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ORIGINAL ARTICLE

In vitro embryo culture to shorten the breeding cycle in lentil (*Lens culinaris* Medik)

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Abstract Breeding in lentil involves hybridization followed by different selection methods and requires 10 years to obtain a cultivar, as only one field generation per year can be produced. To shorten the breeding time it is essential to use biotechnological methods such as in vitro embryo culture combined with SSD method since only one seed is enough to produce the next generation. An efficient in vitro–in vivo system was developed. The best time to extract immature embryos and the best culture medium to obtain their complete development were analyzed. Embryos of Pardina, B1181 (microsperma type), B1051 and A1145 (macrosperma type) were collected at 15, 18, 21, and 24 days after pollination (DAP) and cultured on MS medium with five different concentrations of 6-benzylaminopurine (BAP) (0–0.025–0.05–0.1–0.25 mg L^{-1}). An ANOVA test among genotypes, media, DAP and their interactions was performed. Genotypes, DAP and its interaction showed significant effects on the percentage of shoot production ($F=61.8$; $F=79.3$; $F=8.5$; $p<0.01$) and germination ($F=70.7$; $F=69.8$; $F=3.9$; $p<0.01$). Medium effect was only significant for germination ($F=8.7$; $p<0.01$). The microsperma genotypes gave higher percentages of shoot production (>80 %) and germination (>70 %). Although in vitro culture efficiency increased with DAP, 18 DAP was selected due to its high percentages of germination (13–70 %). The medium without BAP was the most suitable for embryo complete development (41–87 %). All plants obtained were morphologically normal and fertile. Using this approach, four generations per year were obtained allowing a rapid development of RILs.

Keywords Lentil · In vitro culture · Immature seeds · Short generation cycles

Abbreviations

BAP	6-Benzylaminopurine
DAP	Days after pollination
MS	Murashige and Skoog
RILs	Recombinant inbred lines
SSD	Single seed descent

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).

Breeding in lentil involves hybridization followed by different selection methods and requires, at least, 10 years to obtain a new cultivar, as only one field generation per year can be produced.

The length of the breeding cycle from seed to seed is often a limiting factor in plant breeding, for the

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development of recombinant inbred lines (RILs) required for genetic analysis, and the exploitation of molecular marker technology (Ochatt et al. 2002; Ochatt and Sangwan 2008). For these reasons, decreasing the length of the generation cycle will accelerate genetic improvement. To shorten the breeding time it is useful to use biotechnological methods such as in vitro embryo culture combined with single seed descent (SSD) method which allows the attainment of homozygous lines in a relatively short time. The SSD method consists of taking a single seed from each F_2 plant and advancing each seed to the next generation until a desired level of homozygosity is achieved (F_6–F_8), thus saving space and time (Goulden 1939). Homozygosity can be attained by selfing successive generations or by haploidization of hybrids using different in vitro techniques and doubling chromosomes in haploid plants (Croser et al. 2006; Wędzony et al. 2008; Germanà 2011). However, doubled haploid technology has not been widely developed in grain legumes and to date, no robust protocol has been proposed in lentil. Currently, SSD populations are frequently used as alternatives to doubled haploids populations in genetic and genomic studies (Marza et al. 2005; Kuchel et al. 2006).

Modified SSD systems involving a first step of immature seeds in vitro culture for germination and a second step ex vitro for full development have been proposed to significantly shorten the breeding cycles in grain legumes such as pea and lupin (Ochatt et al. 2002; Surma et al. 2013). Recently, in lentil, the length of the generation cycle has been reduced using an in vitro only strategy (Mobini et al. 2014) and an in vivo only strategy (Croser et al. 2014). However, to date, no information was found for accelerating breeding process in lentil using in vitro plus in vivo method.

The aim of the present study was to develop an efficient in vitro–in vivo system for the culture of lentil immature embryos to be used in combination with the SSD technique to accelerate the attainment of RILs.

Materials and methods

Four recombinant inbred lines (RILs) of lentil from our breeding program were used as plant material. Pardina and B1181 belong to the microsperma type and B1051 and A1145 to the macrosperma type. These genotypes were chosen on the basis of differences not only in the seed size but also in days to flowering and maturity and in grain yield (Table 1). Twenty-five mature seeds of each genotype were sown in June 2014 in 1 L pots containing a mixture of peat and perlite, and grown in a greenhouse at the Faculty of Agronomy of National University of Rosario (Zavalla, Argentina; lat. 33°1′S; long. 60°53′W) until pods extraction.

Flowers were tagged at the 3/4 stage of petals to sepals, moment at which anthesis occurs and fertilization takes place (Wilson 1972). Pods were harvested at 15, 18, 21 and 24 days after pollination (DAP) and surface sterilized by immersion for about 5 s in 70 % (v/v) ethanol followed by immersion in 3.5 % (v/v) sodium hypochlorite solution for 10 min, and were then rinsed 4 times with sterile distilled water. Pods were opened aseptically with a scalpel and the immature seeds were extracted, measured in diameter and then cultured on full-strength Murashige and Skoog (1962) (MS) medium with 0.175 mg L^{-1} indole-3-acetic acid (IAA) and 0, 0.025, 0.05, 0.1 or 0.25 mg L^{-1} 6-benzylaminopurine (BAP) supplemented with 1 % sucrose and 0.6 % agar–agar bacteriological grade (Britania) yielding a total of 20 different treatments. In each treatment a total of 20 immature seeds were cultured in glass tubes (four immature seeds per glass tube). Media were always adjusted to a pH of 5.8 prior to autoclaving at 121 °C for 20 min. Cultures were maintained in a growth room at 24 ± 1 °C and a 16:8 h light regime (with a photosynthetic photon flux density of 30 µmol m^{-2} s^{-1} from cool white fluorescent lamps). After a week, embryos were released from the ovular integuments and transferred to glass tubes containing fresh medium of the same composition. Embryos were placed in upright position into the medium.

Table 1 Lentil genotypes used in this study and their main characteristics

Name	Type	Seed size (mm)	Grain yield (g/plant)	Days to flowering in the field[a]	Days to maturity in the field[b]
B1051	Macrosperma	6.0	6.3	78	162
A1145	Macrosperma	6.0	4.2	80	165
B1181	Microsperma	3.8	5.0	110	190
Pardina	Microsperma	4.5	3.7	107	180

Means of data collected during 2013–2015 at the experimental farm of the National University of Rosario at Zavalla (Argentina)

[a]From sowing to flowering without applying the in vitro–in vivo method

[b]From sowing to seed harvest without applying the in vitro–in vivo method

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The percentage of explants producing only shoots i.e. percentage of shoot production was scored after 14 days of culture and the percentage of explants producing shoots and roots i.e. germination percentage was quantified after 21 days of culture.

After in vitro embryos germination, plantlets were removed from culture tubes and washed thoroughly with distilled water to remove the remaining medium. They were planted in plastic pots containing a sterile commercial soil mix (Grow Mix Multipro, Terrafertil®) composed of sphagnum peat and perlite (Horticulture grade P500) and kept in a growth room at 24 ± 1°C and a 16:8 h light regime (with a photosynthetic photon flux density of 30 µmol m^{-2} s^{-1} from cool white fluorescent lamps) for 2 weeks. For the first week, plantlets were covered with transparent polyethylene bags to maintain high humidity and they were watered using half-strength MS salt solution. Then the bags were removed and plantlets were watered with distilled water for another week. Hardened plants were transplanted to 1L pots containing the commercial soil mix (Grow Mix Multipro, Terrafertil®) and grown in a greenhouse for about a month until flowering. The number of feasible generations per year was calculated, based on the number of days elapsed from the date of pollination for R_0 generation up to the date of pollination for the next generation (R_1).

Statistical analysis

The experiment was repeated twice. Data collected from all two repetitions were subjected to analysis of variance (ANOVA) using a randomized complete block design and differences between the means were compared by least significant difference (LSD) test ($P \leq 0.05$) using the Info-Gen software (Balzarini and Di Rienzo 2003).

Results

The effects of genotype, seed age (DAP) and BAP concentration added to MS medium, in relation to the dependent variables percentage of shoot production and germination were studied. Immature seeds measured 2 mm at 15 DAP, 3–4 mm at 18 DAP, 5 mm at 21 DAP and 6 mm at 24 DAP.

Variance analysis revealed that the genotypes, age of embryo (DAP) and its interaction showed significant effects on the percentage of shoot production and germination. Medium effect and its interaction with age of embryo were only significant for germination (Table 2).

As it is shown in Table 3, the microsperma genotypes gave higher percentages of shoot production (>80%) and germination (>70%) than macrosperma genotypes. B1051 genotype gave the lowest percentage of germination (32.5%).

Table 2 Variance analysis showing the effect of genotype, culture media, age of embryo (DAP) and their interactions on the percentage of shoot production and germination

Source of variation	df	Shoot production		Germination	
		Mean square	*F*	Mean square	*F*
Model	80	1539.94	7.45***	2117.98	7.08***
Genotype (G)	3	12775.62	61.83***	21166.61	70.74***
Medium (M)	4	281.18	1.36[a]	2599.43	8.69***
DAP	3	16385.12	79.3***	20896.21	69.83***
Block	1	58.81	0.28[a]	6.81	0.02[a]
G×M	12	361.15	1.75[a]	350.77	1.17[a]
G×DAP	9	1761.61	8.53***	1165.31	3.89***
M×DAP	12	209.27	1.01[a]	609.48	2.04*
G×M×DAP	36	328.62	1.59*	300.95	1.01[a]
Error	79	206.63		299.22	

*Significant at $p < 0.05$, ***Significant at $p < 0.001$

[a]Non significant differences

Table 3 Percentages of shoot production and germination for the different macrosperma and microsperma genotypes tested

Genotypes	Shoot production (%)	Germination (%)
B1051	54.03 ± 5.45[a]	32.50 ± 4.65[a]
A1145	65.10 ± 3.00[b]	44.55 ± 3.93[b]
B1181	80.45 ± 4.74[c]	71.63 ± 5.79[c]
Pardina	94.95 ± 1.92[d]	81.90 ± 3.54[d]

Data are the averages of all the media and seed age (DAP) tested. Means ± SE of two replicates. Mean values with the same letter within a column are not significantly different at P = 0.05 according to the LSD test

Table 4 Percentages of shoot production and germination in relation to genotypes and immature seed age (DAP)

DAP	A1145	B1051	B1181	Pardina
Shoot production (%)				
15	47.70 ± 4.71[a]	9.60 ± 0.27[a]	45.90 ± 10.00[a]	79.80 ± 5.46[a]
18	68.20 ± 6.08[b]	42.60 ± 6.66[b]	76.70 ± 8.59[b]	100.00 ± 0.00[b]
21	71.90 ± 6.53[b]	91.30 ± 2.00[d]	99.20 ± 0.80[c]	100.00 ± 0.00[b]
24	72.60 ± 2.93[b]	72.60 ± 6.26[c]	100.00 ± 0.00[c]	100.00 ± 0.00[b]
Germination (%)				
15	23.90 ± 4.13[a]	3.60 ± 1.48[a]	33.60 ± 9.70[a]	70.90 ± 4.85[a]
18	38.90 ± 7.39[ab]	12.60 ± 3.87[a]	53.70 ± 10.73[a]	70.10 ± 10.26[a]
21	47.60 ± 8.97[b]	52.80 ± 7.82[b]	99.20 ± 0.80[b]	92.60 ± 3.87[b]
24	67.80 ± 1.81[c]	61.00 ± 4.82[b]	100.00 ± 0.00[b]	94.00 ± 4.00[b]

Data are the averages of all the media tested. Means ± SE of two replicates. Mean values with the same letter within a column are not significantly different at P = 0.05 according to the LSD test

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The percentage of shoot production and germination increased with seed maturity and seed size. The shoot production percentage increased significantly up to 18 DAP for A1145 and Pardina genotypes and up to 21 DAP for B1181 genotype and then plateaued (Table 4). For B1051 genotype this percentage increased significantly up to 21 DAP and then declined (Table 4). Regarding the percentage of germination, it increased significantly up to 21 DAP and then plateaued for almost all the genotypes except for A1145 in which continued increasing up to 24 DAP (Table 4), even if the differences were not always statistically significant.

When comparing the culture media, the medium without BAP was observed to be the most effective for the production of plantlets for all seed ages, with percentages higher than 40 % (Table 5).

The in vitro–in vivo system developed in this study is described in Fig. 1, involving a first stage of fertilization and pods extraction (Fig. 1a, b), then a step of immature seeds in vitro culture for germination (Fig. 1c, d) and a final step ex vitro for full development (Fig. 1e).

Lentil plants obtained from immature seeds survived transfer to ex vitro conditions with a success of about 30 %. They started to flower 65 ± 3 days after the beginning of in vitro embryo culture. Moreover, they were morphologically normal and fertile and set at least 10 pods with 1–3 seeds per pod. As only one seed is enough to maintain a population of RILs, this protocol is efficient to be used in combination with a SSD technique to accelerate the attainment of RILs. With the proposed in vitro–in vivo protocol, four generations per year can be obtained for the genotypes tested.

Table 5 Percentages of germination in relation to immature seed age (DAP) and culture media with different BAP concentrations

Media	15 DAP	18 DAP	21 DAP	24 DAP
Germination (%)				
0 BAP	41.25 ± 12.44^{a}	62.25 ± 10.97^{c}	83.13 ± 9.35^{b}	86.75 ± 5.01^{c}
0.025 mg L^{-1} BAP	29.25 ± 12.14^{a}	52.75 ± 15.36^{bc}	74.75 ± 10.19^{b}	76.25 ± 9.33^{ab}
0.05 mg L^{-1} BAP	33.38 ± 10.77^{a}	56.88 ± 9.16^{c}	77.25 ± 10.37^{b}	79.75 ± 8.06^{abc}
0.1 mg L^{-1} BAP	31.88 ± 9.08^{a}	31.63 ± 10.21^{ab}	75.50 ± 8.92^{b}	85.75 ± 6.07^{bc}
0.25 mg L^{-1} BAP	29.25 ± 11.46^{a}	15.63 ± 4.62^{a}	54.63 ± 13.68^{a}	75.00 ± 5.91^{a}

Data are the averages of all the genotypes tested. Means ± SE of two replicates. Mean values with the same letter within a column are not significantly different at P = 0.05 according to the LSD test

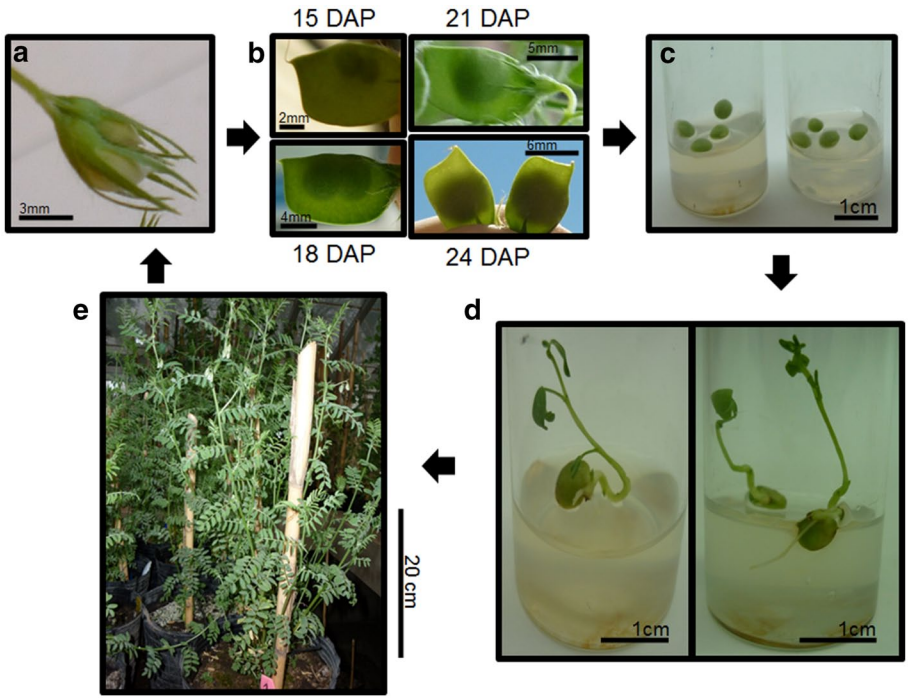

Fig. 1 The in vitro–in vivo system. **a** Moment at which anthesis occurs and fertilization takes place. **b** Pods harvested at 15, 18, 21 and 24 days after pollination (DAP) containing immature seeds. **c** In vitro culture of immature seeds at 18 DAP (four immature seeds per glass tube). **d** In vitro germination of immature seeds producing shoots and roots (one and two immature seeds per glass tube, the other seeds were subcultured). **e** Lentil plants grown in a greenhouse until flowering

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Discussion

In order to shorten the generation cycles in lentil, it is essential to establish the best in vitro conditions for the culture of embryos as the first step in the development of an efficient in vitro assisted single seed descent technique.

As the culture medium replaces the endosperm and provides the nutrients to the developing embryo, its composition is a major factor for successful embryo rescue. In this work, the best time to extract immature embryos and the most effective culture medium for their complete development were analyzed. With regard to the culture media the variance analysis showed that there was significant influence of the different BAP concentrations on germination percentage but not in the shoot production. The highest efficiency of in vitro culture from immature seeds was achieved on MS medium without BAP and the lowest on MS with the highest BAP concentration tested (Table 5). These results could indicate an inhibitory effect of high concentrations of BAP on in vitro rooting. In earlier studies, an inhibitory effect of BAP on lentil root induction has been reported (Polanco et al. 1988; Polanco and Ruiz 1997).

The percentage of shoot production and germination increased with seed maturity and seed size. These results agree with those reported by Polanco and Ruiz (2001) and Fratini and Ruiz (2006). The relationship between the morphogenic potential and developmental stage of embryos has been demonstrated in legumes and other species (Özcan et al. 1993; Colijn-Hooymans et al. 1994).

Although in vitro culture efficiency increased with DAP, and 21 DAP showed higher germination percentages than 18 DAP, this latter was selected as the best time to extract immature embryos because it allowed to reduce the breeding cycle with a germination rate higher than 13 %, which is enough to maintain a RILs population. This percentage is encouraging if we use this immature seed protocol combined with SSD method since only one seed is required to produce the next generation, making this protocol a practical option for generation advancement in lentil.

Polanco and Ruiz (2001) established that seeds in the developmental 4 mm stage (18 DAP) and 5–6 mm stage (21 and 24 DAP) are at an appropriate age for shoot production with a high probability of success. Ribalta et al. (2014) reported that the best germination rates in pea were also obtained by culturing immature seeds between 18 and 20 days after flowering.

Regeneration in plant tissue culture is often a genetically controlled trait (Sugiyama 1999). For legumes such as lentils, regeneration in vitro is highly genotype specific, and only rarely are cultivated varieties amenable to regeneration (Somers et al. 2003). Kasten and Kunert (1991) and Surma et al. (2013) reported that the response of lupin immature embryos cultured in vitro varied depending on cultivar used. Bhattarai et al. (2009) also reported that the absolute germination percentage in tomato would appear to be at least partially under genetic control. In our study, highly significant differences among genotypes were found and all the microsperma lentil genotypes showed higher production potential of shoots and roots from embryo culture than macrosperma genotypes (Table 3). To our knowledge, this is the first study which compares micro and macrosperma types and shows that they not only differ in morphological traits (cotyledon color and flowers, the size of the seed and adaptation to dry environments) but also in the in vitro shoot production and germination ability from immature seeds culture. Significant differences in the germination percentage were also found within the macrosperma genotypes. B1051 genotype gave the lowest percentage of germination (32.5 %) showing the effect of genotype, in agreement with Bermejo et al. (2012) results.

In this study we proposed the reduction of lentil generation cycles based on in vitro culture of embryos and a greenhouse strategy. Ochatt et al. (2002) used this same strategy to reduce the generation cycles in pea and bambara groundnut and Surma et al. (2013) in pea and lupins.

Early and late flowering genotypes were used. The days to flowering of the lentil plants using the conventional method were about 78–80 days for macrosperma and 107–110 days for microsperma genotypes (Table 1). Using the technique optimized in this work the days to flowering could be reduced about to 65 days counted from the date of seed excision indicating that the flowering was accelerated up to 13–15 days for macrospermas and up to 42–45 days for microspermas. Similar results were reported by Surma et al. (2013) in pea and lupin plants which started to flower 1.5–2.5 months after the beginning of in vitro embryo culture. Considering the fact that lentil immature seeds can be rescued with success 18 days from the date of pollination, it took about 83 days in total (65 + 18 days) to obtain a new lentil generation. In the conventional method where the seed is left to mature on the plant, 162–190 days lapsed between sowing and seed harvest (Table 1) whereas the immature seed culture method completed two crops within this time frame. Projecting this outcome, more than four generations per year could be generated using in vitro culture of seed excised at 18 DAP and in vivo flowering. Using an in vitro plus in vivo system 4–6 generations per year for *Pisum*, four generations for *Vigna* and 2.5–3 generations for lupins were obtained by Ochatt et al. (2002) and Surma et al. (2013).

Conclusions

The embryo rescue technique employed in this study using immature seeds cultured at 18 days after pollination in MS medium without BAP allowed us to produce fertile lentil

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plants with a 30 % success rate which is enough in SSD technique, in which only one seed per plant is required. This in vitro–in vivo method was efficient, easy and cheap to perform and it can be applied for attainment of successive generations in the single seed descent technique.

Using this approach, more than four lentil generations can be produced in a year with early to late flowering genotypes in contrast to a maximum of two generations with conventional methods. This accelerates lentil breeding cycles significantly by facilitating rapid development of RILs for mapping key traits, rapid population development for lentil breeding and to quickly introgress key new traits into elite germplasm.

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