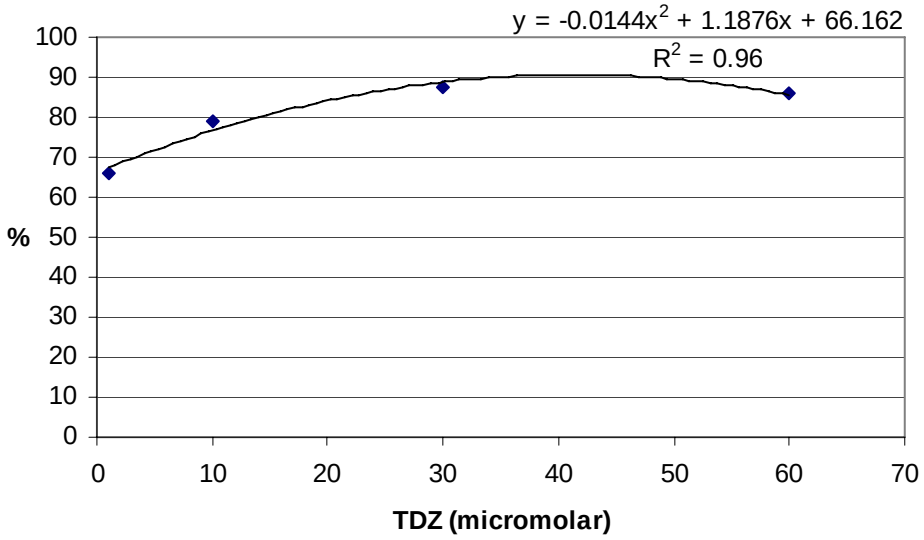

Figure 5-2. Regression curve showing the effect of TDZ concentration on percentage of adventitious bud formation (%) in *A. latifolia* after 60 days of culture.

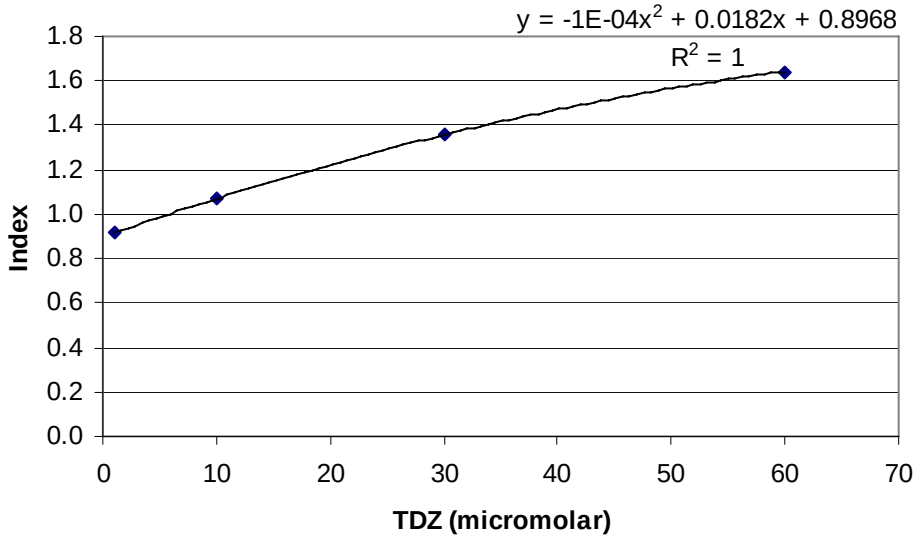

Figure 5-3. Regression curve showing the effect of TDZ concentration on regeneration index (Index) in *A. latifolia* after 30 days of culture.

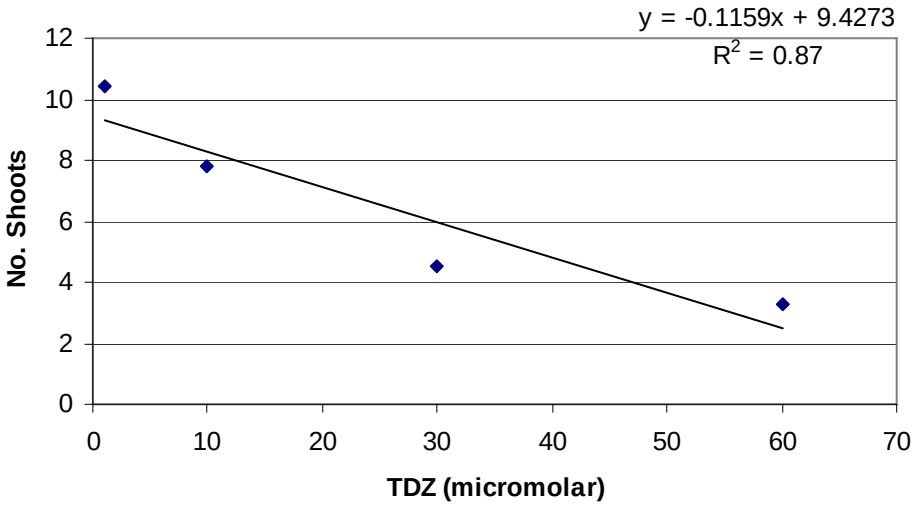

Figure 5-4. Regression curve showing the effect of TDZ concentration on shoot number per explant (No. shoots) in *A. latifolia* after 90 days of culture.

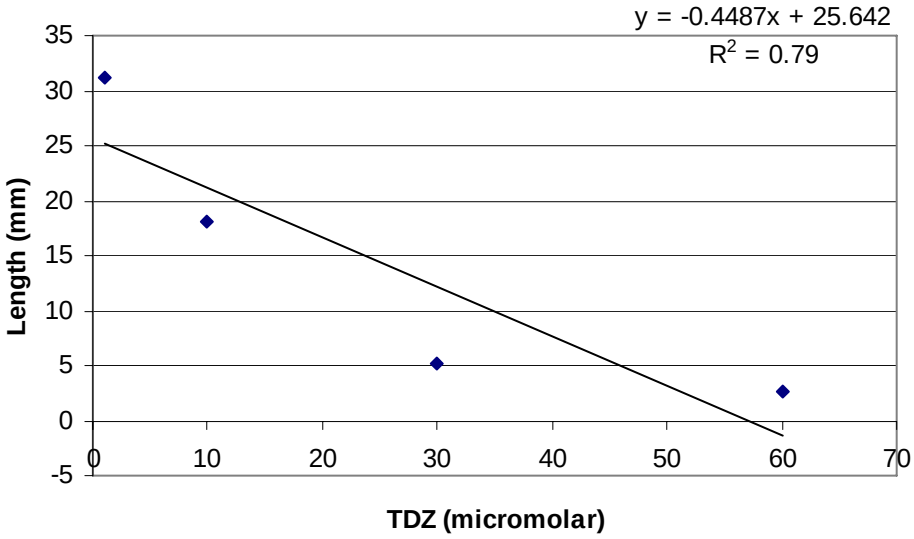

Figure 5-5. Regression curve showing the effect of TDZ concentration on shoot length (Length) in *A. latifolia* after 90 days of culture.

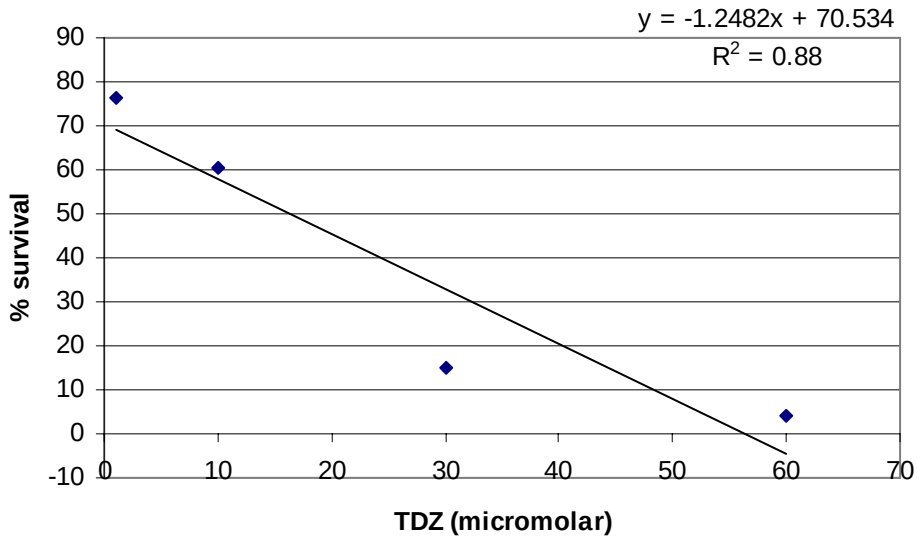

Figure 5-6. Regression curve showing the effect of TDZ concentration on acclimatization rate (% survival) in *A. latifolia* after 30 days of transfer to *ex vitro* conditions.

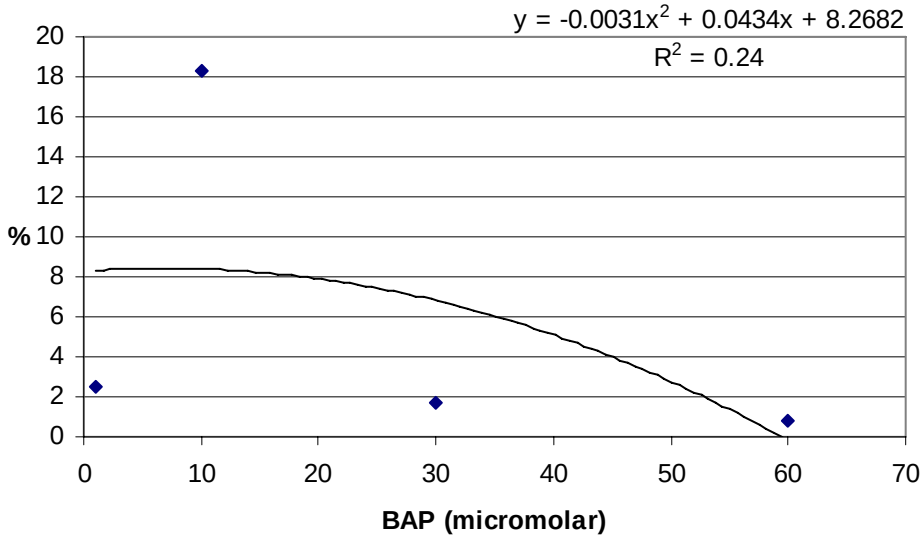

Figure 5-7. Regression curve showing the effect of BAP concentration on percentage of adventitious bud formation (%) in *A. latifolia* after 30 days of culture.

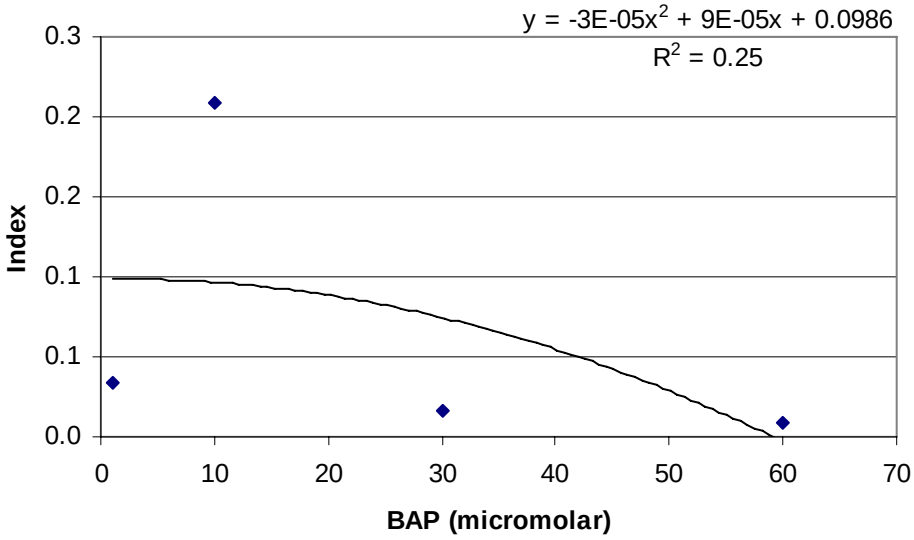

Figure 5-8. Regression curve showing the effect of BAP concentration on regeneration index (Index) in *A. latifolia* after 30 days of culture.

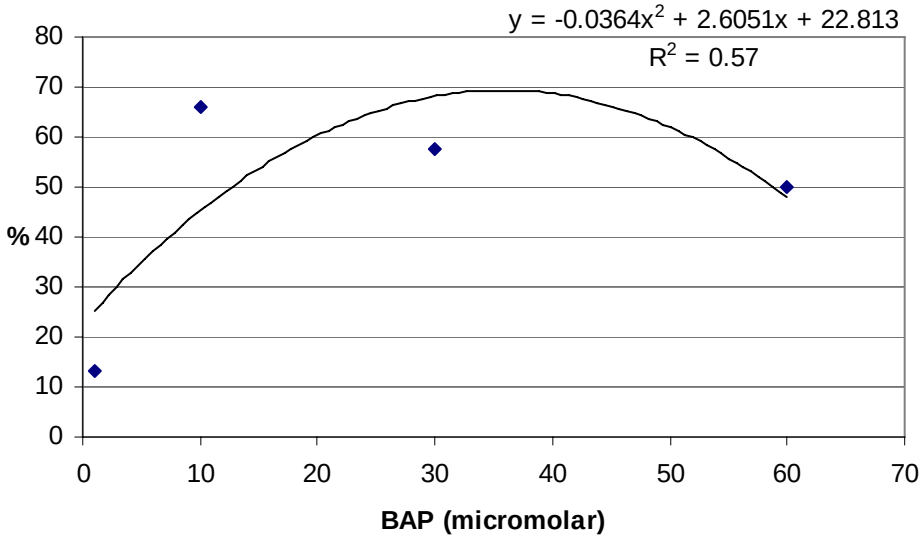

Figure 5-9. Regression curve showing the effect of BAP concentration on percentage of adventitious bud formation (%) in *A. latifolia* after 60 days of culture.

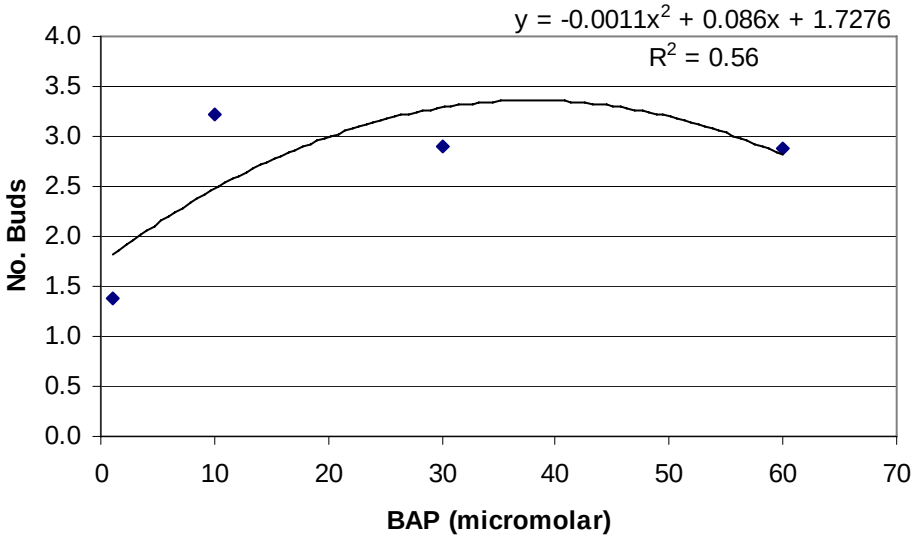

Figure 5-10. Regression curve showing the effect of BAP concentration on mean number of buds per explant (No. Buds) in *A. latifolia* after 60 days of culture.

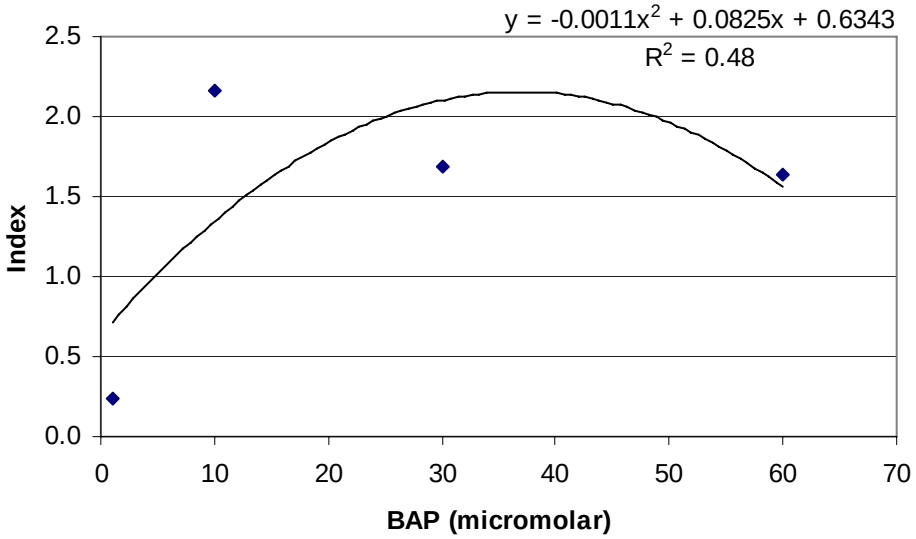

Figure 5-11. Regression curve showing the effect of BAP concentration on regeneration index (Index) in *A. latifolia* after 60 days of culture.

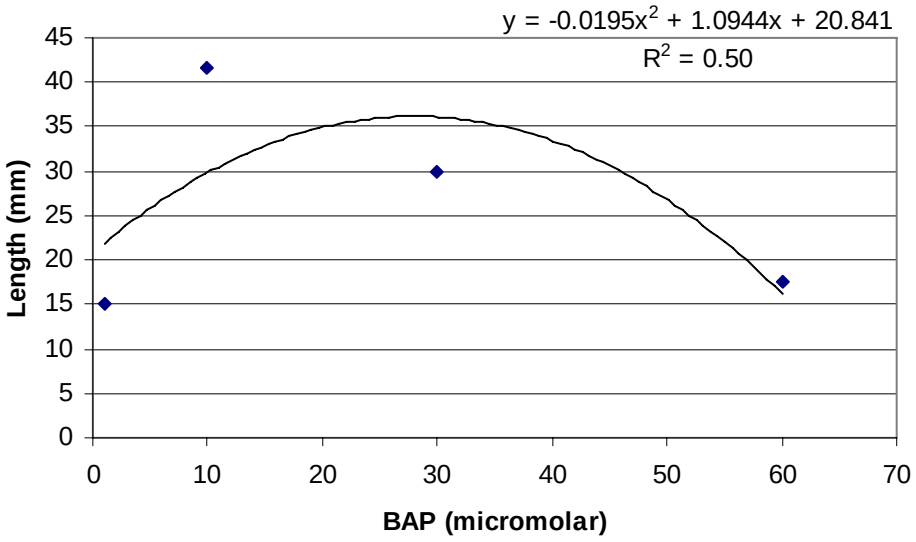

Figure 5-12. Regression curve showing the effect of BAP concentration on shoot length (Length) in *A. latifolia* after 90 days of culture.

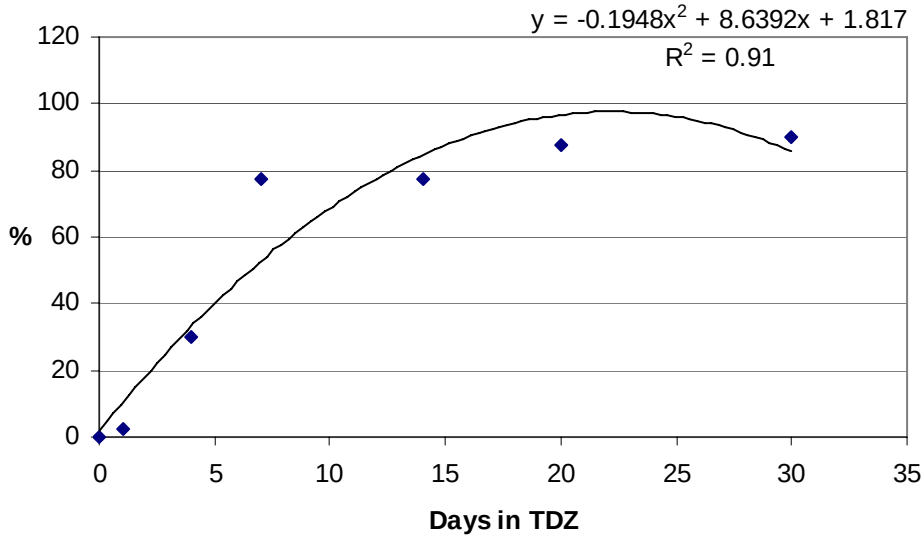

Figure 5-13. Regression curve sowing the effect of exposure to TDZ on bud formation percentage (%) in *A. latifolia* after 60 days of initiation of cultures.

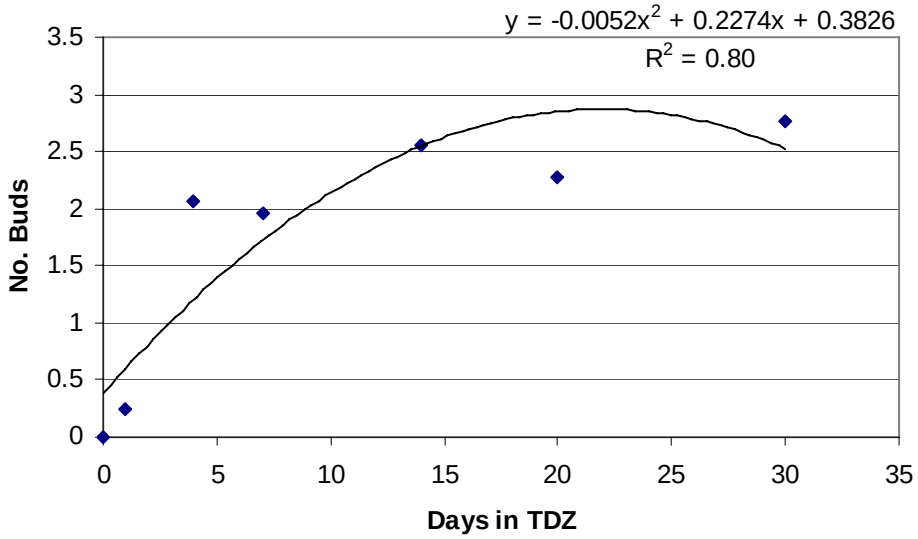

Figure 5-14. Regression curve sowing the effect of exposure to TDZ on mean number of buds per explant (No. Buds) in *A. latifolia* after 60 days of initiation of cultures.

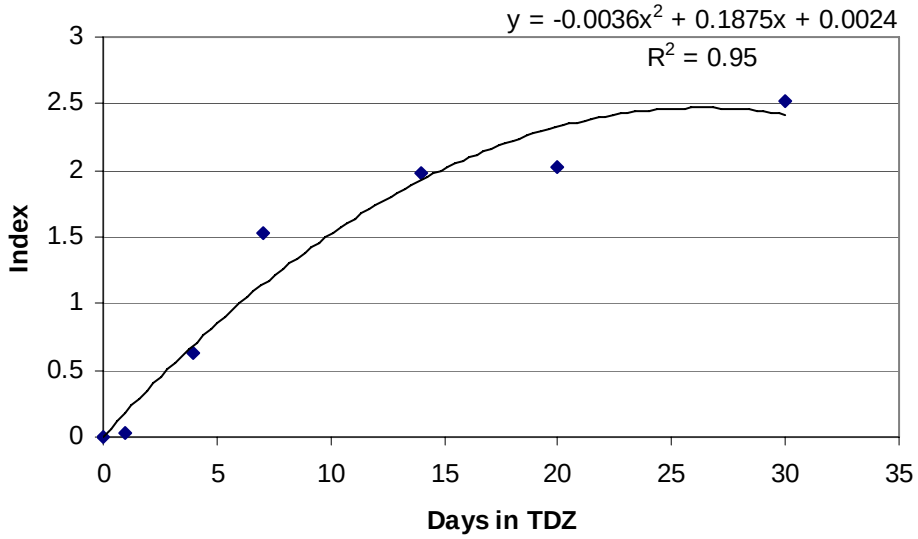

Figure 5-15. Regression curve sowing the effect of exposure to TDZ on regeneration index (Index) in *A. latifolia* after 60 days of initiation of cultures.

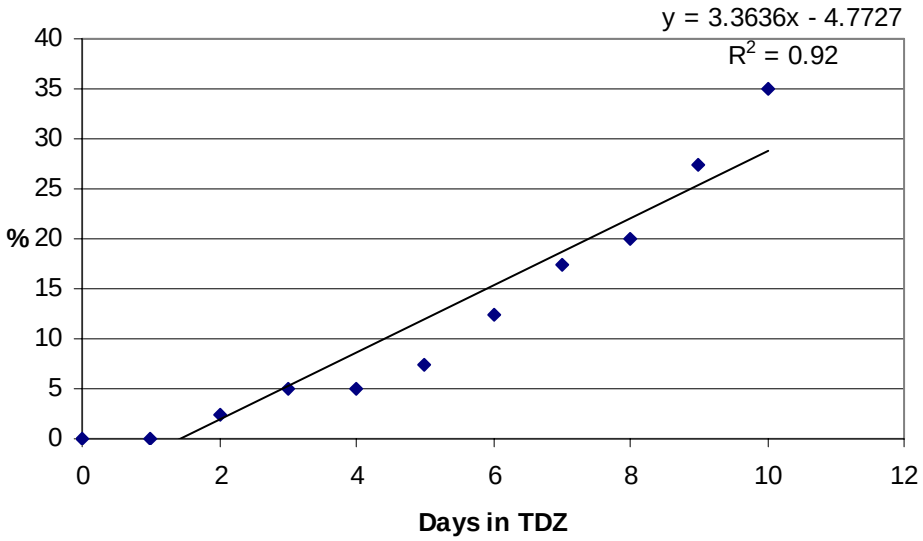

Figure 5-16. Regression curve sowing the effect of short exposures to TDZ on percentage of bud formation (%) in *A. latifolia* after 30 days of initiation of cultures.

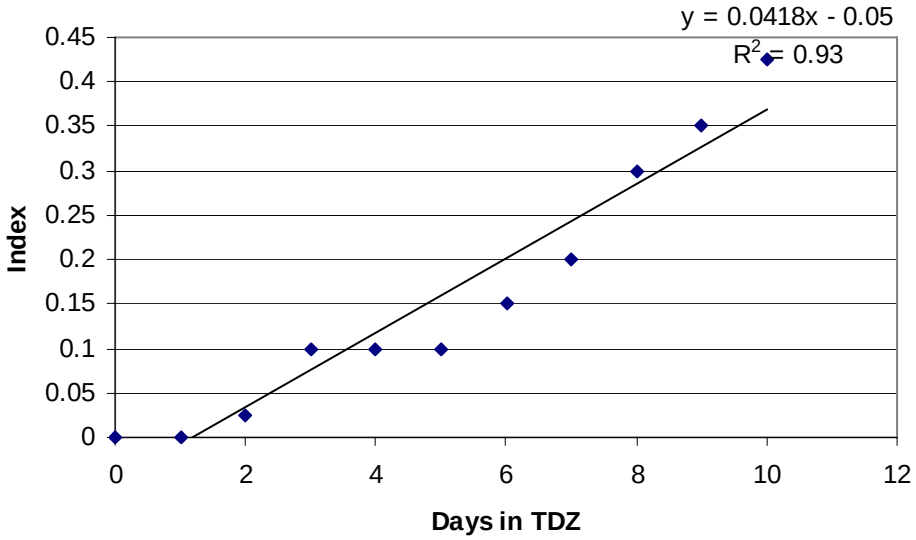

Figure 5-17. Regression curve sowing the effect of short exposures to TDZ on regeneration index (Index) in *A. latifolia* after 30 days of initiation of cultures.

CHAPTER 6
PLANT REGENERATION OF *Lotononis bainesii*

Introduction

The genus *Lotononis* consists of approximately 150 species, from herbs to small shrubs, belonging to the Fabaceae family, tribe Crotalarieae (Jaftha et al., 2002). Their broad distribution from Southern Africa to the Mediterranean region and India indicates that these species grow under dissimilar environments from the climatological and geographical point of view. Among *Lotononis* species, *L. divaricata* (Eckl. & Zeyh.) Benth., *L. tenella* (E. Mey.) Eckl. & Zeyh. and *L. laxa* Eckl. & Zeyh. have forage potential for arid areas, and *L. bainesii* Baker, a perennial herb, is a valuable forage in Australia (Jaftha et al., 2002). The increasing interest in this species has motivated some recent molecular studies to determine its mode of reproduction to assist breeding programs (Real et al., 2004). These studies have reported that, although it has been previously considered a cleistogamous species, it should be treated as an allogamous species in improvement programs. Some seed may be produced by self pollination, but self incompatibility may also be found in some genotypes.

Due to the allogamous nature of *L. bainesii*, seeds from a certain plant may correspond to different genotypes. Therefore, specific genotypes cannot be propagated through seeds and other means of multiplication would be useful. This could be overcome if a plant regeneration protocol is developed that allows the propagation of selected plants. One such protocol has been developed for *L. bainessi* by Bovo et al. (1986), who obtained a low frequency of plant regeneration from cotyledons and leaflets. Moreover, a tissue culture protocol may be used to solve another major constraint in this species, low seedling vigor, through the duplication of

chromosomes using chromosome doubling agents such as colchicine, oryzalin and trifluralin (Quesenberry et al., 2003). Cells with duplicated chromosomes would be induced to follow either organogenesis or somatic embryogenesis, producing solid plants instead of chimeras that would likely result if other techniques were used. In addition, a tissue culture protocol that results in plant regeneration from single cells would open the possibility of genetic transformation of this species (Ozias-Akins & Gill, 2001). The objective of these experiments was to develop an efficient tissue culture protocol for *L. bainesii*, which could then be used in genetic improvement programs of the species.

Materials and Methods

Cotyledon Culture

Seeds of *L. bainesii* cv. INIA Glencoe were scarified and germinated as described in chapter 3. Fifty 1-week-old seedlings were randomly selected and their cotyledons were excised and cut longitudinally along the midrib into two pieces so that four cotyledonary explants were obtained per genotype. Each explant was placed with the abaxial side down onto MS alone or MS with 4.5 μM TDZ, 4.14 μM PIC or 4.52 μM 2,4-D. Five explants were placed per petri dish and the identity of genotypes was maintained. Seedlings without cotyledons were placed onto MS medium and kept *in vitro* for subsequent experiments. After 30 days of culture, regenerated buds were subcultured onto MS medium supplemented with 0.044 μM BAP + 0.049 μM IBA for a month. Developing plants were then transferred to magenta boxes that contained MS lacking growth regulators for the same period of time before acclimatization *ex vitro*. Incubation and acclimatization were the same as those described in chapter 3.

Leaflet Culture

Genotypes used in this experiment were the same as in the cotyledon experiment. Explants consisted of pieces of leaflets (ca. 4 mm^2) including the midvein, harvested from immature

expanding leaves of each genotype growing under aseptic conditions. Treatments, incubation and acclimatization were the same as in the previous experiment.

Type of Explants

One of the genotypes that had performed well in the cotyledon experiment was used as the explant source. Leaflets were excised and placed onto sterile filter paper where they were divided into petiole, petiole tip (in which the three leaflets are inserted) and leaflets. Petiole pieces (divided into 5- to 6-mm portions), the petiole tip and three leaflets corresponding to each trifoliate leaf were placed in a petri dish containing MS supplemented with 10 μM TDZ. This procedure was repeated 10 times for both mature and immature leaves. Thirty days after the initiation of the experiment, explants were subcultured to MS without growth regulators. Incubation conditions were the same as those described in chapter 3. This experiment was statistically analyzed as a factorial arrangement in a completely randomized design (3 parts of the leaf x 2 stages of development) using PROC GLM from PC SAS (SAS Institute, 2003). Tukey's HSD Multiple Range Test at $p \leq 0.05$ was used to compare the means of treatments.

Results and Discussion

Cotyledon Culture

In the absence of growth regulators, cotyledon explants on MS basal medium remained non-responsive and gradually turned brown. Conversely, after 30 days of culture on medium supplemented with PIC, all genotypes produced light brown or light green friable callus of less than 1 cm in diameter. When this callus was transferred onto MS + 0.044 μM BAP + 0.049 μM IBA, these calli did not show further growth and died. Similar results were observed when 2,4-D was used as a growth regulator in the culture medium (Figure 6-1 A,B). However, in six

genotypes, a few short roots were produced from callus on 2,4-D induction medium, but they did not continue growing upon transfer to MS + 0.044 μM BAP + 0.049 μM IBA.

The addition of TDZ to the bud induction culture medium resulted in shoot bud organogenesis in 27 out of 50 genotypes tested (Figure 6-2 A). Callus and bud formation started approximately 7 and 15 days after the initiation of cultures, respectively (Figure 6-1 C,D). Callus was in general dark green with dark brown areas. Considering only responsive genotypes, the mean number of buds per cotyledonary explant was 13.6. Sixty days after culture, 48% of the total number of buds was capable of regenerating plants (177 plants/ 367 buds). In some genotypes, the number of plants after 90 days of culture was superior to the number of buds after 30 days, indicating that during that period new buds were produced and regenerated whole plants (Figure 6-3 A). In other genotypes, not all the buds present after 30 days resulted in plant regeneration. Moreover, nine genotypes produced buds but they did not elongate and finally died.

The lack of elongation and death of buds probably resulted from the hyperhydricity of tissues, which might have been caused by a hormonal imbalance in the culture medium. It was observed that only 39% of plants transferred to *ex vitro* conditions were capable of successful acclimatization 30 days after the transfer (Figures 6-1 E and 6-4 A). This low survival rate is likely associated with the high incidence of hyperhydricity in regenerated plants. Besides the influence of plant growth regulators in the culture medium, this abnormality may also result from high water availability in the culture vessel and low light levels (Hazarika, 2006).

The growth regulator, TDZ, has been used for organogenesis and plant regeneration from embryo- or seedling-derived explants in several species of legumes including *A. hypogaea* (Gill & Ozias-Akins, 1999), *V. radiata* (Mundhara & Rashid, 2006), *Trifolium spp.*, *Medicago sp.p*

(Ding et al., 2003) and *C. cajan* (Singh et al., 2003). Nevertheless, in the latter, somatic embryogenesis was obtained at higher concentrations of TDZ than those that had induced organogenesis. Similarly, there are reports of somatic embryo induction using TDZ in *A. hypogaea* (Murthy et al., 1995). Interestingly, PIC and 2,4-D were not effective for non zygotic embryo induction in *L. bainesii*, but both plant growth regulators were reported to be effective in *A. hypogaea* (Griga, 1999).

Leaflet Culture

Since *L. bainesii* is an allogamous species and not all progeny will necessarily reflect the superior performance of an individual plant, cotyledons are not the most suitable explants when the purpose is propagation of outstanding genotypes. Therefore, the previous experiment was repeated using leaflets as explants, since they are available throughout the year and offer the possibility of large scale propagation of selected genotypes as well as development of an *in vitro* chromosome doubling protocol.

Thirty days after the initiation of cultures it was observed that, when growth regulators were absent, explants remained irresponsive except in 22 genotypes in which roots up to 10-cm long were produced from the cut surface of the midvein. This suggests that the endogenous level of auxins in the leaflets may be enough to induce rhizogenesis in the absence of an exogenous supply of plant growth regulators. Probably, the high levels of auxins are responsible for the formation of vigorous roots in plants maintained *in vitro* not only from basal nodes but also from those not in contact with the culture medium. Some of these genotypes corresponded to those that had produced roots from callus in the previous experiment. When the culture medium contained PIC or 2,4-D, responses were similar to those observed in the cotyledon experiment. Although some calli were larger (up to 1.5 cm in diameter), they did not exhibit further growth

when transferred onto MS + 0.044 μM BAP + 0.049 μM IBA. The addition of 2,4-D resulted in root formation in seven genotypes as well.

The growth regulator, TDZ, was effective in inducing shoot bud organogenesis in 45 out of 50 genotypes tested, four of which were also non-responsive in the cotyledon experiment. However, the mean number of buds (3.8) was considerably lower than that obtained when cotyledon pieces were used (13.6) (Figures 6-1 F and 6-2 B). After 90 days of culture, a 22% increase in the number of regenerated plants compared to the number of buds at 30 days was observed (211 plants at 90 days vs. 173 buds at 30 days) (Figure 6-3 B and 6-1 G). This might be due to buds continuing to be formed on MS + 0.044 μM BAP + 0.049 μM IBA. But since this medium had such a low concentration of plant growth regulators, more probably these buds were already induced before the subculture. Even though the total number of plants obtained from leaflet culture was higher than that for cotyledons, only 21% were successfully acclimatized when transferred *ex vitro* (Figure 6-4 B). This high plant mortality was likely due to hyperhydricity, of which numerous plants showed symptoms; however, manipulation of culture conditions might have resulted in higher survival rates. It is possible that the vigorous growth of *in vitro* regenerated plants resulted in high ethylene accumulation, which has been reported to favor hyperhydricity (Hazarika, 2006).

It is interesting that more genotypes were responsive when explants consisted of leaflets rather than cotyledons. In general, juvenile explants are preferred since they are more likely to undergo organogenesis or somatic embryogenesis. For example, in *A. hypogaea*, seedlings more than 21-days old failed to undergo somatic embryogenesis using TDZ compared to up to 97% of 6-day-old seedlings (Murthy et al., 1995). The lower cotyledon response in *L. bainesii* may have been caused by the scarification/surface disinfection procedures seed received, although this is

unlikely given that much stronger pretreatments have been reported with no negative effects (Bovo et al, 1986). These authors obtained better responses with cotyledons as explants (66% of bud formation in the best culture medium vs. 54% when using leaflets). In the present study, however, a higher frequency of organogenesis was obtained following leaflet culture (90% of explants producing buds vs. 54% of cotyledon pieces producing buds). Additionally, results in this experiment differ from those reported by Bovo et al. (1986) in that shoots readily rooted in spite of being in a culture medium with the potent cytokinin TDZ so rooting was not a critical factor in whole plant regeneration. Veltcheva et al. (2005) suggest that forage legume populations are markedly heterogeneous, resulting in an easier identification of *in vitro* responsive genotypes. Organogenic genotypes of *L. bainesii* cv INIA Glencoe were easily identified, which may be due to a shorter breeding history than those in grain legumes in which the narrow genetic base limits the discovery of regenerating genotypes.

Type of Explant

Since the previous experiment showed that leaflets were capable of producing shoot buds in the presence of TDZ in most of the genotypes tested, another experiment was performed to asses the influence of the part and age of the leaf in shoot organogenesis. Callus formation started within a week of initiation of cultures and shoot buds began to arise after 15 days of culture in the six types of explants used.

For percentage of shoot bud formation, leaf part had a significant effect, whereas leaf age and interaction between leaf part and leaf age were not significant (Table 6-1). Bud organogenesis from leaflets was similar (85.8% from insertion explants and 83.3% from leaflet explants) and higher than petioles (48.3%). The number of buds per explant ranged from 1.1 to 3.4 (Table 6-2) but, similar to bud organogenesis percentage, there was no leaf age by leaf part interaction for number of buds per explant (Table 6-1). In contrast to percentage of bud

organogenesis, the mean number of buds per explant did not differ among parts of the leaf, but did differ due to leaf age. Explants from immature leaves gave a higher number of buds per explant (2.9) than those from mature ones (1.5) (Table 6-2).

Considering the regeneration index, there was a significant effect of the part and age of the leaf but not a significant interaction (Table 6-1). The mean regeneration index for petiole explants (1.1) was significantly lower than the index for leaflet or leaflet insertion area (2.4 in both cases). However, the mean regeneration index for explants from immature leaves (2.4) was significantly higher than the index for explants excised from mature leaves (1.5). Leaflet insertion area from immature leaves had the highest index (3.4) over all combinations.

The advantage of using immature leaves has been reported in *A. hypogaea*, in which the frequency of somatic embryo formation decreased considerably as leaflets unfolded (Baker & Wetzstein, 1998). In *A. villosulicarpa*, the best organogenic frequency and mean number of buds per explant were obtained when mature fully expanded leaves were used as explants (Dunbar & Pittman, 1992). In *L. bainesii*, Bovo et al. (1986) reported up to 54% bud formation when pieces of fully expanded leaflets from greenhouse grown plants were placed onto the best culture medium. Although these authors used larger explants (6 mm^2), organogenesis frequency was lower than in the present experiment. This could be due to the conditions under which mother plants were grown, a factor that greatly affects *in vitro* responsiveness (Radice, 2004).

Conclusions

It was possible to regenerate plants from over 50% of *L. bainesii* cv INIA Glencoe genotypes that were evaluated through cotyledon culture and 90% of genotypes through leaflet culture in a medium composed of MS +4.5 μM TDZ. Bud elongation and rooting was obtained upon transfer onto MS + 0.044 μM BAP + 0.049 μM IBA. Although immature leaflet culture

resulted in a higher number of responsive genotypes, plants regenerated from cotyledons exhibited a higher survival rate when transferred to *ex vitro* conditions. Nevertheless, in both cases survival rates were low and this situation was related with the incidence of hyperhydricity in cultures. When the culture medium was supplemented with either PIC or 2,4-D, friable light green or light brown callus formation was obtained, but this callus did not show further growth when subcultured.

Regarding the type of explants, the experiment carried out with one genotype of *L. bainesii* revealed that leaflet insertion areas from expanding leaves and pieces of leaflets were more efficient for shoot bud induction. For both mature and immature leaves, the lowest frequencies of shoot bud formation were obtained when pieces of petioles were used as explants. Mean number of buds per explant and regeneration index were higher for explants collected from immature leaves.

A suggested protocol for *L. bainesii* plant regeneration is as follows:

1. Use immature leaflets as explants, which are as efficient as leaflet insertion areas but a larger amount of tissue is obtained per leaf

2. Culture on MS + 4.5 μM TDZ for 30 days

3. Transfer to MS + 0.044 μM BAP + 0.049 μM IBA for bud elongation and rooting

Although these experiments have improved the regeneration frequency reported for this species, it is likely that the optimization of other factors besides the type of explant would result in a more efficient protocol. Additional experiments regarding the regulation of plant growth regulator concentrations and combinations, time required for organogenesis induction and control of factors that influence hyperhydricity will be conducted to increase the regeneration frequency and *ex vitro* establishment rate.

Table 6-1. ANOVA table showing the p-values corresponding to the effect of explant type on bud formation percentage (%), mean number of buds per explant (No.) and regeneration index (Index†) in *L. bainesii* after 30 days of culture.

Source	df	p-value		
		%	No.	Index†
Leaf parts	2	0.004	0.606	0.032
Leaf age	1	0.454	0.003	0.048
LP x LA	2	0.120	0.294	0.119
Error	54			

† Index=(% shoot bud formation x mean number of buds) / 100

Table 6-2. Effect of explant type on bud formation percentage, mean number of buds per explant and regeneration index in *L. bainesii* after 30 days of culture.

	Leaf part	Immature	Mature	Mean
Bud formation %	Petioles	38.3	58.3	48.3[b*]
	Leaflet insertion	100.0	71.5	85.8[a]
	Leaflets	90.0	76.7	83.4[a]
	Mean	76.1[A]	68.8[A]	
Number of buds	Petioles	2.2	1.6	1.9[a]
	Leaflet insertion	3.4	1.1	2.3[a]
	Leaflets	3.0	1.8	2.4[a]
	Mean	2.9[A]	1.5[B]	
Regeneration index†	Petioles	1.0	1.2	1.1[b]
	Leaflet insertion	3.4	1.4	2.4[a]
	Leaflets	2.8	1.9	2.4[a]
	Mean	2.4[A]	1.5[B]	

* For each parameter, means within columns (a,b,c) or rows (A,B) with different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= (% shoot bud formation x mean number of buds) / 100

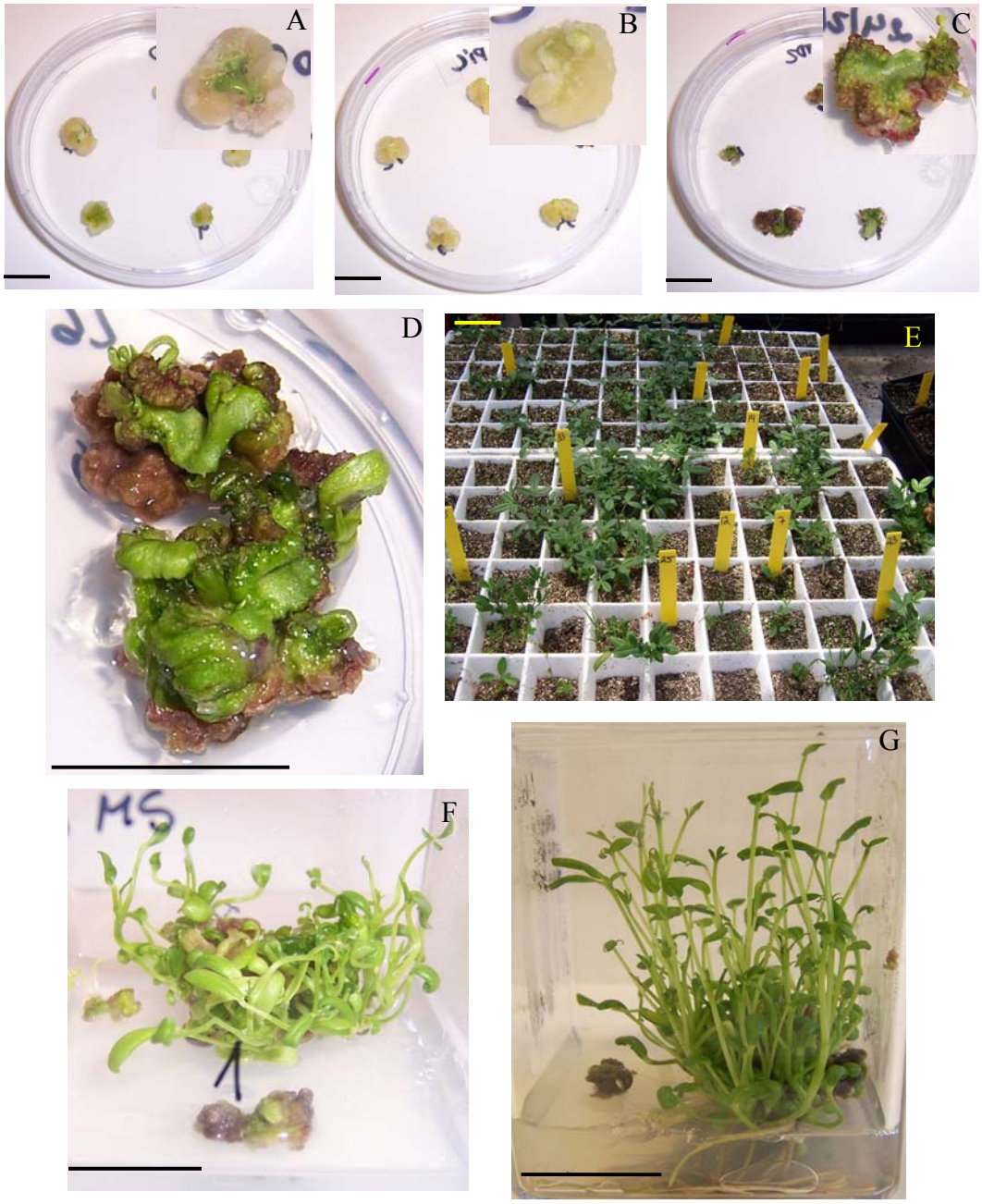

Figure 6-1. Organogenesis in *L. bainesi.* A) Cotyledon cultures 20 days after initiation of experiments in MS basal media supplemented with 2,4-D, B) PIC and C) TDZ (bar: 20 mm). D) Shoot bud formation from cotyledons 45 days after culture (bar: 20 mm). E) Plants successfully acclimatized 45 days after transfer to *ex vitro* conditions (Bar: 50 mm). F), G) Shoot bud proliferation from leaflet explants originated in MS + 10 µM TDZ 60 and 90 days after initiation of cultures, respectively (bar: 20 mm).

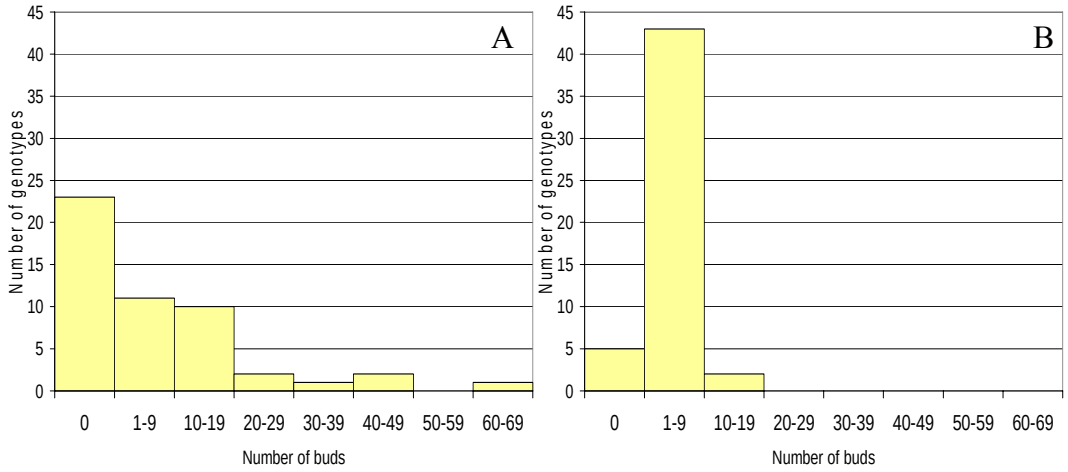

Figure 6-2. Number of buds produced per explant by 50 genotypes of *L. bainesii* after 30 days of culture from A) cotyledon and B) leaflets.

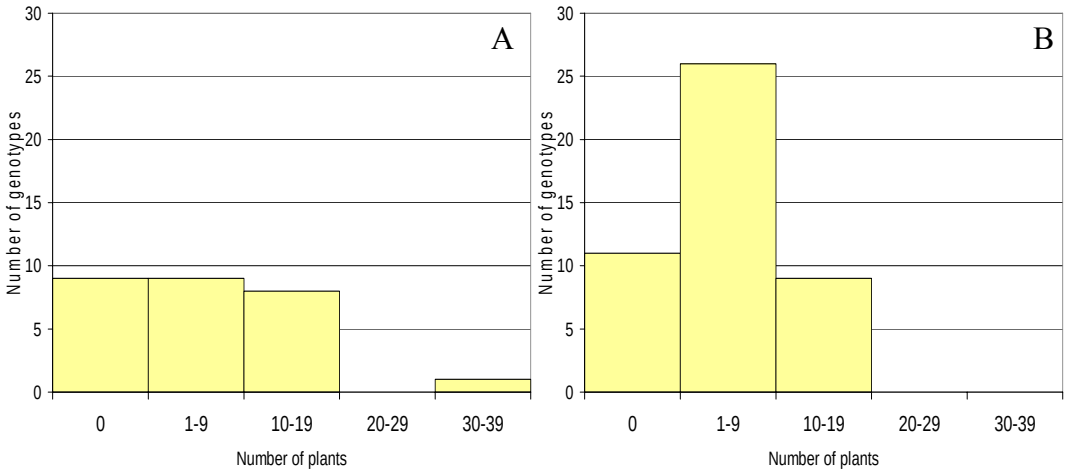

Figure 6-3. Number of plants produced per explant after 90 days of culture by responsive genotypes of *L. bainesii* through A) cotyledon and B) leaflet culture.

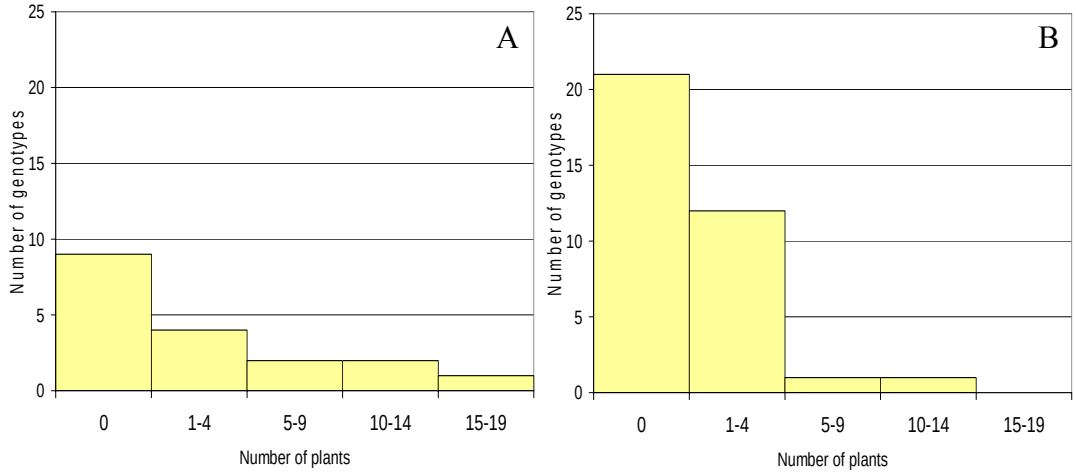

Figure 6-4. Number of successfully acclimatized plants in the genotypes of *L. bainesii* that were capable of plant regeneration through A) cotyledon and B) leaflet culture.

CHAPTER 7
SUMMARY AND CONCLUSIONS

Grain legumes are generally regarded as recalcitrant to *in vitro* plant regeneration due to a narrow genetic base that results in low genetic variability and a more difficult identification of responsive genotypes. It is thought to be easier to identify *in vitro* responsive genotypes of forage legumes, because they have usually undergone fewer selection cycles and populations are more heterogeneous (Veltcheva et al., 2005). Results reported here concur with this concept since shoot bud organogenesis was achieved in 20 to 90% of genotypes in four out of five forage legume species evaluated.

Petiole culture of *T. polymorphum* on three different basal media supplemented with TDZ was not effective to induce bud organogenesis, at least for those genotypes tested. Possibly, this species is more recalcitrant to tissue culture than the others. Further studies will be conducted to determine the most suitable conditions for *in vitro* plant growth (using these aseptically grown plants as an explant source) as well as to determine the culture conditions required for shoot bud induction, i.e., basal medium composition, and plant growth regulator type, concentration and combinations. In *T. carolinianum,* an annual native clover species, shoot bud formation was achieved for the first time through cotyledon culture. Additional experiments are being conducted to achieve further elongation of shoots and rooting, before regenerated plants are transferred to *ex vitro* conditions to evaluate the establishment rate.

In *A. bicolor, A. latifolia* and *Lotononis bainesii*, plant regeneration was achieved via organogenesis in 54 to 90% of the genotypes tested. Nevertheless, additional

experiments should be conducted to improve the frequency of bud formation and to reduce the incidence of hyperhydricity in culture, which should result in higher *ex vitro* survival rates.

Overall, MS was a suitable basal medium for shoot bud induction in all species tested, except for *T. polymorphum* where the only response was callus formation on B5 basal medium. In *A. latifolia*, L2 was as effective as MS for shoot bud organogenesis, but plants regenerated in L2 showed a lower establishment rate when transferred to *ex vitro* conditions. Among plant growth regulators tested, TDZ was efficient in all species except *T. polymorphum*, where it only induced callus formation. In *A. latifolia*, shoot bud formation was observed in TDZ concentrations ranging from 1 to 60 μM. The induction time experiment in *A. latifolia* revealed that 20 days of culture on medium containing TDZ was sufficient to obtain shoot organogenesis. In agreement with other literature, immature leaf tissues were in general more responsive in *L. bainesii* and *Adesmia spp*. Immature rachises proved to be superior explants for organogenesis induction in *A. latifolia,* and leaflet insertion areas or leaflets from immature leaves were more responsive than pieces of petioles in *L. bainesii*. Although cotyledon culture resulted in shoot bud formation in *T. carolinianum* and *L. bainesii*, in *Adesmia spp.* cotyledons remained unresponsive.

Suggested protocols for the studied species are:

T. carolinianum

1. Cotyledon culture on MS + 10 μM TDZ for 30 days

2. Transfer of organogenic cultures on MS + 1 μM TDZ, where new buds continue to arise and short shoots are produced from those buds already differentiated

A. bicolor

1. Immature leaflet culture on MS or L2 + 4.5 μM TDZ for 60 days

2. Transfer to MS devoid of plant growth regulators for 45 days, where buds elongated and rooted.

A. latifolia

1. Immature rachis culture on MS + 10 μM TDZ for 20 days

2. Transfer to MS with no plant growth regulators for bud elongation and rooting

L. bainesii

1. Culture immature leaflets on MS + 4.5 μM TDZ for 30 days

2. Transfer to MS + 0.044 μM BAP + 0.049 μM IBA for bud elongation and rooting

In conclusion, *in vitro* plant regeneration protocols were developed for four promising legume species. These protocols could be used to assist in breeding programs to improve seedling vigor and DM production. This would be important for cattle production in those countries where these species are native. Additionally, this may allow the development of a legume alternative to perennial peanut in the state of Florida, since to-date it is the only introduced forage legume species that has shown long term persistence. The main drawbacks of perennial peanut are its vegetative propagation, which increases the establishment cost, and low production in the year after planting. In contrast, *Adesmia spp*. and *L. bainesii* cultivars could be propagated through seeds, reducing the cost for farmers. Currently, field studies are being conducted to evaluate field performance of *Adesmia spp.* and *L. bainesii* to the Florida environment.

APPENDIX

ADDITIONAL TABLES FOR TWO GENOTYPES OF *Adesmia latifolia*

Table A-1. Effect of different combinations of TDZ and IBA on percentage of adventitious bud formation (ABF) and mean number of buds per explant in *A. latifolia* U18.6 after 30 and 60 days of culture.

	30 days of culture						60 days of culture					
	IBA (μM)						IBA (μM)					
	0		0.1		1		0		0.1		1	
	ABF	Buds	ABF	Buds	ABF	Buds	ABF	Buds	ABF	Buds	ABF	Buds
TDZ (μM)	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
0	0^{a*}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
1	45^{ab}	1.3^{b}	55^{bc}	1.7^{b}	75^{b}	1.7^{b}	70^{b}	3.1^{bc}	60^{bc}	2.2^{b}	85^{b}	2.6^{b}
10	60^{b}	2.8^{cd}	40^{ab}	2^{b}	55^{b}	2^{b}	95^{b}	3.6^{bc}	55^{b}	2.6^{b}	85^{b}	2.4^{b}
30	70^{b}	1.9^{bc}	75^{bc}	2.3^{b}	75^{b}	2.1^{b}	85^{b}	2.1^{b}	80^{bc}	2.8^{b}	85^{b}	2.7^{b}
60	85^{b}	3.1^{d}	100^{c}	1.9^{b}	50^{b}	2.2^{b}	90^{b}	3.7^{c}	95^{c}	2.4^{b}	75^{b}	2.7^{b}

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05

Table A-2. Effect of different combinations of TDZ and IBA on regeneration index (Index†), number of shoots per explant and shoot length in *A. latifolia* U18.6 after 60 days of culture.

	IBA (μM)								
	0			0.1			1		
TDZ (μM)	Index†	Shoots -No.-	Length -mm-	Index	Shoots -No.-	Length -mm-	Index	Shoots -No.-	Length -mm-
0	0^{a*}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
1	2.2^{bc}	10^{b}	25^{b}	1.3^{ab}	11^{b}	25^{b}	2.3^{b}	13^{b}	17.5^{c}
10	3.4^{c}	13^{ab}	13^{b}	1.5^{ab}	10^{b}	23^{b}	2^{b}	8^{ab}	8^{bc}
30	1.8^{ab}	6.5^{a}	5^{ab}	2.2^{b}	9^{a}	6.5^{b}	2.3^{b}	8^{ab}	8^{bc}
60	3.3^{c}	9^{a}	4^{b}	2.3^{b}	5.5^{a}	4^{ab}	2.3^{b}	5^{ab}	7.5^{ab}

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table A-3. Effect of different combinations of TDZ and IBA on percentage of adventitious bud formation (ABF) and mean number of buds per explant in *A. latifolia* U18.8 after 30 and 60 days of culture.

	30 days of culture						60 days of culture					
	IBA (μM)						IBA (μM)					
	0		0.1		1		0		0.1		1	
TDZ (μM)	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.
0	0[a*]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
1	60[b]	2[b]	45[b]	1.4[b]	35[ab]	2.3[b]	70[b]	2.5[b]	45[ab]	3.4[b]	65[b]	2.7[b]
10	80[b]	1.5[b]	45[b]	1.3[ab]	65[b]	1.7[b]	95[b]	2.6[b]	75[b]	2.9[b]	70[b]	2.9[b]
30	80[b]	1.6[b]	50[b]	1.8[b]	55[b]	2.2[b]	100[b]	2.8[b]	80[b]	2.6[b]	95[b]	2.1[b]
60	80[b]	1.8[b]	50[b]	2.3[b]	65[b]	2.5[b]	100[b]	2.9[b]	70[b]	2.1[b]	85[b]	2.4[b]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.05$

Table A-4. Effect of different combinations of TDZ and IBA on regeneration index (Index[†]), number of shoots per explant and shoot length in *A. latifolia* U18.8 after 60 days of culture.

	IBA (μM)								
	0			0.1			1		
TDZ (μM)	Index[†]	Shoots -No.-	Length -mm-	Index	Shoots -No.-	Length -mm-	Index	Shoots -No.-	Length -mm-
0	0[a*]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
1	1.7[b]	11[b]	53[b]	1.4[ab]	7.5[a]	35[a]	1.7[b]	10[b]	33[a]
10	2.5[b]	6[ab]	40[ab]	2.2[b]	5[a]	16[a]	2[b]	5.5[ab]	10[a]
30	2.8[b]	2[a]	8.5[a]	2.1[b]	1.5[a]	3.5[a]	2[b]	0[a]	0[a]
60	2.9[b]	0[a]	0[a]	1.5[ab]	0[a]	0[a]	2.1[b]	0[a]	0[a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at $p \leq 0.05$. † Index= % shoot bud formation x mean number of buds / 100

Table A-5. Effect of different combinations of TDZ and IBA on percentage of *ex vitro* acclimatization (%) in *A. latifolia* U18.6 and U18.8 after 20 days of transfer to *ex vitro* conditions.

	U 18.6 IBA (µM)			U 18.8 IBA (µM)		
TDZ (µM)	0	0.1	1	0	0.1	1
0	0[a*]	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]
1	86 [b]	49 [ab]	96 [b]	83 [b]	63 [a]	83 [b]
10	86 [b]	100 [b]	65 [ab]	25 [ab]	50 [a]	36 [ab]
30	13 [ab]	28 [ab]	50 [ab]	0 [a]	0 [a]	0 [a]
60	0 [a]	0 [a]	25 [ab]	0 [a]	0 [a]	0 [a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05

Table A-6. Effect of different combinations of BAP and IBA on percentage of adventitious bud formation (ABF) and mean number of buds per explant in *A. latifolia* U18.6 after 30 and 60 days of culture.

	30 days of culture IBA (µM)						60 days of culture IBA (µM)					
	0		0.1		1		0		0.1		1	
BAP (µM)	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.	ABF %	Buds No.
0	0[a*]	0	0 [a]	0	0 [a]	0	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]
1	0 [a]	0	5 [a]	1	5 [a]	0.5	5 [a]	1.5[ab]	10 [a]	4 [b]	10[ab]	0.5 [a]
10	10 [a]	0.5	25 [b]	1	0 [a]	0	40 [b]	3.9 [b]	85 [b]	3.5 [b]	45 [b]	2.7 [a]
30	0 [a]	0	5 [a]	0.5	0 [a]	0	45 [b]	3.8 [b]	25 [a]	2 [ab]	40 [b]	2.9 [a]
60	0 [a]	0	0 [a]	0	0 [a]	0	10[ab]	3 [ab]	15 [a]	1.5[ab]	45 [b]	1.8 [a]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05

Table A-7. Effect of different combinations of BAP and IBA on regeneration index (Index†) and shoot length in *A. latifolia* U18.6 after 60 days of culture.

	IBA (μM)					
	0		0.1		1	
BAP (μM)	Index†	Length --mm--	Index	Length --mm--	Index	Length --mm--
0	0[a*]	0	0[a]	0	0[a]	0
1	0.2[ab]	20	0.4[a]	40	0.1[a]	0
10	1.5[bc]	30	3[b]	42.5	1.4[a]	30
30	1.6[c]	20	0.5[a]	5	1.2[a]	50
60	0.3[ab]	20	0.3[a]	0	0.9[a]	15

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table A-8. Effect of different combinations of BAP and IBA on percentage of adventitious bud formation (ABF) and mean number of buds per explant in *A. latifolia* U18.8 after 30 and 60 days of culture.

	30 days of culture						60 days of culture					
	IBA (μM)						IBA (μM)					
	0		0.1		1		0		0.1		1	
	ABF	Buds	ABF	Buds	ABF	Buds	ABF	Buds	ABF	Buds	ABF	Buds
BAP (μM)	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.
0	0[a*]	0	0[a]	0	0[a]	0	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
1	5[a]	0.5	0[a]	0	0[a]	0	20[a]	1[a]	35[ab]	1.3[a]	0[a]	0[a]
10	10[a]	0.5	45[b]	1.3	20[a]	1.2	60[b]	2.8[b]	90[c]	3.9[b]	75[b]	2.5[b]
30	5[a]	0.5	0[a]	0	0[a]	0	80[b]	2.9[b]	65[bc]	3.5[b]	90[b]	2.3[b]
60	5[a]	0.5	0[a]	0	0[a]	0	75[b]	4.8[c]	85[bc]	3.3[b]	70[b]	3[b]

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05

Table A-9. Effect of different combinations of BAP and IBA on regeneration index (Index†) and shoot length in *A. latifolia* U18.8 after 60 days of culture.

	IBA (μM)					
	0		0.1		1	
BAP (μM)	Index†	Length --mm--	Index	Length --mm--	Index	Length --mm--
0	0 [a*]	0	0 [a]	0	0 [a]	0
1	0.4 [ab]	30	0.4 [a]	0	0 [a]	0
10	1.7 [bc]	40	3.5 [b]	58	1.9 [b]	50
30	2.4 [cd]	40	2.3 [b]	33	2.1 [b]	33
60	3.6 [d]	30	2.8 [b]	20	2.1 [b]	20

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table A-10. Effect of different times of exposure to TDZ on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* U18.6 after 30 and 60 days of culture, respectively.

	30 days of culture			60 days of culture		
	ABF	Buds	Index†	ABF	Buds	Index
Days in TDZ	--%--	--No.--		--%--	--No.--	
0	0 [a*]	0 [a]	0 [a]	0 [a]	0	0
1	0 [a]	0 [a]	0 [a]	0 [a]	0	0
4	25 [ab]	1.7 [b]	0.4 [ab]	35 [ab]	2.8	0.9
7	80 [bc]	2.3 [b]	1.8 [c]	85 [bc]	2.3	1.9
14	75 [bc]	1.6 [b]	1.2 [bc]	75 [bc]	2.2	1.7
20	70 [bc]	2.9 [b]	2 [c]	85 [bc]	2.6	2.2
30	90 [c]	1.9 [b]	1.8 [c]	95 [c]	3	3

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table A-11. Effect of different times of exposure to TDZ on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* U18.8 after 30 and 60 days of culture, respectively.

	30 days of culture			60 days of culture		
	ABF	Buds	Index†	ABF	Buds	Index
Days in TDZ	--%--	--No.--		--%--	--No.--	
0	0[a*]	0 [a]	0 [a]	0 [a]	0 [a]	0
1	5 [a]	0.5 [ab]	0.1 [ab]	5 [a]	0.5 [a]	0.1
4	0 [a]	0 [a]	0 [a]	25 [a]	1.3 [a]	0.4
7	35 [b]	1.8 [ab]	0.6 [abc]	70 [b]	1.6 [ab]	1.2
14	65 [b]	1.8 [ab]	1.2 [bc]	80 [b]	2.9 [b]	2.3
20	75 [b]	2.2 [b]	1.7 [c]	90 [b]	2 [ab]	1.9
30	70 [b]	1.6 [ab]	1.1 [abc]	85 [b]	2.5 [b]	2.1

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table A-12. Effect of short exposure to TDZ on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* U18.6 and U18.8 after 30 days of culture.

	U18.6			U18.8		
	ABF	Buds	Index†	ABF	Buds	Index
Days in TDZ	--%--	--No.--		--%--	--No.--	
0	0	0	0	0[a*]	0	0
1	0	0	0	0 [a]	0	0
2	5	0.5	0.1	0 [a]	0	0
3	10	1	0.2	0 [a]	0	0
4	5	0.5	0.1	5 [ab]	1.5	0.2
5	5	1	0.1	10 [ab]	0.5	0.1
6	20	1.2	0.3	5 [ab]	0.5	0.1
7	15	1	0.2	20 [abc]	1.2	0.3
8	25	1.9	0.5	15 [ab]	1	0.2
9	25	1.8	0.4	30 [bc]	1	0.3
10	25	0.7	0.4	45 [c]	1.1	0.5

* Different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

Table A-13. Effect of explant type on bud formation percentage (ABF), mean number of buds per explant and regeneration index (Index†) in *A. latifolia* U18.6 and U18.8 after 30 days of culture.

		U18.6			U18.8		
	Types of explants	ABF --%--	Buds --No.--	Index†	ABF --%--	Buds --No.--	Index
Immature leaves	Petioles	10.3[a*]	1.5 [ab]	0.3 [a]	7.9 [a]	0.1	0
	Rachis	90 [d]	3.5 [bc]	3.5 [b]	70 [c]	1	1
	Leaflets	48.8[bc]	1.3 [ab]	0.8 [a]	27.5[ab]	1.1	0.4
Mature leaves	Petioles	16 [a]	0.8 [a]	0.2 [a]	0 [a]	0	0
	Rachis	80 [cd]	4.3 [c]	4.3 [b]	50 [bc]	1	1
	Leaflets	37.5 [ab]	1.1 [a]	0.6 [a]	3.8 [a]	0.9	0.1

* Within columns, different letters indicate significant differences according to Tukey's HSD Multiple Range Test at p≤0.05. † Index= % shoot bud formation x mean number of buds / 100

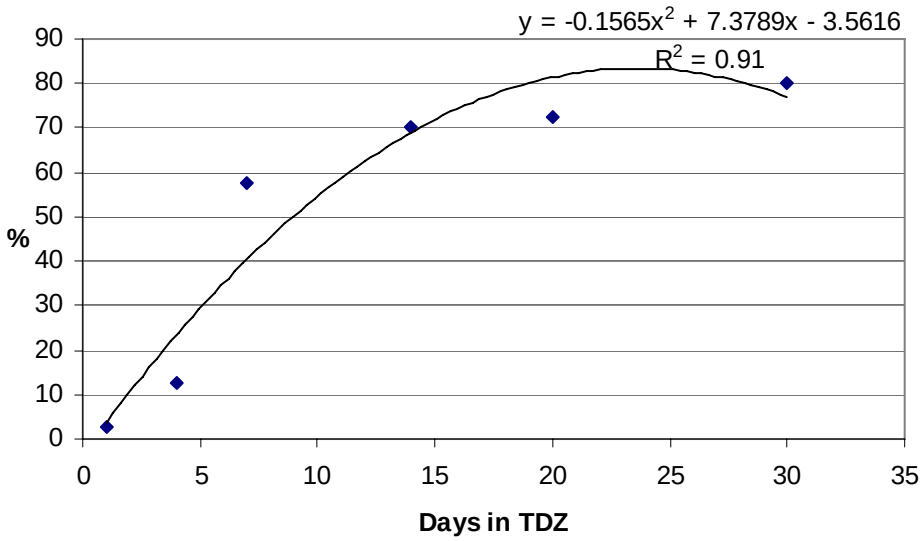

Figure A-1. Regression curve sowing the effect of exposure to TDZ on bud formation percentage (%) in *A. latifolia* after 30 days of initiation of cultures.

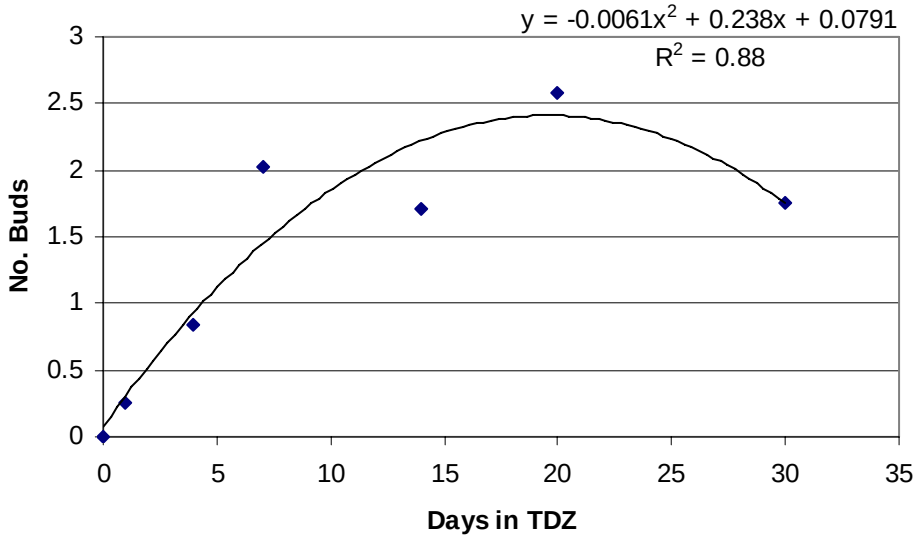

Figure A-2. Regression curve sowing the effect of exposure to TDZ on mean number of buds per explant (No. Buds) in *A. latifolia* after 30 days of initiation of cultures.

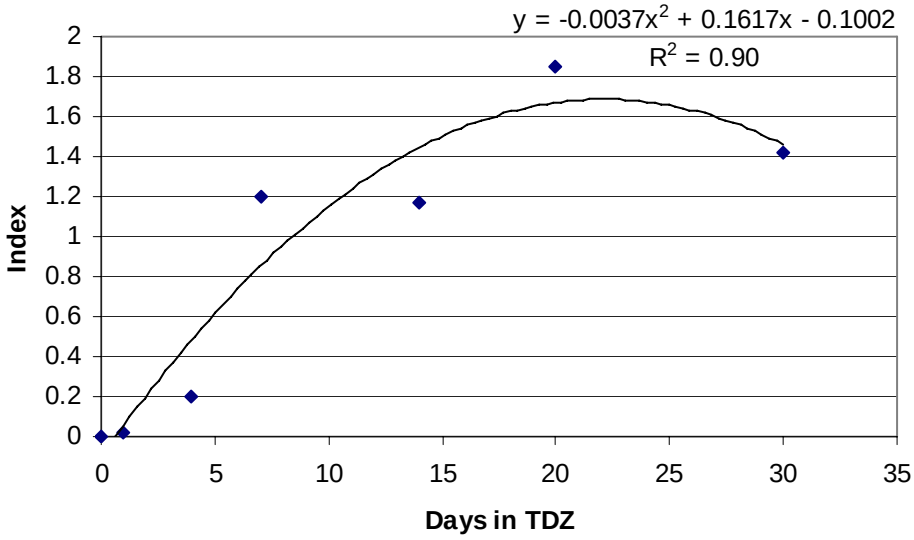

Figure A-3. Regression curve sowing the effect of exposure to TDZ on regeneration index (Index) in *A. latifolia* after 30 days of initiation of cultures.

LIST OF REFERENCES

Agrawal V & Sardar PR (2006) *In vitro* propagation of *Cassia angustifolia* through leaflet and cotyledon derived calli. Biologia Plantarum 50 (1): 118-122

Akasaka Y, Daimon H & Masahiro M (2000) Improved plant regeneration from cultured leaf segments in peanut (*Arachis hypogaea* L.) by limited exposure to thidiazuron. Plant Science 156: 169-175

Akashi R, Kawano T, Hashiguchi M, Kutsuna Y, Hoffmann-Tsay S & Hoffmann F (2003) Super roots in *Lotus corniculatus*: A unique tissue culture and regeneration system in a legume species. Plant and Soil 255: 27-33

Bajaj YPS, Labana KS & Dhanju MS (1980) Induction of pollen-embryos and pollen-callus in anther cultures of *Arachis hypogaea* and *A. glabrata*. Protoplasma 103: 397-399

Bajaj YPS, Ram AK, Labana KS & Singh H (1981) Regeneration of genetically variable plants from the anther-derived callus of *Arachis hypogaea* and *Arachis villosa*. Plant Science Letters 23: 35-39

Baker CM & Wetzstein HY (1998) Leaflet development, induction time, and medium influence somatic embryogenesis in peanut (*Arachis hypogaea* L.). Plant Cell Reports 17: 925-929

Barik DP, Mohapatra U & Chand PK (2005) High frequency *in vitro* regeneration of *Lathyrus sativus* L. Biologia Plantarum 49 (4): 637-639

Bhojwani SS (1981) A tissue culture method for propagation and low temperature storage of *Trifolium repens* genotypes. Physiologia Plantarum 52: 187-190

Bovo OA, Mroginski LA & Rey HY (1986) Regeneration of plants from callus tissue of the pasture legume *Lotononis bainesii*. Plant Cell Reports 5: 295-297

Caponetti JD, Gray DJ & Trigiano RN (2004) History of plant tissue culture. In: Trigiano RN & Gray DJ (eds) Plant development and biotechnology, (pp. 9-15). CRC Press, Boca Raton, Florida, USA

Chakraborti D, Sarkar A & Das S (2006) Efficient and rapid *in vitro* plant regeneration system for Indian cultivars of chickpea (*Cicer arietinum* L.). Plant Cell, Tissue and Organ Culture 86 (1): 117-123

Cheng M, Hsi DCH & Phillips GC (1992) *In vitro* regeneration of Valencia-type peanut (*Arachis hypogaea* L.) from cultured petiolules, epicotyl sections and other seedling explants. Peanut Science 19: 82-87

Chengalrayan K, Mhaske VB & Hazra S (1998) Genotypic control of peanut somatic embryogenesis. Plant Cell Reports 17: 522-525

Chengalrayan K, Hazra S & Gallo-Meagher M (2001) Histological analysis of somatic embryogenesis and organogenesis induced from mature zygotic embryo-derived leaflets of peanut (*Arachis hypogaea* L.). Plant Science 161: 415-421

Clarke HJ, Wilson JG, Kuo I, Lulsdorf MM, Mallikarjuna N, Kuo J & Siddique KHM (2006) Embryo rescue and plant regeneration *in vitro* of selfed chickpea (*Cicer arietinum* L.) and its wild annual relatives. Plant Cell, Tissue and Organ Culture 85: 197-204

Coelho LGM & Battistin A (1998) Meiotic behaviour of *Adesmia* DC. (Leguminosae-Faboideae) species native to Rio Grande do Sul, Brazil. Genet. Mol. Biol. 21 (3)

Collins GB & Phillips GC (1982) *In vitro* tissue culture and plant regeneration in *Trifolium pratense* L. In: Earle ED & Demarly Y (eds), Regeneration from cells and tissue culture, (pp. 22-34). Praeger Scientific Publishing, New York

Crews TE & Peoples MB (2004) Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. Agriculture, Ecosystems & Environment 102: 279-297

Delgado-Sanchez P, Saucedo-Ruiz M, Guzman-Maldonado SH, Villordo-Pineda E, Gonzalez-Chavira M & Fraire-Velazquez S, Acosta-Gallegos JA & Mora-Aviles A (2006) An organogenic plant regeneration system for common bean (*Phaseolus vulgaris* L.). Plant Science 170: 822–827

Ding YL, Aldao-Humble G, Ludlow E, Drayton M, Lin YH, Nagel J, Dupal M, Zhao G, Pallaghy C, Kalla R, Emmerling M & Spangenberg G (2003) Efficient plant regeneration and *Agrobacterium*-mediated transformation in *Medicago* and *Trifolium* species. Plant Science 165: 1419-1427

Dita M.A, Rispail N, Prats E, Rubiales D & Singh KB (2006) Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. Euphytica 147: 1-24

Dodd MB & Orr SJ (1995) Seasonal growth, phosphate response, and drought tolerance of 11 perennial legume species grown in a hill-country soil. New Zealand Journal of Agricultural Research 38: 7-20

Dunbar KB & Pittman RN (1992) Adventitious shoot formation from mature leaf explants of *Arachis* species. Crop Science 32: 1353-1356

Dunbar KB, Pinnow DL, Morris JB & Pittman RN (1993a) Virus elimination from interspecific *Arachis* hybrids. Plant Disease 77: 517-520

Dunbar KB, Pittman RN & Morris JB (1993b) *In vitro* culture of embryonic axes from *Arachis* species for germplasm recovery. Journal of Seed Technology 17(2): 1-8

Ezura H, Nukui N, Yuhashi KI & Minamisawa K (2000) *In vitro* plant regeneration in *Macroptilium atropurpureum*, a legume with a broad symbiont range for nodulation. Plant Science 159: 21-27

Frame J (2004) Forage legumes for temperate grasslands. Food and Agriculture Organization of the United Nations. Science Publishers, Inc. Enfield, USA. 309 pp

Furuya H, Pfahler PL & Quesenberry KH (2001) Diploid- autotetraploid comparisons in red clover: seed weight and temperature effects on seedling growth. Agronomy Abstr. CD-ROM, c01-pfahler131856-0

Gagliardi RF, Pacheco GP, Valls JFM & Mansur E (2002) Cryopreservation of cultivated and wild *Arachis* species embryonic axes using dessication and vitrification methods. CryoLetters 23: 61-68

Gagliardi RF, Pacheco GP, Carneiro LA, Valls JFM, Vieira MLC & Mansur E (2003) Cryopreservation of *Arachis* species by vitrification of *in vitro*-grown shoot apices and genetic stability of recovered plants. CryoLetters 24: 103-110

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50:151-158

Gill R & Ozias-Akins P (1999) Thidiazuron-induced highly morphogenic callus and high frequency regeneration of fertile peanut (*Arachis hypogaea* L.) plants. In Vitro Cellular and Developmental Biology- Plant 35: 445-450

Graham PH & Vance CP (2003) Legumes: Importance and constraints to greater use. Plant Physiology 131: 872-877

Griga M (1999) Somatic embryogenesis in grain legumes. In: Strnad M, Pec P & Beck E (eds) Advances of Regulation in Plant Growth and Development, (pp. 223-250). Peres Publ., Prague

Grosser JW & Collins GB (1984) Isolation and culture of *Trifolium rubens* protoplasts with whole plant regeneration. Plant Science Letters 37: 165-170

Hazarika BN (2006) Morpho-physiological disorders in *in vitro* culture of plants. Scientia Horticulturae 108: 105-120

Heath LC, Chin SF, Spencer D & Higgins TJV (1993) *In vitro* regeneration of commercial cultivars of subterranean clover. Plant Cell, Tissue and Organ Culture 35: 43-48

Hou SW & Jia JF (2004) Plant regeneration from protoplasts isolated from embryogenic calli of the forage legume *Astragalus melilotoides* Pall. Plant Cell Reports 22: 741-746

Huetteman CA & Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue and Organ Culture 33: 105-119

Jaftha JB, Strijdom BW & Steyn PL (2002) Characterization of pigmented methylotrophic bacteria which nodulate *Lotononis bainesii*. Systematic and Applied Microbiology 25: 440–449

Kane ME (2004) Shoot culture procedures. In: Trigiano RN & Gray DJ (eds), Plant development and biotechnology, (pp 145-157). CRC Press, Boca Raton, Florida, USA.

Kanyand M, Porobo Dessai A & Prakash CS (1994) Thidiazuron promotes high frequency regeneration of peanut (*Arachis hypogaea*) plants *in vitro*. Plant Cell Reports 14: 1-5

Kartha KK (1984) Tissue culture techniques for virus elimination and germplasm preservation. Plant Breeding Reviews 2: 265-282

Konieczny R (1995) Plant regeneration in callus culture of *Trifolium nigrescens* Viv. Acta Biologica Cracoviensia 37: 47-52

Konieczny R (1999) Organogenesis during plant regeneration in cotyledon culture of *Trifolium michelianum* Savi. Acta Societatis Botanicorum Poloniae 68(3): 175-181

Konieczny R (2000) Histological studies on callus and shoot induction in culture of *Trifolium nigrescensi* Viv. *in vitro.* Acta Biologica Cracoviensia 42 (1): 139-143.

Lakshmanan P & Taji A (2000) Somatic embryogenesis in leguminous plants. Plant Biology 2: 136-148

Lange O & Schifino-Wittmann MT (2000) Isozyme variation in wild and cultivated species of the genus *Trifolium* L. (Leguminosae). Annals of Botany 86: 339-345

Li Z, Jarret RL, Pittman RN, Dunbar KB & Demski JW (1993) Efficient plant regeneration from protoplast of *Arachis paraguariensis* Chod. Et Hassl. using a nurse culture method. Plant Cell, Tissue and Organ Culture 34: 83-90

Lian MN, Murthy HN & Paek KY (2002) Effects of light emitting diodes (LEDs) on the *in vitro* induction and growth of bulblets of *Lilium* oriental hybrid 'Pesaro'. Scientia Horticulturae 94: 365-370

Loidl P (2004) A plant dialect of the histone language. Trends in Plant Science 9(2): 84-90

Loiseau J, Michaux-Ferriere N & Le Deunff Y (1998) Histology of somatic embryogenesis in pea. Plant Physiology and Biochemistry 36 (9): 683-687

Lombari P, Ercolano E, El Alaoui H & Chiurazzi M (2003) A new transformation-regeneration procedure in the model legume *Lotus japonicus*: root explants as a source of large numbers of cells susceptible to *Agrobacterium*-mediated transformation. Plant Cell Reports 21: 771-777

Luo JP, Jia JF, Gu YH & Liu J (1999) High frequency somatic embryogenesis and plant regeneration in callus cultures of *Astragalus adsurgens* Pall. Plant Science 143: 93–99

Maheswaran G & Williams EG (1984) Direct somatic embryoid formation on immature embryos of *Trifolium repens*, *T. pratense* and *Medicago sativa*, and rapid clonal propagation of *T. repens*. Annals of Botany 54: 201-211

Mansur E, Lacorte C, Rabello ACG & Cordeiro AR (1993) *In vitro* regeneration of *Arachis villosulicarpa* Hoehne from cotyledon segments, leaves and cell suspension. Pesquisa Agropecuaria Brasileira 28 (10): 1143-1146

McGee JD, Wiliams EG, Collins GB & Hildebrand DF (1989) Somatic embryognesis in *Trifolium*: protein profiles associated with high- and low-frequency regeneration. Journal of Plant Physiology 135: 306-312

Microsoft (2003) Microsoft Excel. Release 11.0. Microsoft, Redmond, Washington, USA

Morris JB, Dunn S & Pittman RN (1995) Plant recovery from embryonic axes of deteriorated peanut seed for germplasm renewal. Peanut Science 22: 66-70

Mroginski E, Rey HY, Gonzalez AM & Mroginski LA (2004a) Thidiazuron promotes *in vitro* plant regeneration of *Arachis correntina* (Leguminosae) via organogenesis. Journal Plant Growth Regulation 23: 129-134

Mroginski LA, Sansberro PA & Flachsland EA (2004b) Establecimiento de cultivos de tejidos vegetales. In: Echenique V, Rubinstein C & Morginski L (eds) Biotecnología y mejoramiento vegetal, (pp 35-42). INTA. Buenos Aires, Argentina

Mundhara R & Rashid A (2006) Recalcitrant grain legume *Vigna radiata*, mung bean, made to regenerate on change of hormonal and cultural conditions. Plant Cell, Tissue and Organ Culture 85(3): 265-270

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497

Murthy BNS, Murch SJ & Saxena PK (1995) Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): Endogenous growth regulator levels and significance of cotyledons. Physiologia Plantarum 94: 268-276

Myers JR, Grosser JW, Taylor NL & Collins GB (1989) Genotype-dependent whole plant regeneration from protoplasts of red clover (*Trifolium pratense* L.). Plant Cell Tissue and Organ Culture 19: 113-127

Oelck MM, Bapat VA & Schieder O (1982) Protoplast culture of three legumes: *Arachis hypogaea*, *Melilotus officinalis*, *Trifolium resupinatum*. Z. Pflanzenphysiol. Bd. 106: 173-177

Olhoft PM, Flagel LE, Donovan CM & Somers DA (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Plants 216 (5): 723-735

Olmos SE, Luciani G & Galdeano E (2004) Micropropagation. In: Echenique V, Rubinstein C & Morginski L (eds) Biotecnología y mejoramiento vegetal, (pp 163-172). INTA. Buenos Aires, Argentina

Ozias-Akins P & Gill R (2001) Progress in the development of tissue culture and transformation methods applicable to the production of transgenic peanut. Peanut Science 28: 123-131

Peters M, Lascano CE, Roothaert R & de Haan NC (2003) Linking research on forage germplasm to farmers: the pathway to increased adoption- a CIAT, ILRI and IITA perspective. Field Crops Research 84: 179-188

Phillips GC & Collins GB (1979) Virus symptom-free plants of red clover using meristem culture. Crop Science 19: 213-216

Phillips GC, Collins GB & Taylor NL (1982) Interspecific hybridization of red clover (*Trifolium pratense* L.) with *T. sarosiense* Hazsl. using *in vitro* embryo rescue. Theoretical and Applied Genetics 62: 17-24

Pittman RN, Johnson BB & Banks DJ (1984) *In vitro* differenciation of a wild peanut, *Arachis villosulicarpa* Hoehne. Peanut Science 11: 24-27

Prasada Rao RDVJ, Pio-Ribeiro G, Pittman R, Reddy DVR & Demski JW (1995) *In vitro* culture to eliminate peanut stripe virus from peanut seeds. Peanut Science 22: 54-56

Pryzwara L, Pirog H, Konieczny R & Jach M (1996) The use of tissue culture methods in interspecific hybridization within the genus *Trifolium* L. Acta Biologica Cracoviensia 38: 53-65

Quesenberry KH & Smith RR (1993) Recurrent selection for plant regeneration from red clover tissue culture. Crop Science 33: 585-589

Quesenberry KH, Call NM, Moon DE & Dunn RA (1997) Response of native Eastern North American *Trifolium* spp. to root-knot nematodes. Crop Science 37: 270-274

Quesenberry KH, Smith RL, Mullaney JM & Blount AR (2003) Development, identification and characterization of tetraploid bahiagrass. CSSA. C01-quesenberry529179-poster

Radhakrishnan T, Murthy TGK, Desai S & Bandyopahyay A (1999) Meristem culture of interspecific hybrids of groundnut. Biologia Plantarum 42 (2): 309-312

Radhakrishnan T, Murthy TGK, Chandran K & Bandyopahyay A (2000) Micropropagation in peanut (*Arachis hypogaea* L.). Biologia Plantarum 43 (3): 447-450

Radice S (2004) Morfogénesis *in vitro*. In: Echenique V, Rubinstein C & Morginski L (eds) Biotecnología y mejoramiento vegetal, (pp 27-33). INTA. Buenos Aires, Argentina

Radionenko MA, Kuchuk NV, Khvedynich OA & Gleba YY (1994) Direct somatic embryogenesis and plant regeneration from protoplasts of red clover (*Trifolium pratense* L.). Plant Science 97: 75-81

Real R, Dalla Rizza M, Quesenberry KH & Echenique M (2004) Reproductive and Molecular Evidence for Allogamy in *Lotononis bainesii* Baker. Crop Science 44: 394-400

Rey HY & Mroginski LA (1996) Regeneration of plants from callus tissue of *Aeschynomene spp*. (Leguminosae). Plant Cell, Tissue and Organ Culture 45: 185-190

Rey HY & Mroginski LA (1997) Regenerationof plants from callus tissue of *Desmodium affine* and *Desmodium uncinatum*. Biologia Plantarum 39(2): 309-313

Rey HY & Mroginski LA (2006) Somatic embryogenesis and plant regeneration in diploid and triploid Arachis pintoi. Biologia Plantarum 50 (1): 152-155

Rodrigues LR, Terra TF, Bered F & Bodanese-Zanettini MH (2004) Origin of embryo-like structures in soybean anther culture investigated using SSR marker. Plant Cell Tissue and Organ Culture 77: 287-289

Rybcznski JJ (1997) Plant regeneration from highly embryogenic callus, cell suspension and protoplast cultures of *T. fragiferum*. Plant Cell Tissue and Organ Culture 51: 159-170

Saini R & Jaiwal PK (2002) Age, position in mother seedling, orientation, and polarity of the epicotyl segments of blackgram (*Vigna mungo* L. Hepper) determines its morphogenic response. Plant Science 163: 101-109

Singh AK & Chand S (2003) Somatic embryogenesis and plant regeneration from cotyledon explants of a timber-yielding leguminous tree, *Dalbergia sissoo* Roxb. Journal of Plant Physiology 160: 415-421

Singh ND, Sahoo L, Sarin NB & Jaiwal PK (2003) The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Millsp). Plant Science 164: 341-347

Speroni G & Izaguirre P (2003) Caracteristicas biologicas de la leguminosa native promisoria forrajera *Trifolium polymorphum* Poir. (Fabaceae, Faboideae). Agrociencia 7 (1): 68-76

Statisical Analysis Institute (2004) SAS/STAT User's Guide Release. Release 9.0. Statisical Analysis Institute, Cary, NC

Taylor NL, Anderson MK, Quesenberry KH & Watson L (1976) Doubling the chromosome number of *Trifolium* species using nitrous oxide. Crop. Sci. 16: 516-518

Tedesco SB, Dall'Agnol M, Schifino-Wittmann MT & Valls JFM (2000) Mode of reproduction of Brazilian species of *Adesmia* (Leguminosae). Genetics and Molecular Biology 23(2): 475-478

Tedesco SB, Stefanello MO, Schifino-Wittmann MT, Battistin A & Dall'Agnol M (2001) Superacao de dormencia em sementes de especies de *Adesmia* DC (Leguminosae). Rev. Bras de Agrociencia 7(2): 89-92

Tilman D, Cassman KG, Matson PA, Naylor R & Polasky S (2002) Agricultural sustainability and intensive production practices. Nature 418: 671-677

Tomlin ES, Branch SR, Chamberlain D, Gabe H, Wright MS & Stewart Jr. CN (2002) Screening of soybean, *Glycine max* (L.) Merrill, lines for somatic embryo induction and maturation capability from immature cotyledons. In Vitro Cellular and Developmental Biology- Plant 38: 543-548

Ulibarri EA & Burkart A (2000) Sinopsis de las especies de *Adesmia* (Leguminosae, Adesmiae) de la Argentina. Darviniana 38 (1-2): 59-126

Veltcheva M, Svetleva D, Petkova S & Perl A (2005) *In vitro* regeneration and genetic transformation of common bean (*Phaseolus vulgaris* L.)- Problems and progress. Scientia Horticulturae 107: 2-10

Vidoz ML, Klusacek P, Rey HY & Mroginski LA (2006) *In vitro* plant regeneration of *Arachis correntina* (Leguminosae) through somatic embryogenesis and organogenesis. Plant Cell, Tissue and Organ Culture 86 (1): 111-115

Webb KJ, Woodcock S & Chamberlain DA (1987) Plant regeneration from protoplasts of *Trifolium repens* and *Lotus corniculatus*. Plant Breeding 98: 111-118

Yamada T, Sakai A, Matsumura T & Higuchi S (1991) Cryopreservation of apical meristems of white clover (*Trifolium repens* L.). Plant Science 73: 111-116

Zohary M & Heller D (1984) The genus *Trifolium*. Israel Academy of Sciences and Humanities, Jerusalem. 606 pp

BIOGRAPHICAL SKETCH

Maria Laura Vidoz was born in Reconquista, Santa Fe, Argentina, on 3 November, 1975. At the age of two, her family moved to Corrientes, Argentina. She attended the Colegio San Jose from 1980 to 1993. In 1994 she began her undergraduate studies at the Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste. In 2002, she received her Agronomy Engineer degree. She worked from 1995 to 1997 at the Instituto de Botanica del Nordeste and then from 1997 to 2004 at the Vegetal Tissue Culture Laboratory, at the same university. She began her graduate career at the University of Florida in January 2005, under the direction of Dr. Kenneth Quesenberry. In December 2006 she was awarded a Master of Science degree in Agronomy with an emphasis in plant breeding and genetics. After graduation, Laura plans to pursue her Ph.D. before returning to Argentina to work in the field plant physiology.