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In vitro plant regeneration from cotyledonary nodes of recombinant inbred lines of lentil

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ABSTRACT

An efficient and reproducible *in vitro* regeneration protocol for lentil was developed. For shoot regeneration, cotyledonary node explants of ten elite genotypes were cultured in an inverted orientation on different shoot regeneration media that consisted of Murashige and Skoog (MS) medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BAP) (M1), 1 mg L^{-1} BAP + 0.45 mg L^{-1} indole-3-acetic acid (IAA) (M2), and 2 mg L^{-1} BAP (M3). High percentages of shoot regeneration ranging from 80 to 100% on M1 and M3 media and from 50 to 100% on M2 medium were induced. M1 was the most efficient shoot regeneration medium for most genotypes tested. For rooting, *in vitro* and *in vitro–in vivo* methods were used. Low and variable rooting percentages ranging from 0 to 45% were recorded with *in vitro–in vivo* method. Efficiency of rooting on *in vitro* medium varied depending on the medium in which shoots had been previously regenerated and the genotype tested. When M1 medium was used, high rooting percentages (over 40%) for most genotypes except for microsperma genotypes were found. When the 10 genotypes were screened for good regeneration performance using M1 medium, 2 main clusters and 3 subgroups within one of the clusters were formed based on similarities respect of the number of regenerated shoots per explant and rooting percentages. Subgroup 1 composed by A1146 genotype produced the highest number of shoots per explant (6.17 shoots) and a high rooting percentage (60%) so was selected for further transformation and use as a potential commercial variety.

1. Introduction

Lentil (*Lens culinaris* Medik.) is an important food legume crop for human consumption due to their outstanding protein and fiber content. Seed protein content varies from 22 to 35%, with relatively high levels of lysine, leucine and sulfur-amino acids (Christou, 1997).

It is widely grown in the Indian subcontinent, Middle East, northern Africa, southern Europe, North and South America, Canada, Australia and New Zealand (Ford et al., 2007). India was the leading producer of lentil until 2007 (FAOSTAT, 2011), producing about one third of the total world production, predominantly for its own domestic usage (Ford et al., 2007). However, since then Canada has become the major world producer and the largest exporter of lentil (FAOSTAT, 2011). The main areas of the world consuming lentils are concentrated in Asia, northern Africa, Western Europe, countries of the Andean Community and Brazil (Sagpya, 2008).

Lentil suffers from poor genetic resources, thus limiting the application of biotechnological tools for crop improvement. One of the problems in Argentina is the narrow genetic base of lentil (4 varieties) which must be broadened through introgression of new genes from exotic germplasm (Crippa et al., 2009) because the maintenance of diversity in agriculture is essential to protect plant genetic resources, to maintain the genotype–environment interaction, and to provide greater production stability.

An alternative approach for the improvement of this crop is to complement traditional breeding methods with biotechnology techniques to regenerate plants from single cells and organized tissues and to transfer desirable genes from other sources. Gene transfer in lentil has been difficult and challenging because of its recalcitrant nature to *in vitro* regeneration (Gulati and Mc Hughen, 2003). Establishment of an efficient and repeatable *in vitro* regeneration protocol is one of the basic prerequisites for gene transformation and plant breeding (Omran et al., 2008). Regeneration and transformation procedures of lentil are not well developed compared to the success achieved in other grain legumes (Sarker et al., 2003). Successful *in vitro* plant regeneration depends on the

Abbreviations: BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; IVS, *in vitro* soil-less; KN, kinetin; MS, Murashige and Skoog; RILs, recombinant inbred lines; TDZ, thiadiazuron.

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determination of critical regeneration parameters such as explant source, genotype and the composition of medium, especially growth regulators (Ahmad et al., 1997). Lentil shoots have been regenerated from shoot apices (Williams and McHughen, 1986; Singh and Raghuvanshi, 1989), nodal explants (Polanco et al., 1988; Fratini and Ruiz, 2003), intact seedlings (Malik and Saxena, 1992), cotyledonary nodes (Gulati et al., 2001; Sarker et al., 2003; Chhabra et al., 2008), epicotyls (Omran et al., 2008), decapitated embryos (Sarker et al., 2003) and immature seeds (Polanco and Ruiz, 2001). Among all these explants, the frequency of shoot regeneration from cotyledonary nodes was very high (Warkentin and McHughen, 1993; Gulati et al., 2001). Shoot regeneration has been obtained using media with kinetin (KN) (Bajaj and Dhanju, 1979; Williams and McHughen, 1986; Singh and Raghuvanshi, 1989), thiadiazuron (TDZ) (Malik and Saxena, 1992), and 6-benzylaminopurine (BAP) (Saxena and King, 1987; Polanco et al., 1988).

Root induction has been conventionally approached by means of studies of different auxins at different concentrations but in most cases, the reported rooting frequencies have been poor (Williams and McHughen, 1986; Polanco et al., 1988; Singh and Raghuvanshi, 1989; Khawar and Ozcan, 2002; Sarker et al., 2003; Khawar et al., 2004).

Fratini and Ruiz (2003) described a procedure that greatly improved rooting of *in vitro* cultured lentil and other hypogeous legumes (pea, chickpea and *Lathyrus*) by simply inverting nodal segments explants in the rooting medium. They showed that inverted explants (apical end in medium) of lentil exhibited higher rooting frequencies and average number of shoots regenerated per explant than explants cultured in a normal orientation (basal end in medium). A limited number of studies have been published in ornamental crops with regard to the relationship of explant orientation, shoot regeneration and capacity to induce root morphogenesis *in vitro* (Pierik and Segers, 1973; Pierik and Steegmans, 1975; Choudhary and Prasad, 1990; Zhao et al., 2007). Furthermore, in a preliminary experiment Bermejo et al. (2009) studied the effect of explant orientation in lentil both on the number of shoots regenerated per explant and rooting. These results showed non-significant shoot number differences between normal (3–4 shoots per explant) and inverted orientation (4–5 shoots per explant) but in inverted orientation the rooting frequencies were higher (100%). Thus the inverted orientation of the explants for shoot regeneration and rooting was chosen in the present work.

The main objective of this work was to develop an efficient and reproducible *in vitro* regeneration protocol for lentil based on cotyledonary node explants cultured in an inverted orientation of ten elite genotypes from our breeding program and then to select those genotypes with the best performance in this regeneration system for further transformation and use as potential commercial varieties.

2. Materials and methods

2.1. Plant material

Nine recombinant inbred lines (RILs) of lentil selected for high seed yields from our breeding program (B1181, B1182, B1051, B1052, A1145, A1146, A1062, B1151, B1153) and a commercial cultivar (Silvina) were used for *in vitro* regeneration studies. B1181 and B1182 varieties are microsperma type whereas the remaining are of a macrosperma type. These RILs derived from an intra-specific cross between ILL 8072, ILL 6972 and local microsperma type parentals.

Healthy seeds were surface sterilized with 70% ethanol for 2 s, then in 3.5% sodium hypochlorite for 15 min, and rinsed 4 times with sterile distilled water. The seeds were placed aseptically on half-strength Murashige and Skoog (1962; MS) medium containing 1.5% sucrose and 0.45% agar in glass vials for germination at 23 ± 2 °C in the dark. Cotyledonary nodes were excised from 3-day-old seedlings by removing the shoot apex and root meristem to within 3–4 mm of the node and cotyledons to within 1 mm of the node.

2.2. Culture media

2.2.1. For shoot regeneration

For shoot regeneration M0, M1, M2 and, M3 media were used. M0 medium was MS medium with 3% sucrose and 0.8% agar without growth regulators. M1, M2 and M3 media consisted of the M0 medium supplemented with 1 mg L^{-1} BAP, 1 mg L^{-1} BAP + 0.45 mg L^{-1} indole-3-acetic acid (IAA), and 2 mg L^{-1} BAP respectively. All these media were adjusted to pH 5.8 prior to addition of agar and autoclaved at 121 °C for 20 min.

Cotyledonary node explants were cultured in an inverted orientation by embedding the epicotyl end onto M1, M2 and M3 media. A total of 20 explants per genotype and medium were cultured individually in culture tubes. The experiment was repeated 3 times. After 15 days all explants were transferred with inverted orientation to M0 medium and then were subcultured with the same orientation on this free hormone medium every 15 days to reduce the inhibition effect of BAP on root formation. Visual observations of the cultures were made periodically until the end of the experiment. The percentage of explants producing shoots *i.e.* percentage of shoot regeneration, the average number of shoots regenerated per explant (from explants which regenerated shoots) and the total number of shoots regenerated per genotype and medium (every 20 explants) were scored after 15 and 43 days of culture.

Cultures were maintained in a growth room at a temperature of 23 ± 2 °C and a 16:8 h light regime (30 μmol m^{-2} s^{-1} from cool white fluorescent lamps).

2.2.2. In vitro and in vitro–in vivo root induction

The percentages of rooted explants (from explants which regenerated shoots) were recorded after 1 and 2 months of culture in M0 medium. In the rooted explants, most regenerated shoots were excised and transferred to RM40 medium with normal orientation (basal end in medium) for root induction. RM40 medium was 1/2 MS with 1% sucrose, 0.8% agar, and 7 mg L^{-1} IAA. The remaining regenerated shoots (1–3) were left attached to the original explant and this rooted explant was transferred to plastic pots containing sterile *in vitro* soil-less (IVS) medium for acclimatization. IVS medium comprised sphagnum peat, coarse river sand (1–3 mm d.), and perlite (Horticulture grade P500) at a ratio of 0.5:2:2 and was sterilized for 40 min at 121 °C prior to use. In the non-rooted explants all the regenerated shoots were cut out and transferred with normal orientation to RM40 for root induction. After a 6-day pulse on RM40 at 23 ± 2 °C in the dark all the excised shoots were transferred with normal orientation to sterilized glass vials containing sterile IVS medium for rooting. Data on percentage of rooted shoots in IVS medium were weekly recorded.

2.3. Acclimatization

After rooting, plantlets were removed from culture tubes and glass vials, washed thoroughly with distilled water to remove the remaining medium and planted in plastic pots containing sterile IVS medium. For the first month, plantlets were covered with transparent polyethylene bags to maintain high humidity. They were watered once a week using half-strength MS salt solution. When the bags were removed plantlets were watered twice a week with distilled water. The plants were kept in a growth room at 23 ± 2 °C and a 16:8 h light regime (30 μmol m^{-2} s^{-1} from cool white fluorescent lamps). Data on percentage survival of plants rooted *in vitro*

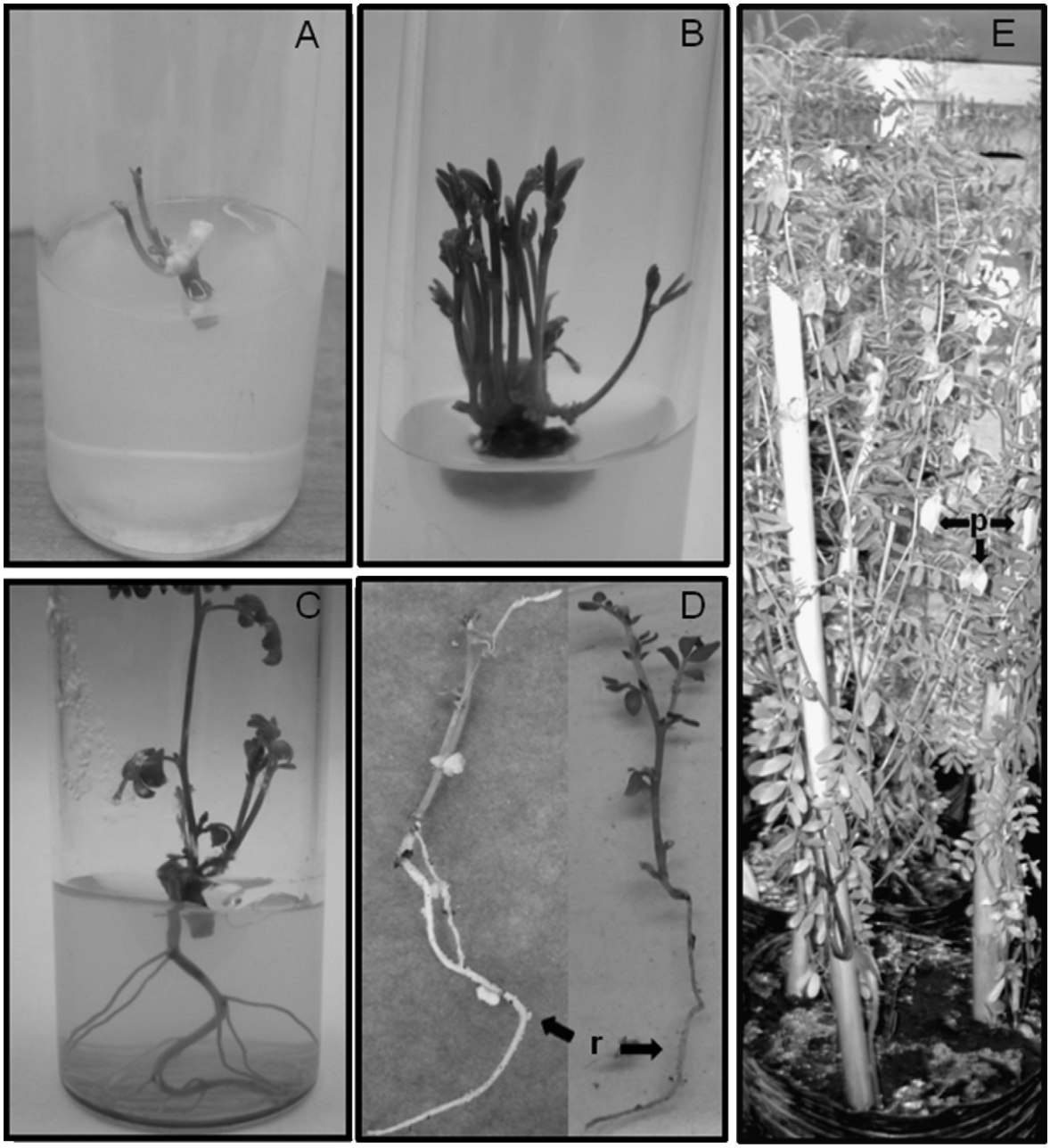

Fig. 1. Lentil plant regeneration from cotyledonary nodes cultured in an inverted orientation (apical end in medium). (A and B) Shoot development from cotyledonary node explants after 8 days of culture on M1 medium and 20 days of culture, respectively. (C) *In vitro* rooting of cotyledonary node explants after 2 months of culture on M0 medium. (D) *In vitro–in vivo* rooting of excised shoots, with roots (r) protruding after 6 days of culture on 7 mg L^{-1} IAA and 8 days of culture on IVS medium. (E) Acclimatization of rooted plants showing normal phenotype with normal pods (p) after 2 months on IVS medium.

and *in vitro–in vivo* were recorded at 2 months after transplantation in IVS medium.

2.4. Data analysis

Data collected from all 3 repetitions were subjected to analysis of variance using a randomized complete block design and differences between the means were compared by Duncan's multiple range test ($P \leq 0.05$) using the general linear model (GLM) of SAS package (SAS, 1985). To establish groups of similar genotypes, a cluster analysis was carried out and a dendrogram was generated using "Ward's minimum variance" through the Info-Gen software (Balzarini and Di Renzo, 2003). Data on percentage of explants producing shoots and percentage of rooted shoots in IVS medium were subjected to arcsine transformation (Snedecor and Cochran, 1967) to satisfy the requirements of normality, before statistical analysis.

3. Results

3.1. Shoot regeneration

After 7–10 days of culture on M1, M2 and M3 media, multiple buds and shoots readily regenerated from cotyledonary nodes of lentil cultured in an inverted orientation (Fig. 1A). All shoots arose in the axils of the cotyledonary petioles. Shoot development always followed normal geotropism with shoots growing upwards. When these explants were transferred to MS medium without

Table 1
Effect of 10 lentil genotypes and 3 different shoot regeneration media (M1, M2 and M3) on the average number of shoots regenerated per explant after 43 days of culture. Means ± SE of 3 replicates. Mean values with the same letter within a column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

Genotypes	Shoot regeneration media		
	M1 No. of shoots/explant	M2 No. of shoots/explant	M3 No. of shoots/explant
Silvina	4.41 ± 0.05^{ab}	4.06 ± 0.70^{ab}	4.56 ± 0.60^{abc}
B1181	5.00 ± 0.56^{ab}	2.73 ± 0.73^{a}	4.66 ± 0.56^{abc}
B1182	4.90 ± 0.25^{ab}	3.14 ± 0.31^{ab}	4.43 ± 0.31^{abc}
B1051	5.44 ± 0.49^{bc}	4.99 ± 0.72^{b}	5.40 ± 0.42^{c}
B1052	4.97 ± 0.18^{ab}	4.13 ± 0.55^{ab}	4.31 ± 0.52^{abc}
A1145	5.35 ± 0.29^{bc}	4.34 ± 0.43^{ab}	3.85 ± 0.39^{ab}
A1146	6.17 ± 0.47^{c}	4.93 ± 0.52^{b}	5.07 ± 0.04^{bc}
A1062	4.09 ± 0.27^{a}	2.96 ± 0.24^{ab}	3.60 ± 0.15^{a}
B1151	5.21 ± 0.16^{abc}	4.59 ± 0.42^{ab}	4.65 ± 0.43^{abc}
B1153	4.80 ± 0.46^{ab}	4.61 ± 0.99^{ab}	4.41 ± 0.51^{abc}

hormones (M0), its buds developed into shoots, the regenerated shoots elongated and new buds were produced (Fig. 1B).

All genotypes and media tested were efficient in the induction of shoots. There was no significant influence of the genotype on the percentage of shoot regeneration at 15 and 43 days of culture, however, an effect of media on shoot regeneration was observed at 15 days of culture ($F = 3.41$; $P < 0.05$). High percentages of shoot regeneration ranging from 80% to 100% on M1 and M3 media and from 50% to 100% on M2 medium were induced. Only 2 genotypes, B1052 and A1145, showed moderate percentages of shoot regeneration on M2 medium, 50% and 60% respectively. No significant differences among media were found at 43 days of culture. The interaction between the 2 independent variables, genotype and medium, was non-significant at 15 and 43 days of culture.

Variance analysis revealed that the average number of shoots regenerated per explant and the total number of shoots regenerated per genotype and medium (every 20 explants) were strongly influenced by genotype ($F = 8.87$, $F = 7.63$ respectively; $P < 0.0001$) and media ($F = 47.27$, $F = 45.52$ respectively; $P < 0.0001$) after 15 days of culture, and also showed highly significant differences among genotypes ($F = 4.14$, $F = 3.98$ respectively; $P < 0.0005$) and media ($F = 11.28$, $F = 12.52$ respectively; $P < 0.0001$) at 43 days of culture while the interaction between genotype and medium was non-significant at 15 and 43 days of culture.

As it is shown in Table 1, when studying the genotype influence on the number of shoots regenerated per explant within each medium, it was observed that on M1 medium, the genotypes that regenerated the highest number of shoots were A1146 (6–7 shoots per explant), B1051, A1145 and B1151 (5–6 shoots per explant). On M2 and M3 media the genotypes that regenerated the highest number of shoots were B1051 and A1146 (4–5 shoots per explant on M2 medium and 5–6 shoots per explant on M3 medium).

As it can be observed in Table 1, the highest mean values for the number of shoots regenerated per explant were obtained on M1 medium for most genotypes tested. Only the commercial variety Silvina regenerated higher number of shoots on M3 medium. However this variety also regenerated a high number of shoots on M1 and M2 media, showing stable behavior in all the tested media (Table 1). From these results M1 medium seems to be one of the most efficient shoot regeneration media in all genotypes tested with a number of shoots regenerated per explant ranging from 4.09 to 6.17 mean values for A1062 and A1146 genotypes respectively (Table 1).

3.2. In vitro rooting

The percentages of rooted explants were recorded after 1 and 2 months of culture in M0 medium. Fig. 1C shows a cotyledonary node explant cultured on M0 medium in an inverted orientation (apical end in medium) with regenerated shoots and roots protruding after 2 months in culture. Root formation always occurred at the basal end. The regenerated roots always maintained normal geotropism with roots growing downwards.

Variance analysis indicated that the efficiency of rooting in M0 medium varied depending on the medium in which shoots had been previously regenerated ($F = 4.10$; $P < 0.05$ and $F = 3.22$; $P < 0.05$ for 1 and 2 months of culture in M0 respectively) and the genotype tested ($F = 3.42$; $P < 0.05$ and $F = 2.92$; $P < 0.05$ for 1 and 2 months of culture in M0 respectively). However, the interaction between these 2 variables was non-significant at 1 and 2 months of culture in M0.

As it is shown in Table 2, the explants with regenerated shoots on M1 and M2 media showed significantly higher percentages of rooting (44.93% and 45.63% respectively) than the explants on M3 medium (32.08%) after 2 months of culture on M0 medium. When M1 shoot regeneration medium was used, high rooting percentages (over 40%) for most genotypes except for microsperma varieties B1181 (20%) and B1182 (10%) were found, and also high rooting percentages ranging from 30% to 70% when M2 medium was tested (Table 2).

3.3. Cluster analysis

Having shown a high percentage of shoot regeneration, a high number of regenerated shoots and a high rooting percentage for all genotypes tested, the M1 medium was selected to study the

Table 2
Rooting percentages after 2 months of culture on M0 medium and 8 days on IVS medium for 10 genotypes of lentil that regenerated shoots on 3 different media M1, M2 and M3. Means ± SE of 3 replicates. Mean values with the same letter within a column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

Genotypes	Shoot regeneration media					
	M1		M2		M3	
	Rooting on M0 [%]	Rooting on IVS [%]	Rooting on M0 [%]	Rooting on IVS [%]	Rooting on M0 [%]	Rooting on IVS [%]
Silvina	68.00 ± 13.32^{b}	23.33 ± 14.53^{a}	44.33 ± 5.67^{a}	4.76 ± 4.76^{a}	66.00 ± 11.37^{c}	13.33 ± 13.33^{a}
B1181	20.00 ± 5.77^{ab}	10.00 ± 5.09^{a}	27.50 ± 18.43^{a}	26.39 ± 18.69^{a}	10.00 ± 5.77^{ab}	45.00 ± 18.93^{a}
B1182	10.00 ± 5.77^{a}	0.00 ± 0.00^{a}	42.17 ± 12.30^{a}	0.00 ± 0.00^{a}	3.70 ± 3.70^{a}	7.87 ± 3.96^{a}
B1051	47.67 ± 20.22^{ab}	9.09 ± 9.09^{a}	58.33 ± 17.40^{a}	23.28 ± 16.66^{a}	53.33 ± 23.33^{bc}	3.00 ± 3.00^{a}
B1052	50.67 ± 10.90^{ab}	19.67 ± 15.39^{a}	47.67 ± 25.67^{a}	13.10 ± 7.24^{a}	37.67 ± 11.78^{abc}	5.67 ± 5.67^{a}
A1145	60.67 ± 8.29^{ab}	0.00 ± 0.00^{a}	42.00 ± 13.32^{a}	16.67 ± 16.67^{a}	21.00 ± 6.66^{ab}	0.00 ± 0.00^{a}
A1146	60.00 ± 15.28^{ab}	7.33 ± 7.33^{a}	48.33 ± 4.41^{a}	0.00 ± 0.00^{a}	48.33 ± 14.81^{bc}	0.00 ± 0.00^{a}
A1062	52.92 ± 21.08^{ab}	11.00 ± 11.00^{a}	33.50 ± 2.18^{a}	0.00 ± 0.00^{a}	37.77 ± 11.76^{abc}	6.67 ± 6.67^{a}
B1151	42.33 ± 22.56^{ab}	0.00 ± 0.00^{a}	46.50 ± 23.62^{a}	13.89 ± 13.89^{a}	24.67 ± 18.12^{abc}	33.33 ± 33.33^{a}
B1153	37.00 ± 17.39^{ab}	28.89 ± 19.75^{a}	66.00 ± 11.37^{a}	6.06 ± 6.06^{a}	18.33 ± 9.28^{ab}	11.00 ± 11.00^{a}
Averages	44.93^{c}	10.93^{a}	45.63^{c}	10.42^{a}	32.08^{b}	12.59^{a}

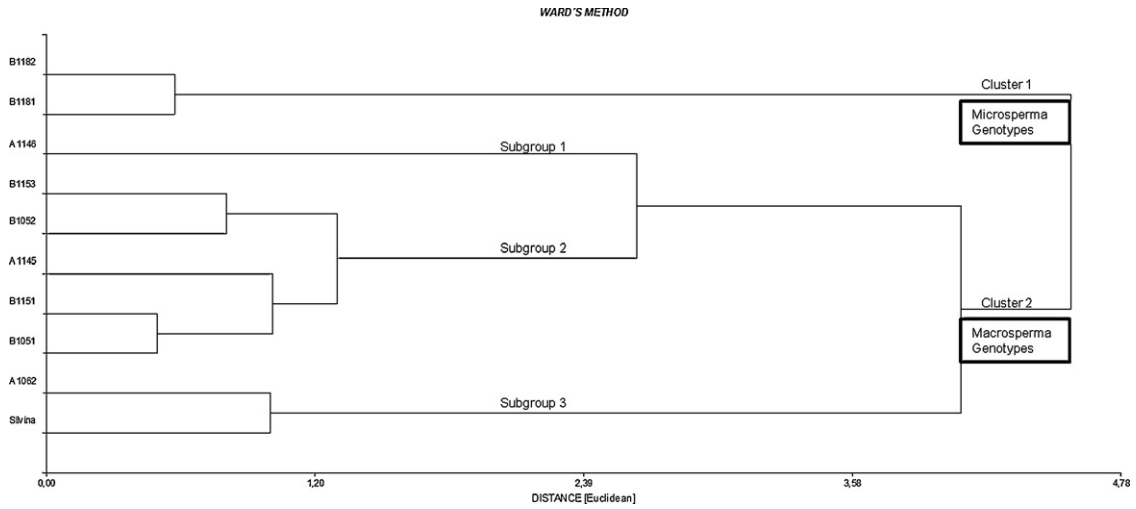

Fig. 2. Dendrogram compiled by Ward's method showing the grouping of 10 lentil genotypes based on the average number of shoots regenerated per explant after 43 days of culture and the rooting percentages after 2 months on M0 medium.

regeneration performance of the ten genotypes. Cluster analysis, based on both the average number of shoots regenerated per explant after 43 days of culture and the rooting percentages after 2 months on M0 medium, allowed the 10 genotypes to be divided into 2 main clusters (Fig. 2). Cluster 1 was composed by the microsperma varieties (B1181 and B1182), which presented the lowest mean value for rooting percentage (15%), while the rest of the genotypes, grouped in Cluster 2, showed high mean values for rooting percentage (>47%). Cluster 2 was divided into 3 subgroups based on the average number of shoots regenerated per explant. Subgroup 1 composed by A1146 genotype produced the highest number of shoots (6.17 shoots) and Subgroup 3, composed by Silvina and A1062 genotypes, the lowest mean value (4.25 shoots) (Table 3 and Fig. 2).

3.4. *In vitro–in vivo* rooting

As we saw earlier, an *in vitro* rooting medium (M0) has been used for explants rooting, but only a few explants (20 explants per genotype and medium) were used and some explants failed to *in vitro* rooting. In order to increase the number of rooted shoots, numerous regenerated shoots from rooted and non-rooted explants were excised and subjected to an *in vitro–in vivo* rooting method. Increasing the number of rooted shoots is essential to obtain more whole plants for further studies.

The excised shoots were pulsed for 6 days on the RM40 root induction medium and then were transferred to IVS medium for rooting. After 8 days on this medium, shoots formed adventitious roots which emerged at the cut end (Fig. 1D). Variance analysis showed that there was no significant influence of the genotypes and shoot regeneration media on rooting percentage in IVS medium. Low and variable rooting percentages ranging from 0% to 45% were recorded on IVS medium (Table 2). B1181 was the genotype that showed the highest rooting percentage on IVS (45%) when M3 shoot regeneration medium was used (Table 2). When comparing the rooting media, M0 medium was observed to be much better for rooting percentage than IVS medium for most genotypes (Table 2). However, B1181 genotype presented higher rooting percentage (45%) on IVS medium as compared to M0 medium (10%) from M3 shoot regeneration medium (Table 2).

Table 3
Mean values of the average number of shoots regenerated per explant on M1 medium after 43 days of culture and the rooting percentages after 2 months on M0 medium for the 2 clusters formed in the cluster analysis. Mean values with the same letter within a column are not significantly different at $P = 0.05$ according to Duncan's multiple range test.

Cluster	Subgroup	No. of shoots/explant	Rooting [%]
1		4.95[b]	15.00[a]
2	1	6.17[c]	60.00[b]
2	2	5.15[b]	47.67[b]
2	3	4.25[a]	60.46[b]

3.5. *Acclimatization*

The rooted plants obtained on M0 and IVS media were acclimatized for 2 months in plastic pots containing sterile IVS medium. These plants were phenotypically normal and produced normal pods and viable seeds (Fig. 1E). As it can be observed in Table 4, when the *in vitro–in vivo* rooting method was used, higher survival percentages (over 50%) in IVS were found for Silvina, B1052, A1062 and B1153 genotypes. When the *in vitro* method was used, B1182, B1051, A1145, A1146 and B1151 genotypes showed superior survival percentages ranging from 11% to 44% in IVS. B1181 genotype had similar behavior in both methods with survival percentages of about 40%. Table 4 also shows the survival percentage data when both methods were used together to complement one another. For Silvina, A1145, A1146, A1062 and B1153 genotypes survival percentages of about 20% were found. B1181, B1182, B1051 and B1052 genotypes showed higher survival percentages of about

Table 4
Percentage of successful transplants in IVS medium when the plants were rooted in an *in vitro* and *in vitro–in vivo* medium (data collected after 2 months of transfer to IVS) (M0 + IVS refers to the results obtained when the 2 media were used together to complement one another).

Genotypes	Percentage of successful transplants [%]		
	In vitro (M0)	*In vitro–in vivo* (IVS)	M0 + IVS
Silvina	18	50	23
B1181	40	44	43
B1182	44	0	36
B1051	42	25	39
B1052	33	67	36
A1145	24	0	19
A1146	26	0	26
A1062	17	100	21
B1151	11	0	9
B1153	0	50	18

40% and B1151 genotype had the lowest survival percentage in IVS of only 9%.

4. Discussion

Regeneration in plant tissue culture is often a genetically controlled trait (Sugiyama, 1999). In our case variance analysis indicated that all genotypes tested showed similar percentages of regeneration. The low genotypic effect observed may be explained by a narrow genetic basis among the genotypes tested but this should be confirmed later by molecular markers. With regard to the media it is known that a high level of cytokinin to auxin ratio favors shoot formation and this has been shown for many plant species (Torrey, 1958). In this study, the M1 and M3 media containing only BAP presented higher regeneration percentages than the M2 medium containing BAP + IAA at 15 days of culture but these differences among media on regeneration capability disappeared at 43 days of culture, maybe because the presence of auxin on M2 medium delayed shoot regeneration.

The genotypes and shoot regeneration media used in this study revealed a strong influence on the number of regenerated shoots and rooting percentage.

Up to date, in lentil (Malik and Saxena, 1992; Polanco and Ruiz, 1997, 2001; Fratini and Ruiz, 2002), as well as in other pulse crops (Mohamed et al., 1992; Gulati and Jaiwal, 1994; Prakash et al., 1994; Sanago et al., 1996; Polisetty et al., 1997; Subhadra et al., 1998), it has been observed that at increasing concentrations of BAP or TDZ in the culture medium, a higher number of shoots can be regenerated. However, shoot length and subsequent rooting was greatly reduced.

In the present study, although all the regeneration media induced high number of shoots, the M1 medium with the lowest BAP concentration (1 mg L^{-1}) regenerated the highest number of shoots per explant ranging from 4.09 to 6.17 mean values in all the genotypes tested. Warkentin and McHughen (1993) and Gulati et al. (2001) also reported multiple shoot regeneration from cotyledonary node explants on MS medium supplemented with 1 mg L^{-1} BAP.

Our procedure is a considerable improvement over the above mentioned procedures because it displays a complete regeneration of shoots and roots with the lowest concentration of BAP (1 mg L^{-1}) for shoot regeneration, and without any growth regulators for rooting (M0). Besides, IVS medium, composed of simple and cheap components, was used for acclimatization. This protocol allows a lower cost for plant regeneration.

Other novelty of the present study is the culture of cotyledonary node explants of lentil in an inverted orientation for both shoot regeneration and rooting. The higher shoot regeneration capability (6–7 shoots per explant) from inverted cotyledonary nodes in this study (Table 1) contrasts with the number of shoots regenerated from axilllary buds of inverted nodal segments (2–3 shoots per explant) in other papers of lentil (Fratini and Ruiz, 2003).

The rooting of regenerated shoots is a very important step and it is often the bottleneck point for the regeneration of whole plants *in vitro* (Polanco and Ruiz, 2001). Traditional *in vitro* rooting studies focus on the application of phytohormones, especially auxins, in order to induce a rooting response (Newell et al., 2006). However, this study focuses on the provision of greater aeration at the proximal end of the explants and regenerated shoots for rooting. The basal end of the explants was exposed to air by inverting cotyledonary nodes on a growth-regulator-free medium (M0). The choosing of this rooting medium and the inverted orientation of the cultured explants was due to the fact that, in previous studies, comparing nodal explants with normal and inverted orientation and different culture media, the highest rooting percentages (100%) were obtained for inverted explants on M0 medium (data not shown). This latter experiment agreed with the Fratini and Ruiz (2003) results. The greater aeration at the basal end of the regenerated shoots was obtained by excising them, retaining its normal orientation but providing an aerobic rooting environment (IVS medium). The benefits of using a generic rooting strategy such as IVS have been demonstrated on a wide range of Australian plants (Newell et al., 2005) and only one cultivar of lentil (Newell et al., 2006). In the present work, this protocol was tested on a wide range of lentil genotypes but the rooting frequencies obtained were poor and they were lower than those obtained with the *in vitro* rooting medium (M0) (Table 2).

In vitro rooting percentages of 40% are usually considered good (Polanco and Ruiz, 2001). In our case, the explants with regenerated shoots on media containing 1 mg L^{-1} BAP (M1 and M2 media) showed significantly higher percentages of rooting (over 40%) than those on medium containing 2 mg L^{-1} BAP (M3 medium). These results indicate an inhibitory effect of high concentrations of BAP on *in vitro* rooting. In earlier studies, an inhibitory effect of BAP on root induction of shoots has been reported (Polanco et al., 1988; Polanco and Ruiz, 1997).

Polanco and Ruiz (1997), who studied the inhibitory effect of BAP on *in vitro* and *in vivo* root formation of lentil, concluded that success in rooting depends on the nature and concentration of cytokinin used for induction of shoots and the time period for which explants are exposed to cytokinin prior transferring to a rooting medium. In the current study, since the time of shoot exposure to cytokinin was short (15 days), the concentration of this hormone on shoot regeneration media was low and the successive transfers of explants to a hormone free medium, the inhibitory effect of BAP was low and successful rooting was achieved.

The higher number of shoots regenerated and higher rooting percentages obtained with M1 shoot regeneration medium make this medium the most efficient one for *in vitro* culture of lentil. So the 10 RILs were screened for good regeneration performance using M1 medium in order to select those genotypes with the highest potential of regeneration. 2 main clusters and 3 subgroups within one of the clusters were formed based on similarities respect of the number of regenerated shoots per explant and rooting percentages (Fig. 2). Cluster 1, composed by the microsperma varieties, showed a lower mean value for rooting percentage (15%) than Cluster 2, composed by the macrosperma varieties (mean value for rooting percentage >47%). To our knowledge, this is the first study which differentiates, compares, and shows that micro and macrosperma types not only differ in morphological traits (cotyledon color and flowers, the size of the seed and adaptation to dry environments) but also in the rooting ability.

Although all the genotypes grouped in both clusters regenerated a high number of shoots in 1 mg L^{-1} BAP, the A1146 genotype (Subgroup 1 of Cluster 2) produced the highest number of shoots (6.17) and a high rooting percentage (60%) (Table 3 and Fig. 2). So this genotype will be selected for further studies of transformation.

Altaf et al. (1998) showed that microsperma lentil needed auxins for root induction in the regenerated shoots. Pulsing with auxin is a recognized strategy for inducing rooting in plants which are difficult to root (Zimmerman and Fordham, 1985). We have shown here that lentil genotypes can also be rooted using a 6-day 7 mg L^{-1} IAA pulse followed by a transfer to IVS medium. This *in vitro–in vivo* rooting method was not influenced by the genotypes or the shoot regeneration media. In this study we combined the *in vitro–in vivo* rooting method with the *in vitro* rooting method in order to increase the production of whole lentil plants.

Transplantation of regenerated grain legume plantlets to soil in pots is usually not successful. Generally, high mortality rates are due to soft, weak stems and roots without good differentiation of vessels that are unable to acquire the nutrients from soil when

transferred from agar medium (Gulati et al., 2001). In our study, the success rate of transplantation showed moderate values ranging from 20% to 40% (Table 4), being an important feature to be improved in further studies.

Plant tissue culture is a cornerstone of modern lentil breeding programs including the raising of interspecific hybrids, genetic engineering, and haploid induction. It is advantageous to have a general effective protocol that does not require individual optimization for each genotype (Newell et al., 2006).

For legumes such as lentils, which have been regarded as recalcitrant to transformation, regeneration *in vitro* is highly genotype specific, and only rarely are cultivated varieties amenable to regeneration (Somers et al., 2003). In our study, all the macrosperma lentil genotypes showed high regeneration potential when both M1 shoot regeneration medium and *in vitro* rooting method were used. Expanding the range of genotypes within a species that undergo the requisite tissue culture process would provide a major contribution to improving the transformation system.

4.1. Conclusions

The results obtained in this experiment implied that an efficient and robust *in vitro* regeneration protocol for lentil could be developed, at a low cost, based on inverted cotyledonary node explants cultured on a shoot regeneration medium containing 1 mg L^{-1} BAP, and using 2 methods of rooting: *in vitro* and *in vitro–in vivo* methods with whole plant establishment on IVS medium. This protocol is an original method to differentiate micro and macrosperma types. Also, the regeneration system developed in this study can be employed in lentil genetic transformation and in propagation of this species. A1146 genotype from our breeding program showed the best performance in this regeneration system so it can be used in transformation studies and as a potential commercial variety.

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