CHAPTER 14

WILD RELATIVES AND BIOTECHNOLOGICAL APPROACHES

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Abstract: Wild species of the genus Lens are an important source of genetic variation for breeding lentil varieties adaptable to new environments and tolerant of biotic and abiotic stresses. The wild species are endemic to a wide range of environments and possess many diverse characteristics. *Lens* species can be divided into three groups, a primary, secondary and tertiary gene pool, according to their inter-crossability. Crosses between members of the different genepools generally fail because the hybrid embryos abort. However, embryo rescue has been used successfully to obtain viable hybrids between groups. It is possible to intercross most of the wild *Lens* species with cultivated lentils using plant growth regulators and/or embryo rescue to allow the growth of hybrid plants. Other biotechnology techniques which may impact on lentil breeding include, micro-propagation using meristamatic explants, callus culture and regeneration, protoplast culture and doubled haploid production. Micropropagation and regeneration from callus culture are relatively well established techniques with further research required for the development of reliable protoplast regeneration and doubled haploid protocols

Abbreviations:

B_5 medium	Culture medium of Gamborg *et al.* (1968)
BAP	6-benzylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	gibberellic acid
IAA	indole-3-acetic acid
MS medium	Culture medium of Murashige and Skoog (1962)
NAA	α-naphthalene acetic acid
RAPD	Randomly Amplified Polymorphic DNA
TDZ	thidiazuron

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1. INTRODUCTION

Lentil is one of the oldest west Asian crops and is still of considerable importance on the Indian subcontinent, in the Middle East, southern Europe, and eastern and northern Africa. On a much smaller scale, it is grown in the New World including Canada, USA and Australia. The total acreage of lentil has grown in the last few years, as has its yield. Production of lentil is estimated at 3.3 million metric tons from an estimated 3.8 million hectares with an average yield of 850 kg/ha (FAOSTAT, 2005). Modern lentil breeding dates back a few decades and is performed at a number of national and international institutions. However, most of the lentil grown by farmers outside the New World is still in the form of land races. These have been selected for adaptation to local conditions and they constitute a valuable source of genetic diversity. Production of widely adapted, high yielding lentil cultivars may cause the extinction of many land races, resulting in an irretrievable loss of genetic diversity.

Another potential source of genetic diversity for the lentil crop is the wild related *Lens* species. The usefulness of these to breeders depends on their genetic relatedness to the cultivated species and the availability of methods for gene transfer. Until recently, only sporadic efforts were made to obtain adequate information on taxonomy, genetics, and evolution of the wild lentil species. This chapter summarises the latest information on taxonomy and genetic variation in the genus *Lens* and includes information on the distribution and ecology of various wild lentil species. It then describes methods for interspecific hybridisation, summarises the interspecific hybrids that have been produced and discusses the potential for improvement of lentils using cell culture technologies.

2. CULTIVATED LENTILS AND WILD RELATIVES

2.1. Taxonomy

The genus *Lens* Miller is a member of the tribe Vicieae, subfamily Papilionacea, family Leguminosae. Beside *Lens*, three other genera are included in the Vicieae: *Vicia* L., *Lathyrus* L., and *Pisum* L. From a morphological point of view, a continuum exists between the genera *Lens* and *Vicia*. However, *Lens* is a much smaller genus, characterised by an annual growth habit, small flowers, calyx deeply divided into subulate, subequal teeth, and a broadly rhomboid compressed legume with one or two orbicular flattened seeds.

The genus *Lens* comprises seven taxa in six species (Ferguson 1998; Ferguson *et al.*, 2000). *Lens orientalis* is the presumed progenitor of *Lens culinaris* and the two species are crossable and produce fully fertile progeny (Muehlbauer *et al.* 2006). According to crossability, phenetic relations and chromosomal diversity, Ladizinsky & Abbo (1993) suggested two biological species in the genus *Lens*: *Lens culinaris* and *Lens nigricans*, with a few subspecies. However, additional information now indicates that some of the proposed subspecies are species in their own right. In 1997, two new species were recognised in genus *Lens*. *Lens*

tomentosus was separated from *Lens culinaris* subsp. *orientalis* on the basis of its tomentose, as opposed to puberulent, pods, and a relatively small asymmetrical chromosome which bears a minute satellite (Ladizinsky 1997). *Lens lamottei*, originally described by Czefranove (1971) was found to be the same as a differentiated cytotype identified within *Lens nigricans* by Ladizinsky *et al.* (1983, 1984) and is now recognised as a separate taxon (van Oss *et al.* 1997). Thus as a result of combined evidence of crossability, phenetic relations and morphological markers (Ferguson and Erskine 2001; Ferguson *et al.* 2000), the genus *Lens* consists of the six species listed in Table 1.

From the standpoint of crossability for use in breeding, the *Lens* species can be divided into three groups: *L culinaris* and *L odemensis* make up the primary genepool, *L ervoides* and *L nigricans* belong to the secondary genepool and *L lamottei* and *L tomentosus* belong to the tertiary genepool. (Muehlbauer and McPhee, 2005). Crosses between members of the different genepools generally fail because the hybrid embryos abort. However embryo rescue (see section below) has been used successfully to obtain viable hybrids between groups (Ladizinsky *et al.* 1985). The basic chromosome number of the genus *Lens* is $n = 7$. All the *Lens* species share more or less the same karyotype, which includes three pairs of metacentric, or submetacentric chromosomes, a pair of metacentric chromosome with a secondary constriction very close to the centromere, and three pairs of acrocentric chromosomes (Ladizinsky & Abbo 1993).

2.2. Geographic Distribution

The main distributional range of the wild lentil species extends from latitude 27 °S to 45 °N and from longitude 70 °E to 15 °W. It includes the Mediterranean basin and extends farther east, up to Tadijikistan. Wild lentils grow almost exclusively in primary habitats where they are not subjected to competition by aggressive colonizer plants. They usually form loose stands in small disjunct populations. The density of plants per site may vary dramatically between years apparently because of climatic conditions.

Table 1. Species of the genus *Lens*

Lens culinaris Medikus
ssp. *culinaris*
ssp. *orientalis* (Boiss.) Ponert
Lens odemensis Ladizinsky
Lens tomentosus Ladizinsky
Lens lamottei Czefranove
Lens ervoides (Brign.) Grande
Lens nigricans (M. Bieb) Godron

Lens ervoides is confined mainly to the Mediterranean region and is relatively common in Israel, Syria, Turkey, the Adriatic coast of Yugoslavia, southern Italy, and more restricted in Spain and Algeria. Isolated populations are also known from Ethiopia and Uganda. *Lens ervoides* usually grows in shady or partially shaded habitats, under a canopy of trees or among shrubs. Ecologically, *Lens ervoides* differs from other wild *Lens* species but may grow close to them when their habitats coincide. *Lens ervoides* has been found adjacent to ssp. *orientalis* in Israel and Turkey and adjacent to *Lens odemensis* in one location in Israel and to *Lens nigricans* in two locations in Yugoslavia.

Lens nigricans is essentially a Mediterranean species, occurring mainly in southern Europe. To the east, it extends to the Crimean Peninsula and Georgia and to the west to La Palma in the Canary Islands, which is also the southern border of this species. This species also occurs sporadically in Algeria, Morocco and on the Italian and French Alps. *Lens nigricans* grows in two different ecological niches: in primary, open or partially shaded and stony habitats, together with other short stature annual legumes, mainly clovers and medics; on limestone, granite and basalt bedrock, from sea level on the Adriatic coast of Yugoslavia up to 1200 m in southern Spain.

The other habitat is typically man-made: abandoned plantations and terraces in Greece, Yugoslavia, France and Spain, in terraced vineyards in the Italian Alps and around ruins in Italy, France and Spain. The populations in these secondary habitats are extremely localised and never extend to adjacent primary habitats which are presumably suitable to *Lens nigricans* (Ladizinsky *et al.* 1985). *Lens odemensis* has only recently been described and was first identified in two locations in Israel, then in Turkey and later identified in herbarium material from other two locations in Turkey and from Chios, the Aegean island. *Lens odemensis* recently was also collected from Syria. *Lens odemensis* grows mostly in open herbaceous habitats together with other annual legumes such as vetch, medic and clover. In Israel and Syria, it occurs on shallow soil and gravel originating from basalt, at altitudes of 700 to 1400 m. In west Turkey, it occurs on calcareous bedrocks, in partially shaded habitats mostly in pine groves from sea level up to 800 m. In southern Turkey, it grows on gravel of basalt and metamorphic rocks.

Lens culinaris ssp. *orientalis* is the wild progenitor of the cultivated lentil. The two are inter-fertile and share the same diagnostic morphological features. Accessions of ssp. *orientalis* occupy the distributional range from Turkey to Tadjikistan and from Iran to the Crimean Peninsula. It is restricted to primary, open or partially shaded habitats on shallow stony soils originating from calcareous, metamorphic and basalt rocks at altitudes ranging from 500 to 1700 m. Mostly it is accompanied by annual vetches, clovers, medics and lathyrus species. Subspecies *orientalis* is also common in the Turkemenian side of the Kopet Dag range, in Uzbekistan and Tadjikistan. The distribution range of ssp. *orientalis* overlaps with those of *Lens odemensis*, *Lens nigricans* and *Lens ervoides*, but they rarely grow side by side. It was found adjacent to *Lens ervoides* in Israel and Turkey but never with *Lens odemensis* or *Lens nigricans* (Ladizinsky 1989).

2.3. Genetic Variation

Several studies have been carried out to evaluate phenetic relations within the genus *Lens*. Conflicting results have emerged which appear to depend on the germplasm and technique used to measure genetic variation (Ahmad and McNeil 1996, Ferguson 1998). Morphologically *Lens lamottei* is closely related to *Lens odemensis* and is practically equally associated with *Lens odemensis* and *Lens culinaris* on the basis of isozyme evidence (Hoffman *et al.* 1986, Ferguson and Robertson 1996); it does however, appear to be the taxon most distantly related to all other taxa according to RAPD marker analysis (Ferguson 1998). Evaluation of species relations of *Lens tomentosus* by biochemical or molecular techniques have not been reported. High genetic diversity has been reported within *Lens nigricans*, *Lens odemensis* and ssp. *orientalis* relative to cultivated lentil. *Lens lamottei* and *Lens ervoides* are the only species reported as having a similar or more restricted genetic base than cultivated lentil (Ferguson 1998).

The geographical distribution of genetic variation as revealed by molecular techniques has been mapped in four wild *Lens* taxa. Centres of diversity as well as areas of low genetic diversity have been identified (Ferguson 1998). For *Lens culinaris* ssp. *orientalis*, two centres of diversity exist, one in south-eastern Turkey and north-western Syria, the other in southern Syria and northern Jordan. *Lens culinaris* ssp. *orientalis* accessions from Iran, central Asia and northern Turkey are genetically all very similar and correspond to the common cytotype identified by Ladizinsky *et al.* (1984). The centre of diversity of *Lens odemensis* overlaps with the southern centre of diversity of *Lens culinaris* ssp. *orientalis* in southern Syria and northern Jordan. A region of high genetic diversity exists for *Lens ervoides* along the eastern Mediterranean coast, but the populations from the coastal region of the former Yugoslavia have a particularly narrow genetic base.

A clear centre of diversity exists for *Lens nigricans* in south-west Turkey with areas of low diversity along the coast of former Yugoslavia, France and Spain. Centres of diversity for *Lens* are also characterised by high population density.

2.4. Morphological Features

Morphological traits are the most useful criterion for species identification. The main differential characteristics of *Lens ervoides*, *L nigricans*, *L odemensis, L culinaris* ssp. *orientalis* and *Lens culinaris* ssp. *culinaris* are listed in Table 2 (Ahmad *et al.* 1997a).

2.5. Domestication

Barulina (1930) was the first to suggest that small seeded cultivated lentil originated from *Lens orientalis* and also suggested that the centre of origin of the cultivated lentil was in the mountainous regions of the Hindo-Kush and Himalayas. Lentil

Table 2. Morphological differences between *Lens* species

Morphological trait	*Lens ervoides*	*Lens nigricans*	*Lens odemensis*	*Lens culinaris* ssp. *orientalis*	*Lens culinaris* ssp. *culinaris*
Plant habit	single or branched column, ascending-erect	single or branched column, decumbent-ascending	single or branched column, decumbent-ascending	single or branched column, decumbent-ascending or erect	single or branched column, ascending-erect or erect
Leaves	4–6 leaflets per leaf, rachis 5–15 mm, ending in leaflet in lower leaves and in tendril in upper leaves	6–8 leaflets per leaf, rachis 8–25 mm, ending in tendril in the upper leaves	6–8 leaflets per leaf, rachis 8–20 mm, ending in tendril in the upper leaves	6–8 leaflets per leaf, rachis 5–25 mm, ending in tendril in the upper leaves	10–16 leaflets per leaf, rachis 20–50 mm, ending in tendril in the upper leaves
Stipules	linear to semi-hastate, 1–3 mm	semi-hastate, 3–5 mm, strongly dentate at the base, perpendicular with parallel position to the stem	semi-hastate, 2–4 mm, slightly dentate at the base, usually horizontal to the stem	lanceolate entire, 2–3 mm	lanceolate entire, 2–4 mm, sometimes with a slight appendage at the base
Peduncle	1–2 flowered, rarely aristate, 22–40 mm	1–3 flowered, aristate, 20–40 mm	1–2 flowered, aristate, 15–35 mm	1–3 flowered, aristate, 12–30 mm	1–3 flowered, aristate, 32–35 mm
Calyx	2–3 mm, teeth shorter than corolla	5–8 mm, teeth as long or longer than corolla	4–6 mm, teeth as long as corolla	4–6 mm, teeth little shorter than corolla	4–7 mm, teeth longer or shorter than corolla
Pod	pubescent or glabrous, rhomboid 8–11 × 3.5–5 mm, 1–2 seeds	glabrous, rhomboid 9–12 × 4–6 mm, 1–2 seeds	glabrous, rhomboid 7–11 × 4–6 mm, 1–2 seeds	glabrous, rarely pubescent, rhomboid 7–11 × 4–6 mm, 1–2 seeds	glabrous, rarely pubescent, rhomboid 1–20 × 4–12 mm, 1–2 seeds
Seed	gray-brown, diameter 2–3 mm	mottled black-brown, diameter 2.5–3.5 mm	mottled gray-brown, diameter 2.5–3.5 mm	mottled gray-brown, diameter 2.5–3.5 mm	variety of colours mottled or plain, diameter 3–9 mm

was utilised by man during the early stages of the Neolithic Revolution. Remains of lentil seeds in archaeological digs suggest that it was one of the first plants to be exploited by man (Zohary and Hopf 1988). The oldest seed remains come from the Middle East, hence the prevailing idea that lentil domestication occurred here, together with that of other pulses and cereals.

The wild progenitor ssp. *orientalis* is at least as common in central Asia as in the Middle East, if not more. All the analysed material from that region are of the common crossability group and share the standard chromosome arrangements, which could be taken as support for Barulina's view that central Asia was the centre for lentil domestication but Zohary (1972) and Williams *et al.* (1974) argued that cultivated lentil had its origin in the Near East arc where it was cultivated with other vegetables as early as the 7th millennium B.C. This evidence for the Near East origin comes from archaeological remains.

2.6. Potential as Genetic Resources

Wild relatives are an integral part of the gene pool of crop plants. They may possess genetic diversity, such as resistance to various diseases and better tolerance to environmental stresses, which is lacking in cultivated crops. Rational exploitation of the wild gene pool depends on the genetic affinities between the crop plant and its wild relative, and on the availability of methods for gene transfer.

The genetic potential of the wild lentil gene pool has not yet been thoroughly estimated. Sources of resistance to the major foliar disease of lentil, rust, the most important soil-borne disease of lentil, vascular wilt and ascochyta blight have been identified in the wild gene pool (Ahmad *et al.* 1997b). Resistance to vascular wilt and ascochyta blight have also been found in *Lens culinaris* ssp. *orientalis* (Bayaa *et al.* 1994, 1995). Greater resistance to cold tolerance has been found in *Lens culinaris* ssp. *orientalis* than in the cultivated lentil (Hamdi *et al.* 1996). The wild gene pool, particularly *Lens odemensis* and *Lens ervoides* also show greater resistance to drought in terms of low relative reduction in yield with drought stress (Hamdi and Erskine 1996).

3. INTERSPECIFIC HYBRIDIZATION

3.1. Crossability Potential

Crossability is defined by Ladizinski (1992) as the potential for intercrossing individuals belonging to different taxa and for producing embryos or seeds that can give rise to an F_1 plant. The potential to cross within the genus *Lens* is hampered by crossability barriers within the species as well as between species (Ladizinsky & Abbo 1993, Ladizinsky 1997, Ferguson *et al.* 2000). *L culinaris* ssp.*orientalis* is considered to be the wild progenitor of the cultigen and most accessions are readily cross-able within the species (Ladizinsky *et al.* 1984). However,

exceptions were reported by Ladizinsky & Abbo (1993) and van Oss *et al.* (1997) who identified *L culinaris* ssp.*orientalis* accession S76 from Syria as compatible with only two accessions (S74 and S138) but incompatible with all other accessions due to hybrid embryo abortion.

Table 3 presents an overview of all the different crosses attempted in the genus *Lens*, the success of hybrid production, fertility of hybrids and factors critical for success. Partially fertile hybrids can be obtained from many crosses within the genus (see Table 3) with varying degrees of hybrid fertility. In many cases, application of GA_3 (gibberellic acid) or rescue techniques due to embryo abortion was required. Only four crosses have not resulted in hybrids to date, *L culinaris ssp orientalis* × *L ervoides or L nigricans* (Ladizinsky *et al.* 1984), *L culinaris ssp tomentosus* × *L lamottei* (van Oss *et al.* 1997), and *L culinaris ssp odemensis* × *L ervoides* (Ladizinsky *et al.* 1984). In all of these crosses, either GA_3 was not applied or embryo rescue techniques were not attempted at the time.

3.2. Crossability Barriers

Ladizinsky (1992) explained that success in lentil crosses depends on the interaction between the parental genomes in the hybrid zygote, embryo or endosperm and between the hybrid tissue and the surrounding maternal tissue. The crossability between lentil and its wild relatives is hampered by pre- and post-fertilization barriers. Problems arise with chromosome pairing in many crosses, for example between *L culinaris* × *L tomentosis* (Ladizinsky 1997). Another common problem is that hybrid embryos cease to grow about 7–14 days after pollination due to endosperm degeneration and thus need rescuing in order to obtain viable hybrids. Hence, *L culinaris* × *L ervoides* or *L culinaris* × *L nigricans* crosses need embryo rescue techniques in order to develop mature hybrid plants (e.g. Abbo and Ladizinsky 1991, Cohen *et al.* 1984). In some *L culinaris* × *L culinaris* ssp *orientalis* crosses, the hybrid embryo ceased growing but the endosperm shows no sign of disintegration (Ladizinsky 1992). In contrast, Abbo and Ladizinsky (1991) observed that the endosperm was found to be either abnormal or lacking in *L culinaris* × *L culinaris* ssp *orientalis* crosses. Hybrids showed varying degrees of fertility usually due to chromosome translocations and subsequent problems with chromosome pairing at meiosis (Ladizinsky *et al.* 1984). These problems can occur in the F_1 and also persist into later generations causing partial or complete sterility. For example, in crosses of *L culinaris* cv. Eston × *L ervoides* L01-827A, 150 F_1 seeds were obtained but only 85 (57%) could be advanced to F_2 (Fiala 2006). Fertility is often very low with little viable pollen produced in anthers and varies depending on the accession in *L culinaris* × *L culinaris* ssp *orientalis* crosses from 2–69% (Ladizinsky *et al.* 1984. Ladizinsky *et al.* (1984). Albino seedlings can occur in the F_1 generation and thus also prevent hybridization success (Ladizinsky & Abbo 1993).

Table 3. Intra- and inter-specific crosses in the genus lentil[1]

Parent 1	Parent 2	Hybrid Status	Critical factors	References
culinaris	*orientalis*	Mostly fertile	Karyotype Embryo rescue GA_3 Environment	Ladizinsky *et al.* 1984 Cohen *et al.* 1984 Abbo and Ladizinsky 1991 Ladizinsky & Abbo 1993 Ahmad *et al.* 1995 van Oss *et al.* 1997 Fratini and Ruiz 2004
culinaris	*odemensis*[2]	Partially fertile	GA_3 Embryo rescue	Goshen *et al.* 1982 Ladizinsky *et al.* 1984 Fratini and Ruiz 2006
culinaris	*tomentosus*[3]	Partially fertile	Karyotype Embryo rescue	Ladizinsky & Abbo 1993
culinaris	*ervoides*	Partially fertile	Embryo rescue GA_3	Cohen *et al.* 1984 Ladizinsky *et al.* 1985 Ahmad *et al.* 1995 Fiala 2006 Fratini and Ruiz 2006
culinaris	*lamottei*	Partially fertile	Embryo rescue	Fiala 2006
culinaris	*nigricans*	Partially fertile	Embryo rescue GA_3	Cohen *et al.* 1984 Ladizinsky *et al.* 1985 Ahmad *et al.* 1995 Fratini and Ruiz 2006
orientalis	*odemensis*	Partially fertile		Ladizinsky *et al.* 1984 Goshen *et al.* 1982
orientalis	*tomentosus*[4]	Sterile	Karyotype Embryo rescue Temp. $> 28\,C$	Ladizinsky & Abbo 1993 Ladizinsky 1997 van Oss *et al.* 1997
orientalis	*ervoides*	None obtained		Ladizinsky *et al.* 1984
orientalis	*nigricans*	None obtained		Ladizinsky *et al.* 1984
tomentosus	*lamottei*	None obtained	Not attempted	van Oss *et al.* 1997
odemensis	*ervoides*	None obtained		Ladizinsky *et al.* 1984
ervoides	*nigricans*	Partially fertile		Ladizinsky *et al.* 1984 Ladizinsky & Abbo 1993
ervoides	*lamottei*	Partially fertile		Ladizinsky *et al.* 1984
nigricans	*lamottei*	Sterile		Ladizinsky *et al.* 1984 van Oss *et al.* 1997

[1] Accessions are listed as Parent 1 or 2 regardless of the direction of the crosses

[2] Initially described as *L nigricans* with horizontal stipule type but later designated as *L culinaris* ssp *odemensis* (Ladizinsky *et al.* 1984, Ferguson *et al.* 2000)

[3] Initially described as *L culinaris* ssp *orientalis* with tomentose pods and later designated as *L culinaris* ssp *tomentosus*

[4] Initially described as *L culinaris* ssp *orientalis* No. 133 and later designated as *L culinaris* ssp *tomentosus* (Ladizinsky & Abbo 1993)

3.3. Hybrid Embryo Rescue Protocols

Ahmad *et al.* (1995) reported obtaining viable hybrids between *L culinaris* × *L ervoides, L culinaris* × *nigricans, L culinaris* × *L culinaris* ssp *odemensis,* and *L culinaris* ssp. *culinaris* × *L culinaris* ssp *orientalis* by applying 50 – 400 ppm GA_3 to the developing pods four and ten days after pollination. Hybrid embryos from interspecific lentil crosses often abort 7–14 days after pollination due to hybrid endosperm breakdown or chromosome abnormalities, resulting in shriveled, non-viable seeds. Cohen *et al.* (1984) were the first to report that hybrid embryos could be rescued by culturing the ovules on an agar solidified MS medium supplemented with 100 g l^{-1} sucrose, 0.2 mg l^{-1} IAA (indole-3-acetic acid), 0.5 mg l^{-1} GA_3, and 0.5 mg l^{-1} zeatin. Seven to 10 days later, embryos were excised and transferred to MS medium with 30 g l^{-1} sucrose and 0.3 mg l^{-1} zeatin and sub-cultured on the same medium 2 weeks later (Ladizinsky *et al.* 1985). Fratini and Ruiz (2006) developed a protocol in which hybrid ovules were rescued 18 days after pollination using a medium consisting of MS salts, 1 μM IAA, 0.8 μM kinetin, 1% sucrose and 0.8% agar. Two weeks later, embryos were excised and cultured on the saume medium for another 2 weeks followed by transfer to culture tubes until plantlet development. They obtained 6 hybrids between *L culinaris* × *L culinaris* ssp *orientalis*, 2 *L culinaris* × *L nigricans* hybrids, and 1 *L culinaris* × *L ervoides* plant. The authors compared different techniques for obtaining lentil interspecific hybrids including crossing without embryo rescue, crossing without rescue but applying GA_3 to the developing pod (Ahmad *et al.* 1995) and embryo rescue using an improved embryo rescue medium (Cohen *et al.* 1984). Even though the number of hybrids obtained with their improved medium was low, all other methods failed to produce mature hybrids except for one *L culimaris* × *L culinaris* ssp *odemensis* hybrid obtained with the rescue medium of Cohen *et al.* (1984). Fiala (2006) also obtained *L culinaris* × *L ervoides* hybrids using the Cohen *et al.* (1984) protocol. In addition, one viable *L culinaris* ssp *culinaris* × *L lamottei* hybrid was also produced in this study. However, the hybrid plantlet could not be rooted directly and was subsequently rooted via micrografting (Gulati *et al.* 2001). Improving the embryo rescue protocol to obtain an improved crossing efficiency seems to be a critical step in overcoming hybrid embryo abortion in the genus *Lens*.

3.4. Shoot Regeneration

Due to the difficulties in obtaining interspecific lentil hybrids, a technique is often required to quickly multiply shoots prior to attempting root induction. *In vitro* propagation from apical meristems of lentil was first reported by Bajaj (1979) who found that shoot regeneration occurred on MS medium (Murashige and Skoog, 1962) supplemented with 2 mg l^{-1} IAA plus 0.5 mg l^{-1} kinetin. Shoot regeneration from meristematic explants using BAP (6-benzylaminopurine) was later reported by Polanco *et al.* (1988) where seedling shoot tips, first nodes and immature seeds cultured on MS medium containing BAP at 2.25 or 0.225 mg l^{-1}

with NAA (α-naphthalene acetic acid) at 0.186 or 0.0186 mg l^{-1} and with or without GA_3(1 mg l^{-1}), produced multiple shoots.

Malik and Saxena (1990) observed that TDZ (thidiazuron) was more effective than kinetin or zeatin for the induction of multiple shoots in axenic seedling cultures with greatest numbers of shoots and greatest percentage regeneration occurring between 10 to 30 μM TDZ. Further optimisation of the shoot regeneration protocol was made by Ahmad *et al.* 1997b) who found that optimal shoot regeneration was obtained using MS medium lacking sucrose and containing 2.89 μM GA_3 plus 1.11 μM BAP.

Ye *et al.* (2002) confirmed that BAP and TDZ induce multiple shoot formation in axenic seed cultures and also found that MS salts produced more shoots and larger shoots than Gamborg's B_5 medium (Gamborg *et al.* 1968) and that an additional 750 mg l^{-1} $CaCl_2$ was necessary to minimise shoot tip necrosis. Fratini and Ruiz (2002) reported higher numbers of shoots using TDZ but subsequent rooting was inhibited after the use of this growth regulator. Hence, the authors recommended zeatin for shoot induction probably due to the lower carry-over effect of the natural cytokinin.

3.5. Root Regeneration and Grafting

The induction of root growth in *in vitro* lentil cultures, which is critical for obtaining whole plants after embryo rescue, has proven more difficult than with many other plant species. In the first report of lentil tissue culture (Bajaj and Dhanju, 1979) no details were provided about root growth. Later reports on embryo rescue (Cohen *et al.*, 1984; Ladizinsky *et al.* 1985) described root growth from rescued embryos on MS medium containing 0.2 mg l^{-1} IAA, 0.2 mg l^{1} IAA and 30 g.l^{-1} sucrose. In the first report of plant regeneration from lentil callus tissue (Williams and McHughen, 1986) roots were not obtained *in vitro* but shoots produced *in vitro* were successfully rooted on sand in a mist chamber.

Polanco *et al.* (1988) found that roots could be regenerated from shoots on media containing either 2 mg l^{-1} IAA or 0.186 mg l^{-1} (1 μM) NAA. Root induction varied between 0 and 86% depending on genotype and explant. Ahmad *et al.* (1997b) found that MS medium with 5.37 μM NAA produced optimal rooting across a range of *Lens* species and their F_1 interspecific hybrids. Polanco and Ruiz (1997) reported that BAP had a strong inhibitory effect on root growth and that 2 mg l^{-1} (11.42 μM) IAA induced roots on 4.6 – 39.3% of shoots cultured.

Fratini and Ruiz (2002) found that both TDZ and BAP inhibited root initiation when used during the shoot induction phase. They recommended reducing the time that shoots are exposed to these growth regulators to a minimum. In contrast, Ye *et al.* (2002) observed no inhibition of rooting after shoot induction with BAP. In this study, shoot tips from the cultivated and wild lentil developed roots on medium with 1.5 mg/l NAA. However, differences between rooting capacities of different species was observed with *L ervoides* shoots rooting at 83% whereas *L nigricans* shoots only rooted at 52%. In an earlier study, Ye *et al.* (2000) obtained 70% and

74% rooting of *L culinaris* × *L ervoides* and *L culinaris* × *L culinaris* ssp*orientalis* hybrids, respectively.

Fratini and Ruiz (2003) determined that by placing the apical end of nodal stem segments in the culture medium ("inverted") rather than the basal end, the frequency of root induction was significantly increased. The highest rooting percentage was 95% which was obtained with inverted stem segments placed on MS medium containing 3% sucrose, 5 μM IAA and 1 μM kinetin. This compares with 11% rooting when stem segments were placed in the normal orientation on the same medium.

A later study by Newell *et al.* (2006) clearly demonstrated that aeration, rather than shoot orientation, is the critical factor resulting in increased rooting frequency. They showed that up to 100% of lentil shoot cuttings could produce roots if the proximal cut end was well aerated.

Gulati *et al.* (2001) developed a micrografting method in which lentil shoots were inserted into decapitated seedling root stock, lining up the exposed vascular tissues. The advantages of this technique are that a short time (less than two weeks) is required for rooting and that any growth regulator can be used during shoot induction, giving success rates of 84 to 96%. This technique was used to root hybrids from crosses involving *L culinaris* × *L lamottei* with 53% efficiency (Fiala 2006).

3.6. Callus Culture and Somatic Embryogenesis

Callus culture and subsequent regeneration by somatic embryogenesis or organogenesis is necessary for genetic transformation, enhanced recombination between genomes of interspecific hybrids and for *in vitro* selection at the cellular level. The initial report of lentil tissue culture by Bajaj and Dhanju (1979) also described callus production from excised meristems on MS medium supplemented with 1 to 2 mg l^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid) but no regeneration from callus was observed. The first report of regeneration from callus was by Williams and McHughen (1986) who found that callus could be produced on MS medium with most combinations of 2,4-D, kinetin and GA_3 at concentrations of 0.1, 1 and 10 mg l^{-1}. Shoots from callus tissue were only found to regenerate on media containing 10 mg l^{-1} kinetin and either 1 or 0.1 mg l^{-1} GA_3.

Callus derived from immature embryo tissue was reported by Saxena and King (1987) using medium with between 1 to 10 mg l^{-1} 2,4-D. This callus was observed to be embryogenic. Polanco *et al.* (1988) observed callusing of shoot tip, first node and leaf explants on MS medium supplemented with either 2,4-D, BAP, NAA or IAA with 2,4-D giving the greatest callusing response. Rozwadowski *et al.* (1990) successfully produced callus colonies from epicotyl protoplasts using complex KM8P medium supplemented with a combination of five growth regulators (2.2 μM 2, 4 − D + 2.7 μM NAA + 2.2 μM BAP + 2.3 μM kinetin + 1.4 μM GA_3) or three growth regulators (5.4 μM NAA + 2.2 μM 2, 4 − D + 2.2 μM BAP). However none of these callus colonies regenerated shoots or embryos.

The first report of lentil somatic embryogenesis was by Saxena and King (1987). Immature embryo explants were cultured on either B5A (B5 + 500 mg l^{-1} ammonium nitrate) or MS medium supplemented with 1 – 10 mg l^{-1} 2,4-D. Callus growth was best at 2,4-D concentrations between 1 – 5 mg l^{-1} and the B5A medium produced more organised callus than MS. Callus initiated on medium containing 1 mg l^{-1} 2,4-D and subcultured to medium without 2,4-D but supplemented with 1 mg l^{-1} BAP and 0.25 mg l^{-1} IAA produced club shaped embryoids. The embryoids were transferred to B5A medium without growth regulators and with the addition of 70 mg l^{-1} glutamine to promote embryo development. Embryos that developed well-defined shoot and root axes were able to germinate on a modified B_5 medium (B_5A) free of growth regulators.

3.7. Protoplast Culture and Hybridisation

Somatic hybridization using protoplast fusion has the potential to overcome pre- and post-zygotic barriers to interspecific hybridisation (Davey *et al.* 2005). It is possible to regenerate plants from a number of legume species including *Pisum* (Ochatt *et al.*, 2000), *Trifolium* (Gresshoff, 1980), *Lotus* (Ahuja *et al.* 1983) and *Melilotus* (Luo and Jia, 1998) and asymmetric protoplast fusion has been used for *Medicago* improvement (Tian and Rose, 1999; Yuko *et al.* 2006). However there are no reports of successful growth or regeneration of protoplasts of *Lens* species. Rozwadowski *et al.* (1990) cultured protoplasts from lentil epicotyl tissue and around 6% of protoplasts developed into cell colonies. However there are no reports of successful plant regeneration from lentil protoplasts.

4. HAPLOIDS AND DOUBLED HAPLOIDS

Doubled haploids are an important breeding tool in many crop species including wheat, barley, rice, maize and canola. The implementation of doubled haploids increases selection efficiency and allows new varieties to be bred up to five years faster than with conventional breeding methods alone. Haploids may be produced either from immature pollen cells, immature egg cells or following asymmetric chromosome elimination after interspecific hybridisation. A recent review of the literature on doubled haploid production in the Fabaceae (Croser *et al.*, 2006) indicates that none of these approaches have been successful to date for producing lentil haploid plants, but the early stages of isolated microspore division have been observed.

5. SUMMARY

The wild relative species of cultivated lentils are a significant source of genetic variation available for improvement of the relatively narrow genetic base of this crop. The wild species are endemic to a wide range of environments and possess

many diverse characteristics including disease resistances and abiotic stress tolerances which may benefit cultivated lentils. It is possible to intercross most of the wild *Lens* species with cultivated lentils using plant growth regulators and/or embryo rescue to allow the growth of hybrid plants. There is enormous potential to exploit these hybrids for the improvement of cultivated lentil germplasm. Other biotechnology techniques including doubled haploid production and regeneration from protoplast culture are much less developed but there has been significant groundwork done to expect that these technologies, particularly doubled haploids, may be of benefit to lentil improvement programs within the next decade.

REFERENCES

Abbo S and Ladizinsky G (1991) Anatomical aspects of hybrid embryo abortion in the genus Lens L. Bot Gaz 152 (3): 316–320.

Ahmad M, and DL McNeil DL (1996) Comparison of crossability, RAPD, SDS-PAGE and morphological markers for revealing genetic relationships within and among *Lens* species. Theoretical and Applied Genetics 93: 788–793.

Ahmad M, Fautrier AG, McNeil DL, Burritt DJ and Hill GD. (1995) Attempts to overcome postfertilization barrier in interspecific crosses of the genus *Lens*. Plant Breeding 114:558–560.

Ahmad M, McNeil DL and Sedcole JR (1997a) Phylogenetic relationships in Lens species and their interspecific hybrids as measured by morphological characters. Euphytica 94(1):101–111.

Ahmad M, Fautrier AG, McNeil DL, Hill GD, Burritt DJ (1997b) *In vitro* propagation of *Lens* species and their F_1 interspecific hybrids. Plant Cell, Tissue & Organ Cult 47: 169–176.

Ahuja PS, Hadiuzzaman S, Davey MR and EC Cocking EC (1983) Prolific plant regeneration from protoplast derived tissues of *Lotus corniculatus* L. (birdsfoot trefoil). Plant Cell Reports 2:101–104.

Bajaj YPS and Dhanju MS(1979) Rescue of plants from apical meristem tips of some legumes. Current Science 48:906–907.

Barulina, H. (1930) Lentil of the USSR and other countries, Suppl. 40th Bull. Appl. Bot. Genet. Plant Breed. Leningrad.

Bayaa B, Erskine W and Hamdi A (1994) Response of wild lentil to Ascochyta fabae f.sp. lentis from Syria. Genetic Resources and Crop Evolution 41: 61–65.

Bayaa B, Erskine W and Hamdi A (1995) Evaluation of a wild lentil collection for resistance to vascular wilt. Genetic Resources and Crop Evolution 42: 231–235.

Cohen D, Ladizinsky G, Ziv M and Muehlbauer FJ (1984) Rescue of interspecific lens hybrids by means of embryo culture. Plant Cell, Tissue & Organ Cult 3: 343–347.

Croser JS, Lulsdorf M, Davies PA, Clarke H, Bayliss K, Mallikarjuna N, Siddique K (2006) Towards doubled haploid production on the fafaceae: progress and constraints. Critical Reviews in Plant Science 25:139–157.

Czefranove, Z. (1971) Novosti Systematischeski Vyssich Rastenii 8: 184–191.

Davey MR, Anthony P, Power JB and Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnology Advances 23:131–171.

FAO (2005) "FAOSTAT database" Food and Agriculture Organisation of the United Nations. http://faostat.fao.org/faostat/collections?ver-sion = ext&hasbulk = 0&subset = agriculture.

Ferguson, M. (1998) PhD Thesis: *Studies of genetic variation within the genus lens*. School of Biological Sciences, University of Birmingham, Birmingham, UK.

Ferguson ME, and Robertson LD (1996) Genetic diversity and taxonomic relationships within the genus *Lens* as revealed by allozyme polymorphism Euphytica 91: 163–172.

Ferguson ME, Maxted N, van Slageren M and Robertson LD (2000) A re-assessment of the taxonomy of *Lens* Mill. (Leguminosae, Papilionoideae, Vicieae). Bot J Linnean Soc 133: 41–59.

Ferguson ME and Erskine W (2001) Lentils (*Lens* L.). In: Maxted N and Bennett SJ (eds.), Plant Genetic Resources of Legumes in the Mediterranean, pp. 125–131. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Fiala JV (2006) Transferring resistance to *Colletotrichum truncatum* from wild lentil species to cultivated lentil species (*Lens culinaris* subsp *culinaris*). MSc thesis, University of Saskatchewan, Saskatoon, Canada, 131 pp.

Fratini R and Ruiz ML (2002) Comparative study of different cytokinins in the induction of morphogenesis in lentil (*Lens culinaris* Medik). In vitro Cell Dev Biol – Plant 38: 46–51.

Fratini R and Ruiz ML (2003) A rooting procedure for lentil (*Lens culinaris* Medik) and other hypogeous legumes (Pea, chickpea and *Lathyrus*) based on plant polarity. *Plant Cell Reports* 21:726–732.

Fratini R and Ruiz ML (2004) Intra-specific and inter-sub-specific crossing in lentil (*Lens culinaris* Medik.) Can. J. Plant Sci 84: 981–986.

Fratini R and Ruiz ML (2006) Interspecific hybridization in the genus *Lens* applying *in vitro* embryo rescue. Euphytica 150: 271–280.

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

Goshen D, Ladizinsky G, Muehlbauer FJ (1982) Restoration of meiotic regularity and fertility among derivatives of *Lens culinaris* × *L nigricans* hybrids. Euphytica 31: 795–799.

Gulati A, Schryer P, McHughen A (2001) Regeneration and micrografting of lentil shoots. In vitro Cell Dev Biol – Plant 37: 798–802.

Gresshoff PM (1980) *In vitro* culture of white clover: callus, suspension, protoplast culture and plant regeneration. *Bot Gaz* 141:157–164.

Hamdi A and Erskine W (1996) Reaction of wild species of the genus Lens to drought. Euphytica 91:173–179.

Hamdi A, Küsmenoglu I and Erskine W (1996) Sources of winter hardiness in wild lentil. Genetic Resources and Crop Evolution. 43: 63–67.

Hoffman D, Soltis D, Muehlbauer F and Ladizinsky G (1986) Isozyme Polymorphism in *Lens* (Leguminosae). Systematic Botany 11: 392–402.

Ladizinsky G. (1989) Ecological and genetic considerations in collecting and using wild relatives. In: Brown A.H.D,. Marshall D.R., Frankel O.H., and Williams L.T., (Eds.), The Use of Plant Genetic Resources, pp. 297. Cambridge.

Ladizinsky G (1992) Crossability relations. Monograph on Theoretical and Applied Genetics pp. 15–31.

Ladizinsky G (1993) Wild Lentils. Critical Rev Plant Sci. 12(3): 169–184

Ladizinsky G (1997) A new species of Lens from south-east Turkey. Botanical Journal of the Linnean Society 123: 257–260.

Ladizinsky G, Braun D and Muehlbauer FJ (1983) Evidence for domestication of Lens nigricans (M. Bieb.) Godron in southern Europe. Botanical Journal of Linnean Society 87: 169–176.

Ladizinsky G, Braun D, Goshen D and Muehlbauer FJ (1984) The biological species of the genus *Lens* L. [*Lens nigricans*]. Bot Gazette 145 (2): 253–261.

Ladizinsky G, Cohen D and Muehlbauer FJ (1985) Hybridization in the genus *Lens* by means of embryo culture. Theor Appl Genet 70: 97–101.

Ladizinsky G and Abbo S (1993) Cryptic speciation in *Lens culinaris*. Genet Res & Crop Evol 40: 1–5

Luo JP and Jia JF (1998) Plant regeneration from callus protoplasts of the forage legume *Astragalus adsurgens* Pall. Plant Cell Reports 17:313–317.

Malik KA and Saxena PK (1990) Thidiazuron induces high-frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). Aust J Plant Physiol 19:731–740.

Muehlbauer FJ and McPhee KE (2005) In: Genetic resources, chromosome engineering and crop improvement, Vol. 1, Grain Legumes, Ch. 8, Lentil (*lens culinaris* Medik), pp 219–230. Eds. RJ Singh, and PP Jauhar. Taylor & Francis, Boca Raton.

Muehlbauer FJ, Cho S, Sarker A, McPhee KE, Coyne CJ, Rajesh PN and Ford R (2006). Application of biotechnology in breeding lentil for resistance to biotic and abiotic stress. Euphytica 147 (1–2): 149–165.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.

Newell C, Growns D and McComb J (2006) Aeration is more important than shoot orientation when rooting lentil (*Lens culinaris* Medik) cv. 'Digger' microcuttings *in vitro*. In vitro Cell. Dev. Biol. – Plant 42:197–200.

Ochatt SJ, Mousset-Déclas C, and Rancillac M (2000) Fertile pea plants regenerate from protoplasts when calluses have not undergone endoreduplication. Plant Science 156:177–183.

Oss H van, Aron Y and Ladizinsky G (1997) Chloroplast DNA variation and evolution in the genus *Lens* Mill. Theor Appl Genet 94: 452–457.

Polanco MC and Ruiz ML (1997) Effect of benzylaminopurine on *in vitro* and *in vivo* root development in lentil, *Lens culinaris* Medik. Plant Cell Reports 17:22–26.

Polanco MC, Pelaez MI and Ruiz ML (1988) Factors affecting callus and shoot formation from *in vitro* cultures of *Lens culinaris* Medik. Plant Cell Tissue Organ Culture 15:175–182.

Rozwadowski KL, Saxena PK and King J (1990) Isolation and culture of *Lens culinaris* Medik. Plant Cell Tissue Organ Culture 15:175–182.

Saxena PK and King J (1987) Morphogenesis in lentil: plant regeneration from callus cultures of *Lens culinaris* Medik. via somatic embryogenesis. Plant Science 52:223–227.

Tian D and Rose RJ (1999) Asymmetric somatic hybridization between the annual legumes *Medicago truncatula* and *Medicago scutellata*. Plant Cell Reports 18:989–996.

Williams .T, Sanchez AMC and Jackson MT (1974) Studies on lentils and their variation, I. The taxonomy of the species. SABRAO Journal 6, 133–145.

Williams DJ and McHughen A (1986) Plant regeneration of the legume *Lens culinaris* Medik (lentil) *in vitro*. Plant Cell Tissue Organ Culture 7:149–153.

Ye G, McNeil DL, Conner AJ and Hill GD (2002) Multiple shoot formation in lentil (*Lens culinaris*) seeds. New Zealand J Crop & Hort Sci 30: 1–8.

Ye G, McNeil DL, Conner AJ and Hill GD (2000) Improved protocol for the multiplication of lentil hybrids without genetic change by culturing single node explants. SABRAO J Breeding & Genet 32 (1): 13–21.

Yuko M, Mitsuru K, Takamizo T, Kanbe M, Inami S and Hattori K (2006) Interspecific hybrids between *Medicago sativa* L and annual *Medicago* containing alfalfa weevil resistance. Plant Cell Tissue Organ Culture 84:79–88.

Zohary D (1972) The wild progenitor and the place of origin of the cultivated lentil *Lens culinaris*. Economic Botany 26, 326–332.

Zohary D and Hopf M. (1988) Domestication of Plants in the Old World. Clarendon Press, London.