African Journal of Biotechnology Vol. 11(41), pp. 9724-9731, 22 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2680
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Plant regeneration of *Lotononis bainesii* Baker (Fabaceae) through cotyledon and leaf culture

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Accepted 3 January, 2012

***Lotononis bainesii* Baker is a promising perennial forage legume for subtropical regions. The development of tissue culture methods for *in vitro* plant regeneration is useful, for example, for the propagation of selected plants and germplasm conservation. In addition, it could also facilitate crop improvement methods. For this last purpose, we performed experiments to develop a tissue culture protocol for different genotypes within a cultivar and from different explants of *L. bainesii*. Plant regeneration was obtained for over 50% of *L. bainesii* cv. INIA Glencoe genotypes evaluated via cotyledon culture and 90% of genotypes evaluated by leaflet culture in a medium composed of Murashige and Skoog medium (MS) + 4.5 µM thidiazuron (TDZ). Bud elongation and rooting were obtained upon transfer onto MS + 0.044 µM 6-benzyladenine (BA) + 0.049 µM indolebutyric acid (IBA). Although immature leaflet culture resulted in a higher number of responsive genotypes than cotyledon culture, plants regenerated from cotyledons exhibited a higher survival rate when transferred to *ex vitro* conditions. Culture medium supplementation with either Picloram (PIC) or 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in friable callus, that did not grow when subcultured. Immature leaflet insertion areas (petiole tip area where the three leaflets are attached) from expanding leaves and pieces of immature leaflets were the most efficient explants for shoot bud induction. A recommended protocol for *L. bainesii* plant regeneration would be placing immature leaflet explants on MS + 4.5 µM TDZ for 30 days; followed by transfer onto MS + 0.044 µM BA + 0.049 µM IBA for bud elongation and rooting.**

Key words: Explant type, forage legume, organogenesis, plant regeneration, shoot bud, thidiazuron.

INTRODUCTION

The family Fabaceae comprises between 18,000 and 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 and Vance, 2003). According to Crews and Peoples (2004), legumes are a more environmentally friendly source of nitrogen than are synthetic fertilizers due to their ability to fix nitrogen which reduces the risk of eutrophication and contamination of subterranean water.

The genus *Lotononis* belongs to the Fabaceae family, tribe Crotalarieae and consists of approximately 150 species, from herbs to small shrubs (Jaftha et al., 2002). Their broad distribution from Southern Africa to the

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Mediterranean region and India indicates that these species grow under dissimilar climatological and geographical environments. Among *Lotononis* species, *L. divaricata* (Eckl. and Zeyh.) Benth., *L. tenella* (E. Mey.) Eckl. and Zeyh. and *L. laxa* Eckl. and 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 these species for breeding programs has lead to the determination of their mode of reproduction using molecular techniques (Real et al., 2004). These studies have reported that, contrary to previous observations, *Lotononis* species should be regarded as allogamous. This implies that seeds from a given plant may correspond to different genotypes and specific genotypes cannot be propagated through seed. Therefore, other means of multiplication are required when several plants of a particular genotype are desired. A possible solution would be the development of a plant regeneration protocol that allows the propagation of selected plants. One such protocol has been developed for *L. bainessi* by Bovo et al. (1986). In this report, a low frequency of plant regeneration was obtained from cotyledons and leaflets. Moreover, a tissue culture protocol may be used to overcome the low seedling vigor of this species through the duplication of chromosomes using agents such as colchicine, oryzalin or trifluralin (Greplová et al., 2009).

After such treatment, cells with duplicated chromosomes need to be induced to follow either organogenesis or somatic embryogenesis, in order to produce non-chimeric polyploid plants. 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 and Gill, 2001). Only a subset of target cells are typically transformed after gene transfer procedures and not all of them survive and regenerate into plants. Since it is possible that transformed cells may not be competent for regeneration, it is essential to develop efficient tissue culture protocols through the adjustment of culture conditions such as culture medium and type of explant (Garcia et al., 2007). In many legumes, low responsiveness to *in vitro* culture hampers efficient genetic transformation (Arellano et al., 2009). The low level of investment in research applied to legumes, other than soybean, and the lack of efficient regeneration protocols have been blamed for the slow progress in legume biotechnology and tissue culture (Grewal et al., 2009).In addition to being used in crop improvement, successful regeneration protocols are valuable in functional genetics to develop gene identification strategies through plant transformation (Somers et al., 2003), and also in germplasm banks, for the recovery of material with low germination ability (Kanchiswamy and Maffei, 2008). The objective of these experiments was to develop a tissue culture protocol for different genotypes within a cultivar and from different explants of *L. bainesii* that could be used in future genetic improvement programs of this species.

MATERIALS AND METHODS

Cotyledon Culture

Seeds of *L. bainesii* cv. INIA Glencoe were scarified using concentrated sulphuric acid (98%) for 5 min and then rinsed for 10 min in running tap water. Subsequently, seeds were surface disinfected by immersion in a solution of sodium hypochlorite containing 0.57% w/v available chlorine for 5 min and rinsed three times with distilled sterile water. Disinfected seeds were placed on half-strength Murashige and Skoog (1962) (MS) basal medium (Sigma M5524), with 15 g/L sucrose and 7 g/L agar (Sigma A-1296) in 100 mm diameter × 15 mm deep Petri plates. 50 one-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 full strength MS alone or with 4.5 µM thidiazuron (TDZ) (Sigma P6186), 4.14 µM picloram (PIC) (Sigma P5575) or 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma D6679). These plant growth regulators were used in order to test the genotypes for either organogenesis (medium with TDZ) or somatic embryogenesis (media with PIC or 2,4-D). All media contained MS vitamins (Sigma M7150) and 30 g/L sucrose. The pH of the media was adjusted to 5.8 using 1 N KOH or 1 N HCl before the addition of 7 g/L agar (Sigma A-1296). Culture media were sterilized by autoclaving for 20 min at 0.103 Mpa.

Five explants were placed per Petri plate and the identity of genotypes was maintained throughout the experiment. After cotyledons were removed, seedlings were placed onto MS medium with 30 g/L sucrose and 7 g/L agar, and maintained *in vitro* for subsequent experiments. After 30 days of culture, regenerated buds were subcultured onto MS medium supplemented with 0.044 µM 6-benzyladenine (BA) + 0.049 µM indolebutyric acid (IBA) for an additional 30 days to achieve bud elongation and root formation. Developing plants were then transferred to Magenta boxes that contained MS lacking growth regulators for the same period of time before acclimatization *ex vitro*. All cultures were kept in a growth chamber at 26 ± 2°C with a 16 h 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 ± 2°C with a 14 h photoperiod. Plants were watered daily to keep humidity levels high 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.

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 as indicated before. Treatments, incubation and acclimatization were the same as in the previous experiment.

Type of explant

A genotype that had performed well in the cotyledon experiment

was used as the explant source for leaflet culture. Leaflets were excised and placed onto sterile filter paper where they were divided into petiole, petiole tip (region where 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 on a Petri plate containing MS supplemented with 10 μM TDZ. This procedure was repeated 10 times for both mature and immature leaves. 30 days after the initiation of the experiment, explants were subcultured to MS without growth regulators. Incubation conditions were the same as those described above.

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. The regeneration index was calculated for each replication separately and the mean value is reported here. This index is similar to the index used by Vila et al. (2007) for somatic embryogenesis. This experiment was statistically analyzed as a factorial arrangement in a completely randomized design (three parts of the leaf × two 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 treatment means.

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 (Figure 1A). When these calli were transferred onto MS + 0.044 μM BA + 0.049 μM IBA, they 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 1B). However, for 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 BA + 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 2A). Callus and bud formation started approximately seven and 15 days after the initiation of cultures, respectively (Figures 1C and D). In general, callus was dark green with dark brown areas. Considering only responsive genotypes, the mean number of buds per cotyledonary explant was 13.6. 60 days after culture, 48% of the total number of buds was capable of regenerating plants (177 plants/ 367 buds). In five out of 27 genotypes, the number of plants produced after 90 days of culture was superior to the number of buds produced after 30 days, indicating that during that period new buds were produced and regenerated whole plants (Figure 2C). In other genotypes, not all the buds present after 30 days resulted in plant regeneration. Moreover, nine genotypes produced buds, but they failed to elongate and finally died.

The lack of elongation and death of buds could have resulted from the hyperhydricity of tissues which may have been caused by a hormonal imbalance in the culture medium. Only 39% of plants transferred to *ex vitro* conditions were capable of successful acclimatization 30 days after the transfer (Figures 1F and 2E). This low survival rate is likely associated with the high incidence of hyperhydricity observed in regenerated plants. In addition to the influence of plant growth regulators in the culture medium, this abnormality may also have resulted 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 *Arachis hypogaea* L. (Gill and Ozias-Akins, 1999), *Vigna radiata* (L.) R. Wilczek (Mundhara and Rashid, 2006), *Trifolium* sp., *Medicago* sp. (Ding et al., 2003) and *Cajanus cajan* (L.) Huth (Singh et al., 2003). 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). In *Phaseolus lunatus* L., TDZ was used to induce shoot organogenesis, but several subcultures in different culture media were required before plant regeneration (Kanchiswamy and Maffei, 2008). In addition, it was observed that cotyledons were less efficient in regeneration when compared to hypocotyls or epicotyls, whereas in our experiment, they proved to be a good explant source for *in vitro* regeneration. Under our conditions, PIC and 2,4-D were not effective for somatic embryo induction in *L. bainesii*. Canhoto et al. (2006) have observed that these compounds have also failed to induce somatic embryogenesis in *Ceratonia siliqua* L., a leguminous tree. In contrast, 2,4-D and PIC were reported to be effective in other legume species such as *A. hypogaea* and *Cicer arietinum* L. and *Leucaena leucocephala* (Lam.) de Wit (Griga, 1999; Rastogi et al., 2008; Kiran Ghanti et al., 2009).

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 geno-

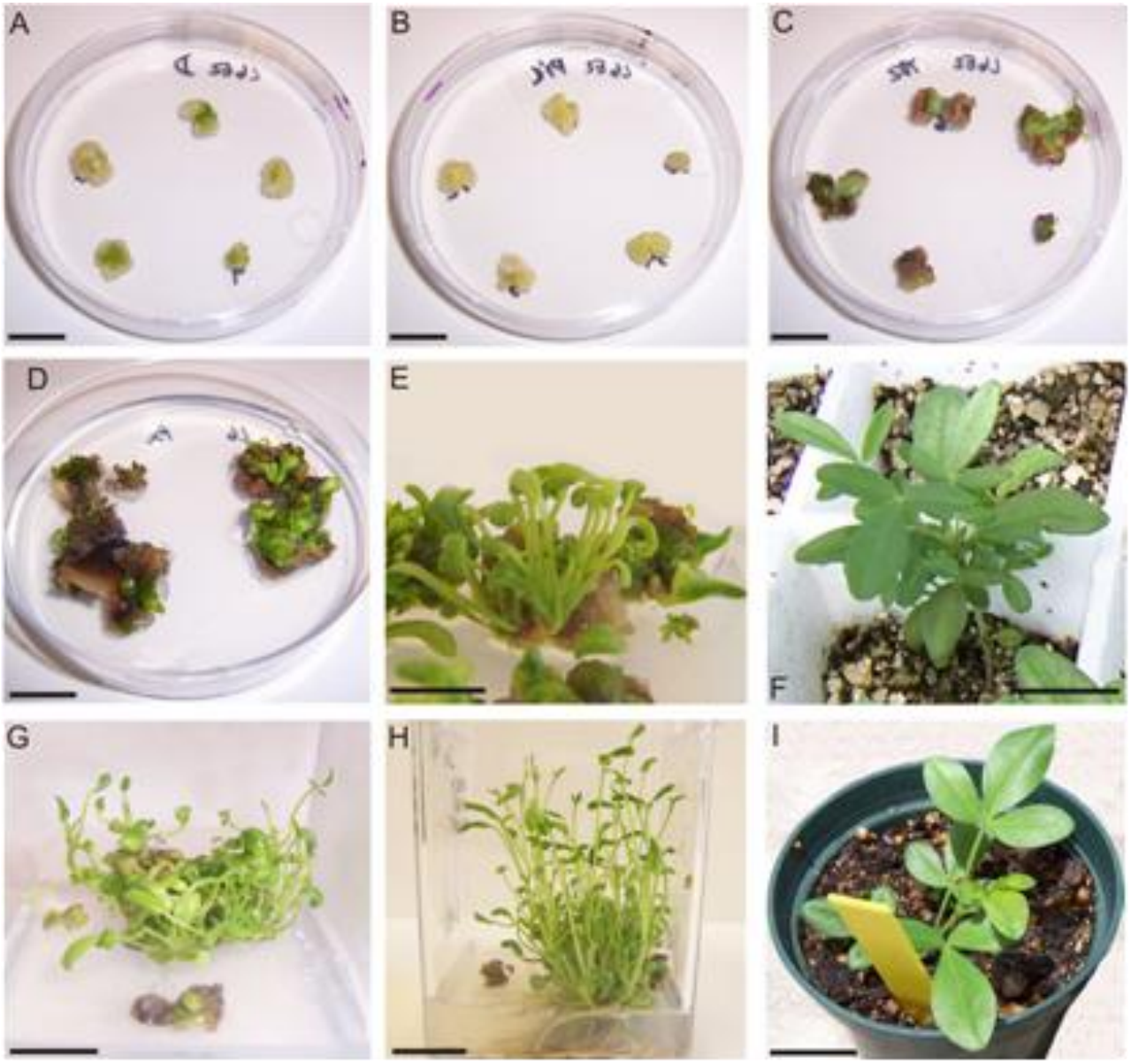

Figure 1. Organogenesis in *L. bainesii.* A) Cotyledon cultures 20 days after initiation of experiments in MS basal media supplemented with 2,4-D, B) PIC and C) TDZ (bar: 20 mm). D) Shoot bud formation from cotyledons 45 days after culture in MS + 0.044 µM BA + 0.049 µM IBA (bar: 20 mm). E) Elongated shoot buds 60 days after initiation of culture (bar: 15 mm). F) Plants successfully acclimatized 30 days after transfer to *ex vitro* conditions (bar: 15 mm). G-H) Shoot bud proliferation from leaflet explants originated in MS + 10 µM TDZ 60 and 90 days after initiation of cultures, respectively (bar: 20 mm). I) Acclimatized plant 30 days after transfer to *ex vitro* conditions (bar: 25 mm). 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; IBA, indolebutyric acid; PIC, picloram, 4-amino-3,5,6-trichloropicolinic acid; TDZ, thidiazuron, 1-phenyl-3-(1,2,3- thiadiazol-5-yl)urea.

types, as well as development of an *in vitro* chromosome doubling protocol.

Thirty days after the initiation of cultures, when growth regulators were absent, explants remained unresponsive except in 22 genotypes in which roots up to 10 cm long were produced from the cut surface of the midvein. This may suggest that the endogenous level of auxins in the leaflets were sufficient to induce rhizogenesis in the absence of an exogenous supply of plant growth regulators. 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 BA + 0.049

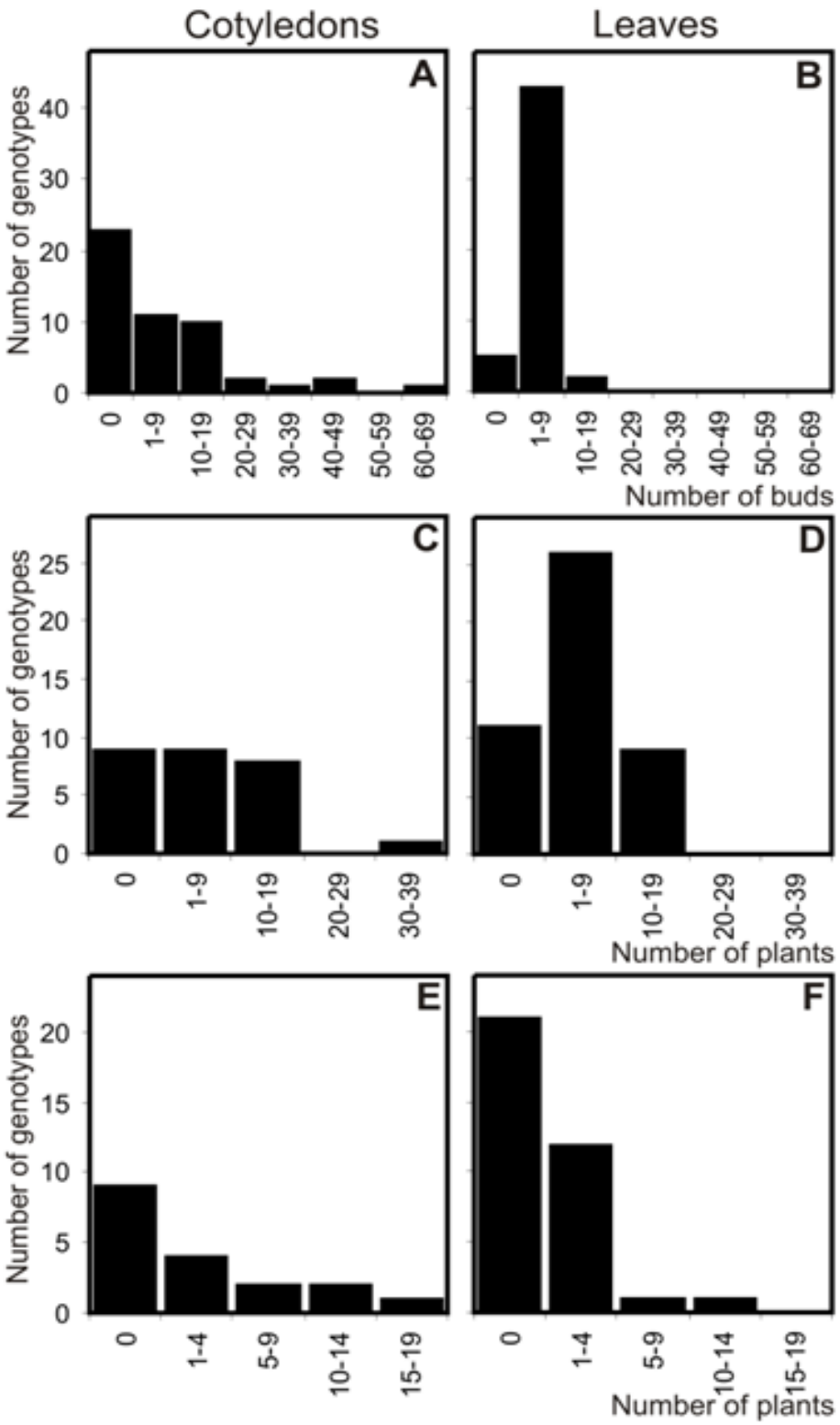

Figure 2. Histograms showing bud formation, plant regeneration and *ex vitro* acclimatization in *L. bainesii*. (A-B) Number of buds produced per explant by 50 genotypes of *L. bainesii* after 30 days of culture from cotyledon and leaflets, respectively. (C-D) Number of plants produced per explant after 90 days of culture by responsive genotypes through cotyledon and leaflet culture. (E-F) Number of successfully acclimatized plants in the genotypes that were capable of plant regeneration through cotyledon and leaflet culture, respectively.

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 out of five non-responsive explants also showed no response 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 1G and 2B). 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) (Figures 1H and 2D). This increment could have resulted from the fact that buds continued to be formed on MS + 0.044 μM BA + 0.049 μM IBA. Nevertheless, since this medium had such a low concentration of plant growth regulators, it is more probable that 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% of plants regenerated from leaflets was successfully acclimatized when transferred *ex vitro* (Figures 1I and 2F). This high plant mortality was likely due, once again, to hyperhydricity. 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). Similarly, Abogadallah and Quick (2010) have obtained lower regeneration rates and *ex vitro* survival in *Trifolium alexandrinum* L. plants regenerated in medium containing TDZ. These authors have attributed this inability of plants to acclimatize to a TDZ effect that could cause hyperhydricity and reduced growth. In our case, *in vitro* rooting was successfully achieved in culture medium without plant growth regulators, and therefore, it was not limiting for *ex vitro* acclimatization, In contrast, this step has been difficult to attain *in vitro* in *Phaseolus vulgaris* L., where efficient rooting was only obtained after transfer to culture media devoid of cytokinins (Kwapata et al., 2010).

It is interesting that more genotypes were responsive as leaflet explants rather than as cotyledon explants. 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-day-old failed to undergo somatic embryogenesis using TDZ compared to up to 97% of six-day-old seedlings (Murthy et al., 1995). Nevertheless, in another legume species, *Albizia odoratissima* (L. f.) Benth, cotyledons failed to regenerate shoots whereas plant regeneration was possible from both epicotyl- and petiole-derived explants (Rajeswari and Paliwal, 2008). In our experiments, the lower cotyledon response in *L. bainesii* may have been caused by the scarification/surface disinfection procedures the seeds 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 this 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, therefore rooting was not a critical factor in whole plant regeneration.

Interestingly, 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 explants

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 assess 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 (P = 0.004), whereas leaf age and interaction between leaf part and leaf age were not significant (P = 0.45 and 0.12, respectively). Bud organogenesis from leaflets was similar to that from leaflet insertion areas (85.8% from leaflet insertion explants and 83.3% from leaflet explants) and higher than that from petioles (48.3%). The number of buds per explant ranged from 1.1 to 3.4 (Table 1) but, similar to bud organogenesis percentage, there was no leaf age by leaf part interaction for number of buds per explant (P = 0.29). In contrast to percentage of bud organogenesis, the mean number of buds per explant did not differ among parts of the leaf (P = 0.60), but did differ due to leaf age (P = 0.003). Explants from immature leaves gave a higher number of buds per explant (2.9) than those from mature ones (1.5) (Table 1).

Considering the regeneration index, there was a significant effect of the part and age of the leaf but not a significant interaction (P = 0.03; 0.05 and 0.12, respectively). 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).

Table 1. 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.

Parameter	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 $P \leq 0.05$. † Index = (% shoot bud formation x mean number of buds) / 100.

Leaflet insertion area from immature leaves had the highest index (3.4) over all combinations. As proposed before by Pacheco et al. (2008) in *Arachis stenosperma* Krapov and W.C. Gregory, the different *in vitro* response among the three parts of the leaf tested might have been due to a difference in the endogenous levels of plant growth regulators or to a different sensitivity to them.

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 and Wetzstein, 1998). In contrast, with *Arachis villosulicarpa* Hoehne, the best organogenic frequency and mean number of buds per explant were obtained when mature fully expanded leaves were used as explants (Dunbar and 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 this present experiment. This could be due to the conditions under which mother plants were grown, a factor that greatly affects *in vitro* responsiveness (Debergh and Maene, 1981).

In conclusion, 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 BA + 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 to the incidence of hyperhydricity. 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. The experiment carried out with one genotype of *L. bainesii* and different explant types 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: (a) use immature leaflets as explants, which are as efficient as leaflet insertion areas but a larger amount of tissue is obtained per leaf; (b) culture on MS + 4.5 µM TDZ for 30 days; and (c) transfer to MS + 0.044 µM BA + 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.

ACKNOWLEDGEMENT

This research was supported in part by USDA-CSREES grant no. 2005-34135-16010 for Tropical and Subtropical Agricultural Research.

Abbreviations

2,4-D, 2,4-Dichlorophenoxyacetic acid; **BA,** 6-benzyladenine; **IBA,** indolebutyric acid; **PIC,** picloram, 4-amino-3,5,6-trichloropicolinic acid; **TDZ,** thidiazuron, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea; **MS,** Murashige and Skoog medium.

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