Hybridization of Cultivated Lentil *Lens culinaris* Medik. and Wild Species *Lens tomentosus* Ladizinsky

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Abstract

Suvorova G. (2014): **Hybridization of cultivated lentil** *Lens culinaris* **Medik. and wild species** *Lens tomentosus* **Ladizinsky**. Czech J. Genet. Plant Breed., **50**: 130–134.

Cultivated lentil *L. culinaris* was crossed to the wild species *L. tomentosus* ILWL90 and ILWL120. An ovule rescue technique was used to overcome interspecific incompatibility. Out of 296 hybrid ovules being planted *in vitro* 27 explants began to grow and three hybrids were recovered. A hybrid between *L. culinaris* and *L. tomentosus* accession ILWL90 was obtained by means of ovule recovery only. F_1 plant and next generations of the hybrid were either sterile or partly fertile. Hybridization with *L. tomentosus* accession ILWL120 was achieved by ovule culture as well as in a usual way i.e. without ovule culture. Seed progenies of these hybrids were fertile in both cases. Breeding lines recombinant in flower, seed coat and cotyledon coloring were developed as a result of multiple regular selection for highly productive plants in F_2–F_7 (*L. culinaris* × *L. tomentosus* ILWL120).

Keywords: interspecific hybridization; *Lens culinaris*; *Lens tomentosus*; lentil; ovule rescue

Currently, most researchers following Ladizinsky define seven taxa in the genus *Lens* Mill.: *L. culinaris* (ssp. *culinaris* and ssp. *orientalis*), *L. odemensis*, *L. ervoides*, *L. nigricans*, *L. tomentosus* and *L. lamottei* (van Oss *et al*. 1997; Cubero *et al*. 2009).

Lens tomentosus Ladizinsky was delimitated as a new species by Ladizinsky: one population of *L. tomentosus* has been known for some time as a variant of *L. culinaris* ssp. *orientalis*; additionally, two populations were discovered in the Mardin area of southeastern Turkey. Morphologically *L. tomentosus* was closer to *L. culinaris* ssp. *orientalis* than to any other taxon, but it was distinguished by a hairy pod, a modified karyotype and cpDNA restriction pattern (van Oss *et al*. 1997). Afterwards data of molecular analysis supported the independent species status of *L. tomentosus* (Galasso 2003; Sonnante *et al*. 2003; Duran & Perez de la Vega 2004).

When crossing cultivated lentil *L. culinaris* with the wild species *L. tomentosus*, pod and seed setting is observed. But with time growing embryos are gradually degraded and shrivelled, and non-viable seeds are formed. So an embryo rescue technique is required in this case.

For the first time the interspecific lentil hybrid with the use of embryo culture *in vitro* was obtained by Ladizinsky in the cross of *L. culinaris* with *L. ervoides* (Ladizinsky *et al.* 1985). Spanish researchers developed an efficient protocol to recover lentil embryos which yielded a few hybrids with *L. odemensis*, *L. ervoides* and *L. nigricans* (Frantini & Ruiz 2006). In Canada a breeding program was initiated to introgress the anthracnose genes from *L. ervoides* into the high-yielding cultivars of *L. culinaris* (Fiala *et al.* 2009; Tullu *et al.* 2013). We have noted the success in the cross of *L. culinaris* and *L. tomentosus* reported (Tullu *et al*. 2011).

In the present study the procedure for obtaining interspecific hybrids between *L. culinaris* and *L. tomentosus* is described in detail and the perspective of hybrid use in practical breeding is discussed.

MATERIAL AND METHODS

The plant materials used were four cultivars of *Lens culinaris* (cvs: Rausa, Obraztsov Chiflik 7, Svetlaya, Vekhovskaya 1) and two accessions of *L. tomentosus* (ILWL90, ILWL120) received from ICARDA gene

bank. Parental plants were grown in pots with soil in a greenhouse and crosses were done in spring or autumn. Flowers on maternal plants were emasculated in buds and pollen from a paternal plant was put into the stigma immediately. The pollinated flowers were marked with colored threads but no isolators were used. In 7–20 days after pollination the pods were taken off the plants and sterilized in 0.25% sodium chlorhexidine digluconate solution for 7 min. Ovules were aseptically taken out from the pods and put *in vitro* onto the surface of a nutrient medium.

Nutrient media contained mineral salts of MS, vitamin B5, 30 g/l of sucrose, 100 mg/l of inositol, 1000 mg/l of casein hydrolyzate, 5.5 g/l of agar, 1 mg/l of BAP (6-benzylaminopurine), 0.2 mg/l of IAA (indole-3-acetic acid). Sometimes BAP was replaced by zeatin. Rooting media included 1 mg/l IBA (indole-3-butyric acid). To optimize the rooting procedure the method of shoot culture in inverted orientation demonstrated on legumes by Frantini and Ruiz (2003) was used.

The genomic DNA was isolated from leaves of young plants by the CTAB method after grinding in liquid nitrogen (Doyle & Doyle 1990). Amplification reactions were performed in 25 µl volume with the following program: one cycle of 93°C for 5 min; 40 cycles of 93°C for 50 s, 34°C for 35 s, 72°C for 2 min, followed by the incubation at 72°C for 5 min and the soaking at 4°C. The primer used was CS4 (GACTTCCTGT). The amplified products were analysed by electrophoresis in agarose gel.

RESULTS AND DISCUSSION

In the cross of *L. culinaris* with *L. tomentosus* a high percentage of pod setting was observed (Table 1). Of 296 immature ovules that were planted onto nutrient media only 27 started to develop. Ovular integuments dying and embryo emerging accompanied the development. Embryos developed directly into plantlets as well as in the way of multiple shoot formation. Direct plantlet formation usually occurred on media with zeatin (Figure 1A), but BAP induced abnormal embryo development as a rule. In the case of direct embryogenesis a seedling was taken out from the medium and transferred into the soil. When a morphogenic tissue was formed, the period of hybrid recovery took a longer time. The morphogenic tissue was periodically passed to fresh media containing 0.6 mg/l BAP (Figure 1B), the shoots were detruncated and passed to rooting media. Only three hybrids out of 27 ovules developed on nutrient media and were recovered.

Each recovered hybrid was characterized by its own unique history. The first hybrid, cv. Obraztsov Chiflik 7 × *L. tomentosus* ILWL90, was obtained as a result of germination of 8-days-old embryo *in vitro* on a medium containing BAP. In a month the seedling was passed onto hormone-free MS media, and in the next month plantlets were transferred into the soil. F_1 plant formed only one pod with one seed. F_2 plant had 45 pods and 37 well-filled seeds, when the rest of the seeds, about the same number, were not well-filled. Next generations of this hybrid were either sterile or partly fertile.

Hybrid cv. Svetlaya × *L. tomentosus* ILWL120 was obtained from a 19-days-old embryo initially cultivated on a medium with BAP. The process from the morphogenic tissue formation to obtaining a rooting plantlet took about a year. Three seeds were set on F_1 plant, which were planted in a usual way. F_2 plants formed 56, 39 and 36 seeds, respectively. The plants of next generations were fertile.

Hybrid cv. Vekhovskaya 1 × *L. tomentosus* ILWL120 was obtained from 17-days-old embryo in a longer way. A weak rootless seedling formed on the medium with zeatin was passed after 2 months onto the medium with BAP to induce the formation of additional buds. During the 4th passage long shoots were formed, which were periodically transferred into rooting media. But in the long-term culture of lentil tissues there arose a problem of root induction. In this case we optimized the rooting procedure and cultured a shoot segment with axillary bud in inverted orientation with the apical end pointed down in the medium (Figure 1C). The grown shoot

Table 1. Results of interspecific hybridization and ovule rescue in the cross *L. culinaris* × *L. tomentosus*, 2003–2006

Cross	Flowers pollinated	Pods developed	Pod setting (%)	Ovules isolated	Ovules developed	Hybrids recovered
	(No.)			(No.)		
L. culinaris × *L. tomentosus* ILWL90	278	168	60.4	206	21	1
L. culinaris × *L. tomentosus* ILWL120	107	73	68.2	90	5	2

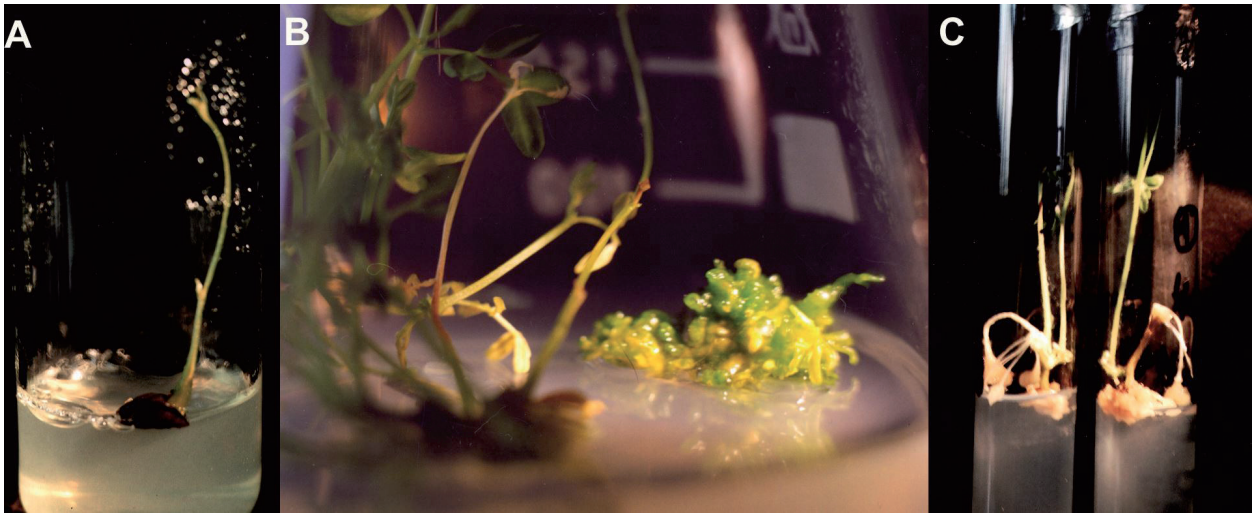

Figure 1. Development of immature ovules of *L. culinaris* × *L. tomentosus in vitro*: (A) – direct plantlet formation from hybrid embryo; (B) – multiple shoot formation; (C) – rooted shoots in inverted orientation

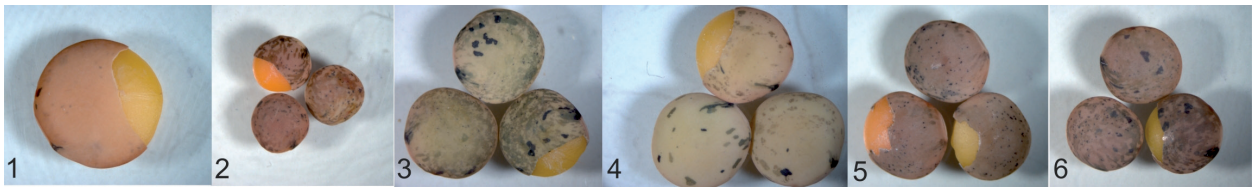

Figure 2. Seeds of lentil interspecific hybrids *L. culinaris* × *L. tomentosus* and their parental species: 1 – *L. culinaris* cv. Svetlaya; 2 – *L. tomentosus* ILWL120; 3, 4, 5, 6 – F_4 (Svetlaya × *L. tomentosus* ILWL120)

began to blossom in one tube and formed one pod. Then the pod ripened and two F_2 seeds fell into the medium, where they successfully germinated. The process from flowering to seed ripening and their following *in vitro* germination were going on for 5 months. On the whole, nearly three years passed from the moment of F_1 immature ovule isolation to taking the F_2 seedlings out from the tube. The seedlings were planted in a pot with soil, where they successfully acclimatized and continued their development like normal plants. There were 68 and 21 seeds formed on two F_2 plants. Next generations of hybrids were fertile.

Highly productive plants with large seeds were regularly selected in F_2–F_7 generations, which led to the creation of breeding lines recombinant in flower, seed coat and cotyledon coloring, with new character combinations (Figure 2). After several cycles of selection lentil lines became closely related to the cultivated species *L. culinaris* in plant habit (Figure 3). The best breeding lines were highly productive and had high protein content. Seed yield of the breeding lines was at the level of 1.51–1.78 t/ha in 2012, and protein content reached 27.4%.

Having repeated the crossing of *L. culinaris* and *L. tomentosus* ILWL120, we unexpectedly found that in some cases the ovules did not die after pollination, but continued to develop into ripe viable seeds. On

Figure 3. Plants of the lentil hybrid *L. culinaris* × *L. tomentosus* and parental species: 1 – *L. culinaris* cv. Vekhovskaya 1; 2 – F_8 (Vekhovskaya 1 × *L. tomentosus* ILWL120); 3 – *L. tomentosus* ILWL120

Table 2. Results of the interspecific cross *L. culinaris* × *L. tomentosus* ILWL120, 2011

Cross	Flowers pollinated	Pods formed	F_1 seeds yielded	F_1 plants obtained
	(No.)			
Obraztsov Chiflik 7 × *L. tomentosus* ILWL120	49	7	9	7
Vekhovskaya 1 × *L. tomentosus* ILWL120	14	1	1	1
Svetlaya × *L. tomentosus* ILWL120	3	0	0	0
Rausa × *L. tomentosus* ILWL120	18	6	6	3
Total	84	14	16	11

the whole, 16 F_1 seeds and 11 plants were obtained in the cross (Table 2). But there was not any success in obtaining hybrid seeds in the usual way without ovule culture using *L. tomentosus* ILWL90 accession.

F_1 plants were characterized by purple epicotyl and violet flower coloring, dominant traits, typical of the paternal species. RAPD analysis also supported the hybrid nature of F_1 plants (Figure 4). All F_1 hybrids had a DNA fragment of about 500 bp in size in their electrophoretic spectra which was specific to *L. tomentosus* ILWL120 and absent in cultivars of *L. culinaris*. As for the breeding lines, obtained in previous crossings with embryo rescue, only one line out of eight included into the DNA analysis saved this fragment. Probably in the process of purposeful selection for seed productivity the elimination of genetic material of the wild parent occurred.

In this work we show the possibility of interspecific hybridization of cultivated lentil *L. culinaris* with the wild species *L. tomentosus*. It is worth mentioning that ILWL120 and ILWL90 accessions of *L. tomentosus* differ from each other in the degree of compatibility with *L. culinaris*. To obtain a hybrid with *L. tomentosus* ILWL90 was possible only using an ovule culture and the hybrid was characterized by complete or partial sterility. Hybridization with *L. tomentosus* ILWL120 was achieved not only by embryo rescue but also in the usual way. Seed progenies of this hybrid were fertile enough. Different degree of compatibility can be explained by heterogeneity of the *L. tomentosus* ILWL120 population, and also by an ambiguity of the systematic position of the accession.

Cases of misclassification are not rare in the systematic history of the genus *Lens*, and researchers came across this from time to time (Ladizinsky 1993; van Oss *et al.* 1997; Fiala *et al.* 2009). On the basis of chromosome morphology and FISH karyotypes it was suggested that two *L. tomentosus* accessions (ILWL149 and ILWL120) were probably misclassified and they were more similar to *L. culinaris* than to *L. tomentosus* (Galasso 2003). According to data on SDS-PAGE of seed storage protein accessions of *L. tomentosus* ILWL90 and ILWL120 were different both from each other and from cultivars

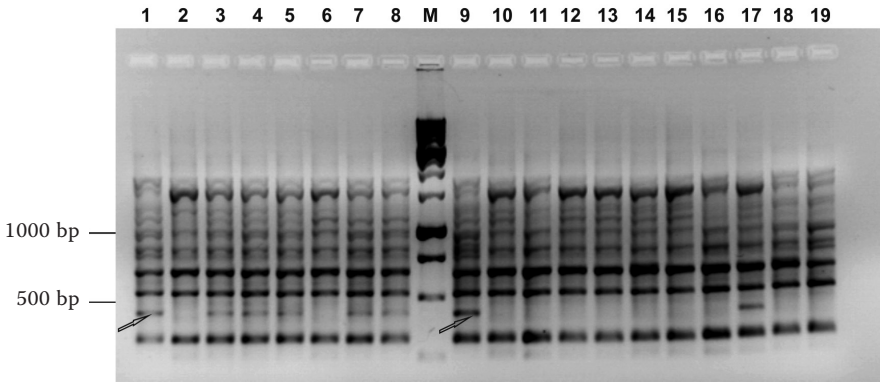

Figure 4. RAPD profile of lentil hybrids *L. culinaris* × *L. tomentosus*, parental species and cultivars with CS4 primer: 1, 9 – *L. tomentosus* ILWL120; 2 – cv. Obraztsov Chiflik 7; 3, 4, 5 – F_1 (Obraztsov Chiflik 7 × *L. tomentosus* ILWL120); 6 – cv. Rauza; 7, 8 – F_1 (Rauza × *L. tomentosus* ILWL120); M – DNA marker; 10 – cv. Vekhovskaya 1; 11, 12, 13, 14, 15 – F_7 (Vekhovskaya 1 × *L. tomentosus* ILWL120); 16 – Svetlaya; 17, 18, 19 – F_7 (Svetlaya × *Lens tomentosus*); the arrow indicates a DNA fragment specific to *L. tomentosus*

of *L. culinaris* (Suvorova & Kornienko 2011). A degree of compatibility – incompatibility cannot be a systematic criterion only; presumably additional researches are needed to define the species status of *L. tomentosus* ILWL120.

The breeding lines, created as the result of repeated individual selection of highly productive plants in F_2–F_7 (*L. culinaris* × *L. tomentosus* ILWL120), are recommended to be used as initial material in practical breeding. Thereby interspecific hybridization in the genus *Lens* could be a powerful tool of increasing the genetic diversity and breeding of the cultivated lentil.

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