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Toward Doubled Haploid Production in the Fabaceae: Progress, Constraints, and Opportunities

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The Fabaceae species have a major role to play in sustainable farming systems, but they have lagged behind other families in respect to the development of doubled haploid protocols for plant improvement. Currently, no plant improvement program uses doubled haploids on a routine basis for any member of the Fabaceae. There has recently been renewed interest in haploid research as the usefulness of doubled haploid material in molecular mapping has become clear. This review provides a comprehensive account of the current information regarding the development of haploid protocols in the Fabaceae. In the Fabaceae crop species there have been isolated reports of haploid plant induction in the phaseoloid clade; soybean, cowpea and pigeonpea, as well as promising progress towards haploidy in peanut and winged bean. As yet there have been no reports of haploid plant production in the galegoid clade, but early stage haploid embryogenesis has been achieved in chickpea, field pea, and lupin. Success in the production of haploid plants has also been reported within the pasture genera *Lotus*, *Medicago*, and *Trifolium* and the arboreal genera *Cassia*, *Peltophorum*, and *Albizzia*. A review of the literature has enabled us to identify some general similarities between the protocols developed for haploid plant induction across the various legumes. These are the culture of intact anthers; use of a cold pretreatment to induce sporophytic development; targeting of microspores at the uninucleate stage of development; and use of MS (Murashige and Skoog, 1962) based nutrient medium with plant growth regulators to encourage continued division following induction. These protocol commonalities will assist researchers to identify approaches suited to their target Fabaceae species. The paucity of research funding for haploid research in most Fabaceae species has highlighted the need for strong collaborative linkages between institutions and researchers.

Keywords legume, anther culture, isolated microspore culture, tissue culture, androgenesis, gynogenesis

Abbreviations: BAP, 6-Benzylaminopurine; IBA, Indole-3-butyric acid; NAA, Naphthalene acetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2-iP, N-(3-methyl-2-butenyl)-1H-purin-6-amine; MS, Murashige and Skoog basal medium as per Murashige and Skoog (1962); B5, Gamborgs basal medium as per Gamborg (1966); DH, Doubled haploid; DAPI, DNA specific fluorochrome 4′,6-diamidimo-2-phenylindole dihydrochloride; Kr, Kilorad; Gy, Gamma radiation—Gray.

I. INTRODUCTION

Doubled haploidy is not used as a routine tool in breeding programs for any leguminous species and, in comparison with many other families of angiosperms, progress towards this goal

has been slow. Research efforts to obtain haploid plants from legumes peaked in the 1980s (Table 1). Molecular marker research became the focus of many legume research programs in the 1990s and with the recognition of the value of haploids to molecular mapping efforts there has been a renewed surge of interest in haploid technology for legumes. We present here a critical overview of past and current attempts to develop haploids in leguminous species, giving particular emphasis to those with economic significance and those in which sporophytic induction from gametophytes has been successful.

Haploid plants provide an excellent example of cellular totipotency (Powell, 1990) as they develop from either the male or female gametes without fertilization. Androgenesis is the development of haploids from the male gametes, parthenogenesis is the development of haploids from the unfertilized egg and apogamy is the development of haploids from the other cells of the mega-gametophyte. As they arise without fertilization, or following fertilization and subsequent paternal chromosome elimination, haploid plants possess either maternal or paternal genetic information. Regardless of the mode of development, doubling of haploid plants either spontaneously via endomitosis or by chemical means, leads to a homozygous "doubled haploid" individual with two identical copies of each chromosome. By using doubled haploids, homozygosity can be achieved in a single generation (Maluszynski *et al.*, 2003).

Guha and Maheshwari (1964, 1966) were the first to demonstrate haploid embryos and plants could be produced by culturing anthers of *Datura*. Kasha and Kao (1970) later reported haploid production via chromosome elimination following wide hybridization in barley and the use of haploid plants in breeding programs began. These two systems—anther/microspore culture and wide hybridization—are the two major methods used in breeding programs today. Wide hybridization methods are limited to potato and the cereals and, while microspore culture should be feasible in most species, it has taken a long time to develop efficient systems in a relatively limited number of crops. Each crop has individual optimal pretreatment regimes and *in vitro* culture requirements and there is a need for extensive research to develop an efficient system (Kasha and Maluszynski, 2003).

It is important to note that the term haploid refers to a plant with the gametic number of chromosomes (n). The gametic number of chromosomes may not be equivalent to the original, or basic, number of chromosomes (x) for that species, leading to a number of different terms for haploid plants. For example, monoploid refers to a haploid derived from a diploid in which "x" is equal to one genomic complement (i.e., $x = n$). Polyhaploid refers to a haploid derived from a polyploid (i.e., $n > x$) and in this case the prefix indicates the genetic complement, e.g., dihaploid.

Doubled haploids have been used in plant breeding programs to produce homozygous genotypes in a number of important species, e.g., tobacco (*Nicotiana tabacum* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), canola (*Brassica napus* L.), rice (*Oryza sativa* L.), and maize (*Zea mays* L.) (Maluszynski *et al.*, 2003). Publications on crops such as rice, wheat, and maize have shown that significant progress in haploid technology is attainable given an intensive research effort. Such an effort has yet to be directed towards leguminous species, predominantly due to their cultivation in developing countries and consequent paucity of research funding. Difficulties have also been posed by the generally small anther size and relatively low numbers of microspores per anther in legume crops. The benefits of doubled haploids in plant breeding has been widely reviewed; readers should refer to the series of five volumes on *In Vitro Haploid Production in Higher Plants* edited by Jain, Sopory, and Veilleux (1996–1997).

The leguminous species are generally classed within the family Fabaceae, sometimes ambiguously referred to as the Leguminosae, which is one of the largest families of flowering plants, numbering some 630 genera and 20,000 species. These genera are divided into the following three subfamilies: Mimosoideae, Caesalpinioideae, and the numerically dominant Papilionoideae. With the exception of peanut (*A. hypogaea* L.—Aeschynomenoid), the economically important crop legumes occur in two Papilionoid clades, the phaseoloid and the galegoid legumes (Choi *et al.*, 2004), which are separated into various tribes. Leguminous species are particularly important in low input and sustainable cropping systems due to their unique ability to fix atmospheric nitrogen via a symbiotic relationship with *Rhizobium* bacteria and their use as plant-based dietary protein for human food and animal feed (Table 2).

II. PAPILIONOIDEAE

A. Progress Towards Haploids within the Tropical and Sub-Tropical (Warm-Season) Grain Legume Crop Species

Of the leguminous species, the tropical and sub-tropical legumes have had the highest level of biotechnology research investment—due to the importance of the soybean (*Glycine max* L. Merr.) crop to the food and industrial sectors. As a result of this, and perhaps a higher responsiveness to tissue culture techniques, haploid research within this group is more advanced than in other legumes. Cytologically confirmed haploid plant production has been achieved for four species of the tribe Phaseoleae: soybean, pigeonpea (*Cajanus cajan* L.), cowpea (*Vigna unguiculata* L. Walp.), and winged bean (*Psophocarpus tetragonolobus* L. D.C.). Haploidy in peanut has also been moderately successful with shoots produced from potentially haploid, anther-derived callus.

1. Tribe Phaseoleae

1.1. Soybean (Glycine max L. Merr.). Cultivated soybean is a diploidized tetraploid ($2n = 40$), important in food and industry as it possesses a complete amino acid profile almost equivalent to meat, milk, or eggs, and both oil and protein can be extracted from the same grain. Due to the large size of the

TABLE 1
Key publications relating to haploid research in species of the Fabaceae

Author/s	Cultivar/line	Target material	Level of success
Soybean *(Glycine max)*			
Tang *et al.*, 1973	Unknown	Anther culture	Callus but no confirmation of ploidy.
Ivers *et al.*, 1974	Hark	Anther culture	Somatic embryogenesis from callus (diploid).
Yin, 1981	Chinese cultivars	Anther culture	Callus derived from anthers containing uninucleate pollen. Sporadic induction.
Yin *et al.*, 1982	Chinese cultivars	Anther culture	Microspore-derived callus and a few shoots. Suggest that the vegetative nucleus of the microspore gives rise to multinucleate cell which is assumed to be precursor of the embryo *in vitro*.
Liu and Zhao, 1986	Chinese cultivars	Isolated microspore	Callus (haploid—no quantitative data given).
Jian *et al.*, 1986	Chinese cultivars	Isolated microspore	Callus induction 36% (haploid). One genotype found which regenerated shoots with frequency of 1% from callus derived embryos.
Kadlec *et al.*, 1991	Unknown	Anther culture	Callus and embryo-like structures. No quantitative data given.
Kiss *et al.*, 1991			Review.
Zhuang *et al.*, 1991	Williams 82; Asgrow A1929	Anther culture	Embryo-like structures, abnormal, "presumed" to be derived via androgenesis.
Kaltchuk-Santos *et al.*, 1993	Decada; IAS 5; Ipagro 21; BR 4	Isolated microspore	Pollen dimorphism (development of small atypical pollen interspersed with 'normal' pollen — observed *in vivo* and *in vitro*).
Ye *et al.*, 1994	Many Chinese cultivars & 1 US line PI486355	Anther culture	Callus induction frequency was 36% in best genotype, of which 84% were haploid if 15% sucrose was used in medium. Seven embryoids induced but did not develop further.
Yao *et al.*, 1996	Unknown		Reference not available.
Hu *et al.*, 1996	Unknown		Review.
Kaltchuk-Santos *et al.*, 1997	IAS 5; RS 7	Anther culture	Frequency of anthers or callus with embryos was 2%.
Zhao *et al.*, 1998	110 tested.	Anther culture	26–60% callus induction; 4–18% embryos; 0–2% plantlets. Plants returned from genotypes: LA88-408814, PI-9501, Buckshot 723, Changnong No. 4, Jilin No. 20, Jilin No. 32, Jilin No. 36, 70203-1. Histological study showed plantlets were haploid.
da Silva Lauxen *et al.*, 2003	Decada; IAS 5; RS 7	Anther culture	Variation in microspore development between genotypes and even within anthers of the same bud.
Rodrigues *et al.*, 2004	BRQ96-3065; BRSMG-Lideranca; and F2 progeny	Anther culture	Embryo-like structures, derived from simultaneous somatic embryogenesis and androgenesis (SSR marker).
Rodrigues *et al.*, 2005	Bragg; IAS 5; Conquista; BRS 133	Anther culture	Histology showed that all viable embryo-like structures arose from callus derived from anther tissues. Induced multinucleate pollen were unviable by 18th day *in vitro*.
Pigeonpea (*Cajanus cajan L.*)			
Bajaj *et al.*, 1980b	T-21	Anther culture	17.4% haploid callus. Pollen embryoids and callus from microspore cultures preconditioned for 3 weeks in the anther.
Mohan Ram *et al.*, 1982	*Cajanus cajan*	Anther culture	Multicellular structures.

TABLE 1
Key publications relating to haploid research in species of the Fabaceae (*Continued*)

Author/s	Cultivar/line	Target material	Level of success
Sudhakar and Moss, 1990	T-7; T-21	Anther culture	Mixoploid callus.
Fougat *et al.*, 1992	NP (WR)-15	Anther culture	Callus regenerated from anthers—haploid in primary culture, but becoming polyploid and aneuploid in subcultures and finally mixoploid through endomitosis.
Kaur and Bhalla, 1998	ICPL 89021	Isolated Microspore	Regeneration frequency of 10–13% haploid plants.
VishnuKumar *et al.*, 2000	cv. Maruti; ICPL 87; TS-3; and GS-1	Anther culture	Callus formation in TS-3 was 26%; followed by Maruthi (25%); and GS-1 (21%). Details of ploidy level not reported.
Cowpea (*Vigna ungicualata*)			
Ladeinde and Bliss, 1977	cv.Texas cream	Anther culture	Callus and roots but no indication of ploidy level.
Mix and Wang, 1988	cv. Pipo cv. Tvu 91 and Tru 1987	Anther culture	Haploid plants were obtained from all the cultivars used in the study. Cultivars Pipo and Tvu 91 produced 2 shoots and Tvu 1987 produced one.
Arya and Chandra, 1989	n/a	Anther culture	Callus with shoot like structures and roots. Origin of callus not known and frequency of callus/shoot/root not reported.
Mung Bean (*Vigna radiata* L.)			
Bajaj and Singh, 1980	G-65, SML-32, cv. Shining Moon No 1.	Anther culture	Haploid callus, multinucleate cells and early stage embryogenesis.
Gosal and Bajaj, 1988	Mash T9 and Mash 1-1	Anther culture	10–15% haploid callus cells, pollen embryogenesis in 0.66% of studied pollen. No further development.
Winged bean (*Psophocarpus tetragonolobus* L. D.C.)			
Brunel *et al.*, 1981	Unknown	Anther culture	Callus but no regeneration.
Mohan Ram *et al.*, 1982	Unknown	Isolated microspore	Callus but no regeneration.
Pal, 1983	Unknown	Isolated microspore	80% haploid cell population. Leafy shoots, but no plantlets.
Trinh *et al.*, 1986	Unknown	Anther culture	Haploid plants from anther callus.
Common Bean *(Phaseolus vulgaris)*			
Peters *et al.*, 1977	cv. Bico de Ouro	Anther culture	Callus induction ion 57% of anthers, of which, ca. 50% were haploid. No shoot or plant regeneration.
Munoz and Baudoin, 2001	*P. vulgaris* cultivars NI922 and X1862 *P. coccineus* cultivars N115 and N116	Anther culture	Haploid anther-derived callus but no regeneration to plants.
Groundnut/Peanut (*Arachis hypogaea* & wild *Arachis* spp.)			
Martin and Rabechault, 1976	*A. hypogaea*	Anther culture	Haploid, diploid and mixoploid callus was produced. In addition weak and albino seedlings were obtained.

(*Continued on next page*)

TABLE 1
Key publications relating to haploid research in species of the Fabaceae (*Continued*)

Author/s	Cultivar/line	Target material	Level of success
Mroginski and Fernandez, 1979	*A.correntina*; *A. villosa*;	Anther culture	Callus with haploid to mixoploid chromosome number, and multicellular microspores in 1% of the cells analyzed.
Mroginski and Fernandez, 1980	*A. lignosa*	Anther culture	Regenerated plants were not haploids.
Bajaj *et al.*, 1981	*A. hypogaea A. villosa*	Anther culture	Haploid to octoploid callus. Frequency of callus induction not reported.
Sastri *et al.*, 1981	*A. hypogaea*	Anther culture	Callus and early stages of pollen embryogenesis. No data on ploidy level or frequency of callus induction.
Pittman, 1981	*Arachis spp* (exact species not detailed)	Anther culture	Shoot regeneration but no haploids
Bajaj, 1983	M13; *A. villosa*(wild species)	Anther culture	Cryopreservation and regeneration of pollen embryos from anthers.
Still *et al.*, 1987	*A. Paraguariensis*	Anther culture	Callus, buds, shoots were obtained from filament of the anther. Plants were obtained by grafting the shoots on groundnut stocks.
Sudhakar and Moss, 1990	R33-1; TMV3; ICGS11.	Anther culture	Identification of most uninucleate and vacuolated stage of microspore development as most appropriate for anther culture.
Willcox *et al.*, 1991	PI 109839	Anther culture	Anthers with microspores at uninucleate stage of development most responsive in producing multinucleate microspores. No further details.
Yeh and Tseng, 1999	cv. Chico; TN11; VA; TN5;VB12; VR34	Anther culture	Callus induction from TN-5 was 94% and 82% from VR-34. Ploidy level not reported.
Tseng and Yeh, 2000	TN11; TN5; VR34	Anther culture	Callus and roots—ploidy level of callus not reported.
Lee and Yeh, 2001	TNS9; TN11	Anther culture	Embryoids regenerated from TNS9 (18.5%). Rate of shoot regeneration from embryoids 47.1%. No confirmation of haploid.
Field Pea (*Pisum sativum*)			
Gupta *et al.*, 1972	Breeding line B22	Anther culture	Microspores were at uninucleate stage. 14% of anthers had callus. 91% of callus cells were haploid. No embryo development.
Gupta, 1975	Breeding line B22	Anther culture	After 21 passages, some embryos and shoots developed. After 39 passages, subcultured callus became mixoploid with predominantly tetraploid cells. No plants were regenerated.
Virk and Gupta, 1984		Matromorphy	Some progeny showing homozygosity, but not clear haploidy.
Gosal and Bajaj, 1988	Bonneville, breeding lines T163 & P88	Anther culture	Cold pre-treatment of anthers at 4–5°C for 72 hours improved induction. Embryoid formation was 0.34% with subsequent dedifferentiation into callus. Callus was mixoploid.
Croser and Lulsdorf, 2004	CDC April, Highlight	Isolated microspore	Cold or heat pre-treatment necessary for induction. Symmetrical microspore nuclei division to multinucleate stage. One diploid plant recovered.
Lentil (*Lens culinaris*)			
Keller and Ferrie, 2002	CDC Robin	Anther culture	Callus induction on 57.4% of anthers with some embryo development.

TABLE 1
Key publications relating to haploid research in species of the Fabaceae (*Continued*)

Author/s	Cultivar/line	Target material	Level of success
	CDC Crimson	Isolated microspore	Cell divisions to the 8-cell stage with microspore culture.
Croser and Lulsdorf, 2004	CDC Robin	Isolated microspore	Cold pre-treatment for 1–5 days necessary. Symmetrical microspore nuclei division.
Lupin (*Lupinus* spp.)			
Sator, 1985	*L. polyphyllus*	Anther culture	Callus returned from anthers and plantlets regenerated but chromosome counts suggested they were diploid.
Ormerod and Caligari, 1994	*L. albus*	Anther culture Isolated microspore	Embryo-like structures (ELS) produced from anthers cultured in liquid medium. Recurrent somatic embryogenesis from the ELS.
Bayliss *et al.*, 2004	*L. albus* cv Kiev Mutant, *L. angustifolius* cv. Marri and cv. Chittick, *L. luteus* cv. Wodjil	Isolated micropsore	Induction of microspores for embryogenesis and production of multicellular microspores/pro- embryos.
Chickpea *(Cicer arietinum)*			
Khan and Ghosh, 1983	B108	Anther culture	Cultured anthers produced callus with roots. Anthers with uninucleate pollen grains produced most callus. Callus-cell chromosome numbers ranged from n = 8 (haploid) to n = 16, with 28.1% being haploid, 38.2% diploid and 37.7% aneuploid.
Altaf and Ahmad, 1986	Unknown	Anther culture	Callus from anthers, but no regeneration to plants.
Bajaj and Gosal, 1987	G 543, L 550, Hare Chhole 1	Anther culture	Anthers with pollen at the uninucleate stage. 72 h cold pretreatment. MS based medium gave up to 60% callus initiation. Rhizogenesis and embryoids. No haploid callus cells after 6–8 weeks of subculture.
Gosal and Bajaj, 1988	G 543, L 550, Hare Chhole 1	Anther culture	Anthers with pollen at the uninucleate stage. 72 h cold pretreatment. MS based medium gave up to 60% callus initiation. Rhizogenesis and embryoids. No haploid callus cells after 6–8 weeks of subculture.
Reddy and Reddy, 1996		In vivo	Production of haploids using a male sterile parent.
Huda *et al.*, 2001	Nabin, Deshi, ICCL 83105, ICCL 85222, Bari Chhola 5	Anther culture	Pollen at mid-late uninucleate stage, cold pretreatment 4°C for 3–7 days, 80% callus formation on B5 based medium. Somatic embryogenesis, shoot differentiation. No confirmation of ploidy level.
Croser, 2002	F_1 crosses, Bumper, Garnet, Kaniva, Macarena, CDC Yuma, Chico, Desiray + breeding lines.	Anther culture Isolated microspore	16 plants regenerated from anther-derived callus but all those tested using flow cytometry were diploid. Androgenic structures from isolated microspore culture, but no regeneration to plants.
Vessal *et al.*, 2002	Pirooz and Karaj 12-60-31	Anther culture	Haploid callus cells and mature embryos were returned from anthers containing uninucleate stage microspores.
Croser *et al.*, 2004	Bumper, CDC Cabri, Rupali	Isolated microspore	32.5°C 16 h pretreatment of buds. Production of proembryos from isolated microspores. Cytological tracking proved androgenic origin of proembryos. No regeneration to mature embryos or plants.

(*Continued on next page*)

TABLE 1
Key publications relating to haploid research in species of the Fabaceae (*Continued*)

Author/s	Cultivar/line	Target material	Level of success
Lucerne *(Medicago sativa)*			
Bingham, 1969	Unknown	Intraspecific hybridization	Dihaploid plants produced by pollinating tetraploids with diploi pollen.
Bingham, 1971	Unknown	Intraspecific hybridization	Dihaploid plants produced by pollinating tetraploids with diploid pollen.
Bingham *et al.*, 1975	Unknown	Intraspecific hybridization	Dihaploid plants produced by pollinating tetraploids with diploid pollen.
Zagorska *et al.*, 1984	Unknown	Anther culture	Mixoploid plants regenerated.
Tanner *et al.*, 1990	Unknown	Anther culture	Microspore division observed.
Zagorska and Dimitrov, 1995	Unknown	Anther culture	Haploid plants regenerated.
Zagorska *et al.*, 1997	Unknown	Anther culture	Haploid plants regenerated.
Berseem clover *(Trifolium alexandrinum)*			
Mokhtarzadeh and Constantin, 1978	Unknown	Anther culture	Haploid plants regenerated.
Lotus *(Lotus corniculatus)*			
Negri and Veronesi, 1989	Unknown	Interspecific hybridization	Dihaploid plant produced by pollinating tetraploid *L corniculatus* with diploid *L tenuis* pollen.
Séguin-Swartz and Grant, 1995	Unknown	Anther culture	Mixoploid plant regenerated.
East Indian Walnut (*Albizzia lebbeck* L.)			
Gharyal *et al.*, 1983a	Unknown	Anther culture	20% callusing, followed by organogenesis and somatic embryogenesis. Two haploid plantlets regenerated.
Cassia fistula			
Bajaj and Dhanju, 1983	Unknown	Anther culture	Multinucleate or multicellular pollen in 1.6% of pollen studied. 0.65% pollen embryos.
Cassia siamea			
Gharyal *et al.*, 1983b	Unknown	Anther culture	Pollen embryogenesis—6–8 celled within the exine but no plantlet regeneration.
Yellow Flame Tree (*Peltophorum pterocarpum*)			
Rao and De, 1987	Unknown	Anther culture	Globular embryogenesis followed by haploid callus (60–70%). Organogenesis followed by plant regeneration, of which 12% were haploid.

soybean industry extensive resources have been invested in crop improvement. The potential of doubled haploid technology for the development of homozygous parents for hybridization programs has been recognized since soybean anthers were cultured in the 1970s and 1980s (Ivers *et al.*, 1974; Tang *et al.*, 1973; Yin, 1981; Yin *et al.*, 1982). Progress towards androgenesis has been made by research groups in China, Brazil, and the United States (reviewed by Hu *et al.*, 1996; Kiss *et al.*, 1991) but so far there is no reproducible system for the routine production of doubled haploid soybean plants.

Early attempts to culture soybean anthers or microspores resulted in mostly callus formation, some of which was diploid

TABLE 2
2004 area, production and productivity for tropical/sub tropical and temperate leguminous crop species that have been the subject of attempts to develop haploid plant production protocols

Crop species	World area (Mha)	Average yield (t ha^{-1})	World production (MMt)	Five highest producers (ranked highest to lowest)
Tropical/ subtropical species				
Soybean (*Glycine max* L.)	91.6	2.2	206.4	US, Brazil, Argentina, China, India
Peanut/ Groundnut (*Arachis hypogaea* L.)	26.4	1.4	36	China, India, Nigeria, US, Indonesia
Common bean (*Phaseolus vulgaris* L.)	26.9	0.7	18.7	Brazil, Indian, China, Myanmar, Mexico
Pigeonpea (*Cajanus cajan* L.)	4.4	0.7	3.2	India, Myanmar, Malawi, Uganda, Kenya
Winged bean (*Phosphocarpus tetragonolobus* L.)	n/a	n/a	n/a	n/a
Mung Bean* (*Vigna radiata* L.)	n/a	n/a	n/a	n/a
Temperate species				
Field pea/Dry Pea (*Pisum sativum* L.)	6.7	1.8	12.1	Canada, France, China, Russian Federation, India
Chickpea (*Cicer arietinum* L.)	10.3	0.8	8.6	India, Turkey, Pakistan, Iran, Mexico
Lentil (*Lens culinaris* Medik.)	4.1	0.9	3.8	India, Canada, Turkey, Australia, Nepal
Lupin (*Lupinus* spp. *L. albus*, *L. angustifolius*, *L. luteus*)	0.8	1.4	1.2	Australia, Chile, France, Russian Federation, Morocco

Source: (FAOSTAT, 2005) (http://faostat.fao.org/).
*Note Mung bean is often included with common bean in FAO statistics.

(Ivers *et al.*, 1974) and some haploid (Jian *et al.*, 1986; Jian *et al.*, 1985; Liu and Zhao, 1986). This has since been clarified by Rodrigues *et al.* (2004) who used a co-dominant molecular marker to screen callus derived from anthers from heterozygous donors and confirmed that both androgenic and somatic embryogenesis was occurring simultaneously. Sporadic development of embryo-like structures, or shoots, was observed in both Chinese and U.S. soybean cultivars and was presumed to be derived by androgenesis (Kadlec *et al.*, 1991; Yin *et al.*, 1982; Zhuang *et al.*, 1991). Subsequent research revealed the importance of selecting early to mid-uninucleate stage microspores (Ye*et al.*, 1994; Yin *et al.*, 1982). More recently, da Silva Lauxen *et al.* (2003) demonstrated that microspores can be at different stages of development in anthers of the same flower, depending on their position in the anther whorl. Secondly, different bud sizes were found to contain microspores at different stages depending on the soybean genotype, with the uninucleate stage being found most appropriate for culture. Variation among soybean genotypes is also reported by Kaltchuk-Santos *et al.* (1993). Kaltchuk-Santos *et al.* (1997) reported the regeneration of well-formed cotyledonary embryos from soybean anthers. Zhao *et al.* (1998) achieved haploid embryo induction from anthers of soybean on a MS based medium supplemented with 2,4-D, NAA, BAP, TDZ (Table 3) followed by haploid plant regeneration. One of the major constraints to the development of routine androgenesis protocols has been the recalcitrance of most soybean tissue to *in vitro* regeneration.

Cytological and histological studies have shown that dimorphism, associated with androgenic ability, occurs during pollen development in soybean. The following three types of pollen are described by Kaltchuk-Santos *et al.* (1993) at the binucleate stage: (a) normal large binucleate grain with two unequal sized cells, i.e., the vegetative and generative cells, (b) atypical small uninucleate grain, and (c) atypical small binucleate grain with two equal-sized cells. Cold treatment had no clear effect on the frequencies of symmetrical-binucleate or multinucleate microspores (Kaltchuk-Santos *et al.*, 1997). Culture temperature, following cold treatment of buds, is an important factor that deserves further study. For example, Zhao *et al.* (1998) reported increases in embryo induction from 2 to 20 percent at a culture temperature of 22°C across an experimental range from 18 to 28°C. Other factors such as increased sucrose concentration promoted haploid calli over somatic calli, and changing from a higher to lower sucrose concentration in the medium promoted embryo development (Ye *et al.*, 1994; Zhao *et al.*, 1998). The presence of activated charcoal combined with low sucrose induced up to 18 percent haploid embryo formation, but germinated embryos must be transferred to a medium without charcoal to achieve 0.5 to 2 percent haploid plantlet formation (Zhao *et al.*, 1998). Genotype was found to be a major determinant of successful haploid plant induction, with seven out of a total of 110 genotypes tested giving rise to plantlets (Zhao *et al.*, 1998) (Table 1).

1.2. Pigeonpea (Cajanus cajan L. Millsp.). Pigeonpea ($2n = 22$) is an integral part of subsistence agriculture in semi-arid regions particularly on the Indian subcontinent and West Africa. Research towards the development of a haploid production protocol has reported the culture of anthers and the division

TABLE 3
Medium details for induction of embryogenesis in the tribe Phaseoleae

	Pigeonpea Kaur and Bhalla, 1998	Soybean Zhao *et al.*, 1998	Cowpea Mix and Wang, 1988
Macronutrients	1/2 MS	MS	MS
Micronutrients	NN	MS	MS
Vitamins and Organics	NN	MS	MS
Carbon	2% Glucose 2% Sucrose	12% Sucrose	6% Sucrose
Hormones	0.10 mg l^{-1} NAA 0.10 mg l^{-1} BAP	2.00 mg l^{-1} NAA 2.00 mg l^{-1} 2,4-D 1.00 mg l^{-1} BAP 0.01 mg l^{-1} TDZ	2.00 mg l^{-1} BAP 1.00 mg l^{-1} NAA
Autoclaved	Yes	No information	Yes
Technique	Microspore culture	Anther culture	Anther culture
Pretreatment	3–7 days 4°C as buds	48h 4°C as buds	None
Osmolarity	240 mOsm	508 mOsm	300 mOsm

in microspores leading to the formation of callus or multicellular structures (Bajaj and Singh, 1980; Mohan Ram *et al.*, 1982; VishnuKumar *et al.*, 2000). Differentiation of these structures was not observed. Kaur and Bhalla (1998) were the first to report successful regeneration of haploid plants using isolated microspore culture. They reported a regeneration frequency of 10 to 13% haploid plants on MS based medium supplemented with NAA and BAP (Table 3). This research is notable for being the first to confirm isolated microspore culture as a possible route to doubled haploidy in the grain legume species.

Interspecific hybridization techniques for doubled haploidy have also been attempted in pigeonpea resulting in a small proportion of sterile progeny from crosses between *Cajanus cajan* and *C. acutifolius*. Pollen fertility studies of the progeny showed some plants with a small proportion of uninucleate microspores (<5%) that had undergone division to the 4–6-cell stage. Division in these microspores was a consequence of the wide cross, as the anthers were not cultured *in vitro* (Nalini Mallikarjuna, pers. comm., 2005). The mode of division was not identified and the multicellular cells occurred in conjunction with sterile pollen grains. This may be a unique way to induce androgenesis from the microspores, which could then be isolated and cultured *in vitro*.

1.3. Cowpea (Vigna unguiculata L. Walp.). Cowpea (2n = 22) is grown throughout the African continent as well as in some parts of South East Asia and Latin America. Abiotic and biotic constraints, as well as the lack of improved cowpea genotypes, have kept farmers' yields low ranging from 350 to 700 kg/ha.

The first report of the production of plantlets from anther-derived callus culture was by Ladeinde and Bliss (1977). There was no record of the ploidy level of the regenerated plants. It was not until more than a decade later that Mix and Wang (1988) reported haploid plants. Donor plants were grown at 30°C day and 22°C night at a humidity of 30 to 40 percent. Immature flower buds 3-mm long yielded white/green anthers with microspores at the uninucleate developmental stage. A temperature of 28°C and dark culture was found to be optimal for anther callus induction on MS medium supplemented with BAP, NAA and 3% sucrose (Table 3). Interestingly, the auxins Picloram and 2,4-D, which are usually associated with callus production from a variety of explant tissue types including anthers, were not suitable for the induction of callus in this species. Calli were transferred to RL-based medium (Collins and Phillips, 1982) for shoot induction. Of 38 regenerated shoots five were cytologically confirmed to be haploid. Many shoots (90%) formed roots after the addition of 3-Aminopyridine to the medium but there was no mention of whether these plantlets were transferred to soil. The authors reported a significant effect of genotype and did not feel that any of the media used in the study was optimal.

Arya and Chandra (1989) reported callus production from young anthers containing microspores at the late uninucleate stage. Greening and leaf-like structures were produced from calli subcultured on liquid MS medium supplemented with 1 mg l^{-1} BAP and 0.5 mg l^{-1} IBA, with rotary shaking and low light levels. Rooting of calli was obtained after subculturing on solid MS medium containing the same growth regulators under continuous light conditions. There was no confirmation of ploidy level of these structures.

1.4. Mung Bean (Vigna radiata L.). Mung bean (2n = 22) are self-pollinating warm season annuals with either an upright or vine growth habit. Mung bean sprouts are high in protein (21–28%) and can replace scarce animal protein in human diets. Bajaj and Singh (1980) observed anthers containing pollen at an early to late uninucleate stage gave the maximum callusing response in

culture. On modified MS medium, 28% of anthers callused and 2% of the pollen underwent androgenesis. Androgenesis took the form of multinucleate cells and early stage embryogenesis. Of the three cultivars tested, G-65 gave the highest frequency of callus and androgenesis. Callus was predominantly haploid but chromosome numbers ranged from 11 to 22. No mature embryos or haploid plants were regenerated.

Gosal and Bajaj (1988) cultured anthers containing microspores at the uninucleate stage from cultivars Mash T9 and Mash 1-1 grown in the field. Anthers were given a 72 h 4°C pretreatment prior to culture on MS medium modified with 4 mg l^{-1} IAA and 2 mg l^{-1} kinetin. Of 2105 pollen, 14 (0.66%) underwent embryogenesis. These embryos occasionally showed further development to the heart-shaped stage but most proliferated to form callus. The cold temperature pretreatment enhanced embryogenesis. The frequency of haploid callus cells was low (10–15%) and this was further reduced in the older cultures.

1.5. Winged Bean (Psophocarpus tetragonolobus L. D. C.). The winged bean (2n = 18) grows abundantly in hot, humid equatorial countries such as Indonesia, India, Sri Lanka, and Papua New Guinea and has seeds which are morphologically similar to soybean and are likewise comparable in composition and nutritional value. There have been three reports regarding haploid research in winged bean. Brunel *et al.* (1981) attempted to regenerate callus from anthers but were unsuccessful. Mohan Ram *et al.* (1982) obtained callus from pollen grains but with no regeneration. Pal (1983) used isolated microspore culture to regenerate callus and cytological analysis confirmed up to 80 percent of these callus cells were haploid. Shoots were regenerated from this callus but there was no confirmation of haploid status. Trinh *et al.* (1986) report the production of mixoploid plants (1n and 2n) from embryoids which developed from callus derived from the culture of anthers and unfertilized ovules; regeneration only occurred after transfer to a culture medium with reduced auxin content. It was not clear if the regeneration was from the anthers or the ovules in this report. Unfortunately the Trinh *et al.* paper did not provide details of medium composition or culture requirements.

1.6. Common Bean (Phaseolus vulgaris L.). Common bean (2n = 22) is a highly polymorphic species native to the Mexican mountains. They are a highly nutritious, relatively low-cost protein food and are the most widely cultivated of all grain legumes worldwide. Peters *et al.* (1977) were the first to produce haploid callus from anthers of this species. Of 40 cells observed with high clarity for chromosome counting, 11 were definitely haploid and 11 were classed as possibly haploid. The remaining cells were diploid (10) or possibly diploid (8). Plants were not returned from this callus.

More recently, Munoz and Baudoin (2001) used modified MS medium to establish haploid callus. The best results were obtained with anthers containing microspores at the uninucleate stage. The anther response was improved by cold pretreatment of the flower buds for one or two days prior to culture. No plants were regenerated from these structures.

2. Tribe Aeschynomeneae

2.1. Peanut/Groundnut (Arachis hypogaea L.). Groundnut or peanut (2n = 40) is a tetraploid native of South America, grown primarily for its high quality edible oil and protein in the seed. The gap between potential and realized yield remains large primarily due to several biotic and abiotic constraints. Attempts to develop doubled haploid plants in *A. hypogaea* began in 1976 with Martin and Rabechault reporting mixoploid chromosome numbers in cells of anther derived callus and albino plants from anther culture (Martin and Rabechault, 1976).

Sudhakar and Moss (1990) and Willcox *et al.* (1991) reported the most effective induction of embryogenesis from microspores isolated and cultured at the uninucleate stage of development. The induction of androgenesis leading to the formation of multicellular pollen grains has been reported from the cultivated species *A. hypogaea* and the wild species *A. villosa*, *A. glabrata*, and *A. lignose* (Bajaj, 1983; Mroginski and Fernandez, 1980; Pittman, 1981; Sastri *et al.*, 1981; Willcox *et al.*, 1991, 1990). More recently, Yeh and Tseng (1999), Tseng and Yeh (2000) and Lee and Yeh (2001) have reported the induction of callus, embryo-like structures and shoot regeneration from anther culture of *A. hypogaea* L. However, the androgenic nature of the regenerants has not been confirmed. Haploid plants have therefore yet to be achieved for peanut but the research to date suggests that anther culture holds much promise for the development of haploid plants in this species.

B. Progress Towards Haploids within the Temperate (Cool-Season) Grain Legume Crop Species

The cool-season grain legumes include field pea (*Pisum sativum* L.), lupin (*Lupinus* spp.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik. subsp. *culinaris*), faba bean (*Vicia faba* L.), and grasspea (*Lathryus sativus* L.). All species except grasspea and faba bean have been subject to attempts to develop haploid production protocols but as yet there has been no successful regeneration of confirmed haploid plants. Research in chickpea, field pea, and lupin is promising, with routine regeneration of microspore-derived early stage embryos in all three species, and a recent report of a single plant regenerated from field pea (Croser and Lulsdorf, 2004).

1. Tribe Vicieae

1.1. Field Pea (Pisum sativum L. subsp. sativum *var.* arvense*).* Field pea is diploid (2n = 14) and self-pollinating with a very low percentage of out-crossing. Major breeding objectives for field pea *viz.* increasing yield, disease resistance and lodging resistance or seed quality (Davies, 1993) are constrained by the absence of biotechnology tools applicable to the crop. Peas were one of the first species to be cultured *in vitro* by William Robbins in 1922 (Robbins, 1922). Despite this, field peas are still considered to be recalcitrant in tissue culture and to biotechnological approaches including doubled haploidy. Genotype effects and poor regeneration rates pose problems to protocol development.

In particular, regeneration competence of cells or tissues into fertile plants remains challenging since all protocols developed to date are either genotype specific and hence not widely applicable, or they are slow and inefficient (Ochatt *et al.*, 2000). In addition, *in vitro* root development can be difficult, often requiring several subculture steps (Tzitzikas *et al.*, 2004) or grafting procedures (Bean *et al.*, 1997).

Gupta *et al.* (1972) published the first attempts to develop an androgenesis protocol for field pea breeding line B22 using anther culture. Microspores were in the uninucleate stage at the time of culture. Anthers were placed on White's (1963) basal medium supplemented with coconut milk and 2,4-D. However, only callus was obtained in this work. Subsequent experiments with the same callus resulted in a few roots, shoots and torpedo-shaped embryos after 36 months if 2,4-D was replaced by NAA (Gupta, 1975). Gosal and Bajaj (1988) also induced callus on anthers from field pea cultivar Bonneville as well as two breeding lines (T163 and P88). Anthers received a 72-hour cold pretreatment at 4°C and were cultured on various MS-based media. Few heart-shaped stage embryos developed but no regeneration was obtained. About 90 percent of the cells were diploid indicating that callus might have developed from maternal anther tissue rather than microspores.

Recently, researchers in Canada and Australia have collaborated with the aim of developing a doubled haploid protocol for field pea using microspore culture (Croser and Lulsdorf, 2004). Cultivars responsive to microspore induction were CDC April (Canadian) and Highlight (Swedish). Depending on the genotype, either heat or cold was found to be an effective pretreatment. A portion of uninucleate stage microspores divided symmetrically on solid medium containing 2,4-D and developed to the multinucleate stage. Microspore nuclear divisions and embryogenesis were tracked using DAPI stain and fluorescent microscopy and microspores were clearly embryogenic. One plant could be regenerated from cultivar Highlight after transfer of the cotyledonary stage embryo to hormone-free medium. The plant was diploid and fully fertile and set seed *in vitro* but rooting could not be accomplished. As the donor plant was a homozygous cultivar, there was no way to determine if this plant was of haploid origin and underwent spontaneous diploidization *in vitro*, or was derived from diploid somatic tissue.

Virk and Gupta (1984) made intergeneric crosses (*P. sativum* × *Lathyrus odoratus*) and intraspecific crosses using delayed pollination, gamma radiation of the pollen and growth regulator application in an attempt to obtain matromorphic plants. Matromorphs are nonhybrid diploid offspring, which originate entirely from the maternal parent. Matromorphs produced by the doubling of the maternal haploid gamete during an early stage of cleavage are completely homozygous. Of the treatments, the following met all the criteria for a purely maternal origin: the progeny of *P. sativum* × *Lathyrus odoratus* following pollination on the same day and ninth day subsequent to emasculation; *P. sativum* × *P. sativum* following pollination on the second day subsequent to emasculation; pollen irradiation with 35 Kr and 2.5 ppm IAA treatment. The results from this study suggested the induction of matromorphs may be a viable proposition for the production of homozygous lines in pea.

Considering the importance of field pea as a grain legume crop, the lack of information on androgenesis is surprising. The progress made to date on embryogenesis or protoplast fusion and regeneration or embryogenesis protocols indicate that the development of a doubled haploid system for field pea should be possible.

1.2. Lentil (Lens culinaris Medik. subsp. culinaris*).* Lentil ($2n = 14$) is diploid and predominantly self-pollinated (Kupicha, 1977; Malaviya and Shukla, 2000; Smartt, 1990). Lentil is primarily used for human consumption but its straw is also a valuable animal feed. A biotechnological approach requires efficient and reproducible *in vitro* protocols and in lentil progress is hampered by genotype specificity, regeneration inefficiency, and low rooting response.

Various genotypes, media, culture, and pretreatment conditions, anther and microspore culture were tested at the National Research Council (NRC), Plant Biotechnology Institute in Saskatoon (Canada) by Keller and Ferrie (2002) with the aim of developing haploid technology for lentil. Some microspores divided into six to eight cells and proembryos could be obtained from anther culture. However, no mature embryos could be regenerated. Recent research at the Crop Development Centre (CDC), University of Saskatchewan focused on developing microspore culture for lentil (Croser and Lulsdorf, 2004). They found that the Canadian lentil cultivars CDC Crimson and CDC Robin were the most responsive to induction after a cold pretreatment of 4°C for one to five days. These embryogenic microspores underwent symmetrical division of the nuclei followed by further cell division to the early embryo stage. However, as with the NRC research, no plant regeneration could be obtained.

Related work at the South Australian Research and Development Institute (SARDI), Adelaide, Australia, attempted to produce haploid lentils through interspecific and intergeneric hybridization using a wide range of forage legume species as pollinators. The most promising results have come from crosses between lentils and field pea. Two putatively haploid plants have been produced using this technique. They appear not to be true hybrids and further investigations into the nature of these plants are currently underway (Philip Davies, pers. comm., 2005).

The lack of information on somatic embryogenesis will hamper the development of an androgenesis protocol for lentil. In addition, doubled haploid protocols require prolonged *in vitro* culture. Polanco and Ruiz (1997, 2001) observed that the inhibitory effect of growth regulators on rooting efficiency increased the longer the lentil shoots were exposed to cytokinins.

2. Tribe Genisteae

2.1. Lupin (Lupinus spp.). Lupin is a major grain legume crop in Australia, mainly targeted for animal feed. The major species of lupin grown for grain production are all old world and include *L. albus* ($2n = 50$), *L. angustifolius* ($2n = 40$), and

L. luteus (2n = 52). One of the major limiting factors to further lupin improvement is the lack of doubled haploid technology adapted for these species. A review of recent (2000–2004) literature indicates that the majority of *in vitro* research in lupin is targeting protoplast culture (Sinha and Caligari, 2004), micropropagation (Pniewski *et al.*, 2002) and the production of transgenic lupins (Li *et al.*, 2000). A drawback to the *in vitro* culture of *L. albus* and potentially other species of lupin is the requirement for grafting of regenerated shoots onto suitable rootstocks. *L. angustifolius* has no such requirement and can be routinely micropropagated, such that it is a good target species for doubled haploid research.

There have been limited efforts to produce doubled haploid lupins via anther and microspore culture. Sator (1985) reported the regeneration of lupin plants following anther culture of *L. polyphyllus, L. hartwegii, L. luteus*, and *L. angustifolius*. While callus induction was high, plant regeneration was reported only from *L. polyphyllus*, and was determined to be diploid. Ormerod and Caligari (1994) reported the production of embryo-like structures from isolated microspore culture in *L. albus*. There was no confirmation of the haploid origin of these structures and attempts by researchers to repeat these experiments were unsuccessful (Kirsty Bayliss, pers. comm., 2005). Bayliss *et al.* (2004) reported the induction methods required for haploid embryogenesis from isolated microspores in *L. albus*, *L. angustifolius*, and *L. luteus* and the subsequent production of multicellular proembryos. Further development beyond this stage appears to be limited by the strong outer exine layer of the proembryo, which must rupture to allow continued embryo growth (Bayliss *et al.*, 2004).

It is likely that if the exine layer is the limiting factor in the development of haploid lupin embryos, this may be overcome following more intensive research. Ogawa *et al.* (1995) reported that the exine layer also inhibited the development of rice embryos; however this problem was eventually overcome by the addition of sucrose to the microspore growth medium. Further research into the chemical nature of the exine may also elucidate more information that could assist embryo development.

3. Tribe Cicereae

3.1. Chickpea (Cicer arietinum L.). Chickpea (2n = 16) is a diploid self-pollinating species. It is a dryland crop requiring low inputs with one of the highest nutritional compositions of any dry edible grain legume. It is also an excellent source of plant-based dietary protein. Breeding efforts are hampered by a low level of genetic variability within the cultivated germplasm and a paucity of biotechnological techniques for genetic improvement adapted to chickpea. This includes *in vitro* doubled haploid production which, despite a number of efforts, has yet to be achieved in chickpea (Altaf and Ahmad, 1986; Bajaj and Gosal, 1987; Croser, 2002; Croser *et al.*, 2004; Croser and Lulsdorf, 2004; Gosal and Bajaj, 1988; Huda *et al.*, 2001; Khan and Ghosh, 1983; Vessal *et al.*, 2002).

Reddy and Reddy (1996) reported the *in vivo* production of haploids from the progeny of a cross between a male sterile mutant and kabuli chickpea line ICC 4973. A total of 51 haploid plants were recovered upon delayed pollination of the male sterile line. The presence of univalents at pachytene confirmed the haploid nature of these plants. Of the 51 haploids, 47 were male sterile and white-flowered like the female parent suggesting they may be maternal haploids; the other four exhibited flower morphology which may have originated from the male gametes.

To date, all attempts to develop *in vitro* doubled haploids in chickpea have targeted the male gametophyte and used intact anthers as explants, with varied success. The reports by Huda *et al.* (2001) and Croser (2002) indicated it was possible to regenerate plantlets from anther-derived callus via somatic embryogenesis, but did not confirm an androgenic origin of the regenerants. In the case of Croser (2002), flow cytometry revealed all regenerants were diploid, indicating either a somatic origin or a high level of spontaneous diploidy during culture.

Croser (2002) undertook preliminary research into the culture of isolated microspores of chickpea and obtained androgenic structures but no plant regeneration. Recent collaborative efforts by Canadian and Australian researchers (Croser *et al.*, 2004) have led to induction of sporophytic division, multinucleate synctiums, early stage embryogenesis, and in some cases caulogenesis from isolated microspores of chickpea. As yet, no haploid plant regeneration has been achieved. In chickpea, as with many other species, microspore culture is most successful when microspores are harvested and cultured at the early to mid uninucleate stage. The optimum mode and intensity of stress pretreatment appears to be determined by the genotype. Cold and heat stress have both been used to induce sporophytic development in chickpea, with heat shock appearing to be the most effective method across the widest range of genotypes.

Interestingly, androgenesis has also been observed as a result of a wide cross between the cultivar *C. arietinum* and the annual wild relative *C. pinnatifidum*. Hybrids between *C. arietinum* × *C. pinnatifidum* were obtained after rescuing the hybrid embryos *in vitro*. Cytological examination of the anthers from these hybrid plants revealed some of the microspores were undergoing division. The number of divisions varied from four to six. This is the first report of induction of androgenesis as a result of wide cross in chickpea (Mallikarjuna *et al.*, 2005).

C. Progress Towards Haploids within the Pasture Legume Species

Pasture legumes include a diverse collection of genera including *Medicago*, *Lotus*, *Trifolium*, *Onobrychis*, *Melilotus*, *Astragalus*, *Coronilla*, and *Stylosanthes*. These species play an important role in temperate agricultural systems, providing protein-rich feed for grazing animals, improving soil fertility through nitrogen fixation and providing a disease break for subsequent cereal crops. Doubled haploid technology is not yet routinely available to produce haploids in the large numbers

required to assist breeding of pasture legume species, although there are reports which indicate that this technology may be developed in the near future.

1. Tribe Trifoleae

1.1. Lucerne/Alfalfa (Medicago sativa L.). The first report of haploid production from tetraploid *M. sativa* L. (2n = 4x = 32) was achieved by crossing tetraploids (2n = 4x = 32) with a diploid pollen parent to yield "haploid" (2n = 2x = 16) progeny (Bingham, 1969, 1971). The best results were achieved using cytoplasmic male sterile tetraploids as donors. This technique has been used for genetic analysis and to breed lucerne with maximum heterozygosity (Dunbier and Bingham, 1975), however it has not succeeded in producing monoploid (2n = 1x = 8) plants.

Anther culture of *M. sativa* diploids (2n = 2x = 16) was first reported by Zagorska *et al.* (1984). Although none of the regenerants were reported as haploid (2n = 1x = 8), some were mixoploid with chromosomes close to the haploid level. Attempts by Tanner *et al.* (1990) to induce *M. sativa* microspore division demonstrated that what they referred to as "nonphysiological" divisions, or divisions not typical of the natural pollen developmental pathway, could be induced in immature *M. sativa* microspores, but sustained divisions were not achieved. Further work by Zagorska and Dimitrov (1995) and Zagorska *et al.* (1997) demonstrated conclusively that haploid plants could be generated from *M. sativa* microspores. Anthers were pretreated for four days at 4°C and treated with 1 Gy gamma radiation prior to culture. The highest percentage of anthers yielding callus (68%) was obtained on Blaydes (1966) medium supplemented with kinetin and highest shoot formation was achieved on Blaydes medium with isopentenyladenosine or myoinositol together with yeast extract. Of 100 regenerants, a total of only four haploid plants were produced indicating the necessity to improve the protocol for haploid production.

1.2. Trifolium *spp.* The only report of haploid plant regeneration from anther culture in *Trifolium* is by Mokhtarzadeh and Constantin (1978) using Egyptian or berseem clover (*T. alexandrinum* L) (2n = 2x = 16). Callus was induced from anthers cultured on MS medium containing NAA, 2,4-D and 2-iP to induce callus growth. Plants were regenerated following transfer of callus to MS medium containing NAA plus kinetin and observed to have the haploid (2n = 1x = 8) chromosome complement.

2. Tribe Loteae

2.1. Lotus corniculatus. Evidence for the generation of haploid *Lotus* plants by androgenesis has been presented by Séguin-Swartz and Grant (1995). *L. corniculatus* anthers produced several cell lines from which plants were regenerated. One of the regenerants was morphologically different to the mother plant (2n = 4x = 24) for several key traits. Examination of root tip cells revealed a mixoploid condition consisting of a small number of haploid (2n = 2x = 12) and octoploid (2n = 8x = 48) cells interspersed with tetraploid cells. Although the origin of this plant is uncertain, it is possible that it may have originated from microspore-derived callus tissue that underwent subsequent polyploidization. There is also evidence for the generation of a haploid *L. corniculatus* plant (2n = 2x = 12) through parthenogenesis after male sterile tetraploid *L. corniculatus* (2n = 2x = 24) was pollinated with *L. tenuis* Wald. et Kit. (Negri and Veronesi, 1989).

There is no certainty about why it has been so difficult to generate haploids from pasture legume species. One possible explanation is that most of these species are outbreeding polyploids that suffer inbreeding depression. Any reduction in ploidy level may result in decreased fitness and the possible unmasking of lethal recessive genes. The fact that there is some evidence for haploid production in *M. sativa* and *L. corniculatus* provides hope for further developments in the future. However, the haploids produced in these species were derived from tetraploid parents and therefore have two sets of chromosomes. It remains unknown whether viable monoploid plants can be produced. A worthwhile objective may be to put further effort into developing *Medicago truncatula* doubled haploids since a large and increasing volume of genetic data is accumulating for this species.

III. MIMOSOIDEAE AND CAESALPINIOIDEAE

A. Progress Towards Haploids within the Arboreal Legume Species

Leguminous trees are becoming increasingly important in agricultural systems and agroforestry due to the nitrogen benefits obtained from interplanting leguminous trees with developing crops in nutrient-poor farmland. There are positive indications of the potential for doubled haploid technology in the arboreal legumes with successful haploid plant production in *Albizzia lebbeck* L. (East Indian Walnut) and *Peltophorum pterocarpum* L. (Yellow Flame Tree), and the induction of pollen embryos in species of the *Cassia* genus.

1. East Indian Walnut (Albizzia lebbeck L.)

Gharyal *et al.* (1983a) reported the production of two haploid plantlets in East Indian Walnut (2n = 26), a member of the Mimosoideae subfamily. Anthers containing microspores at the late uninucleate to the early bicelled stage were cultured on a modified B5 medium (Table 4). Callus masses emerged from about 20 percent of the anthers and shoot buds were initiated from all callus masses. Less frequently, embryoids were developed from the callus. The addition of anti-phenolic compounds to the medium was critical to the production of callus from the anthers. Despite the abundance of shoot buds, well-developed shoots were rare and only two plantlets were regenerated. Root tip squash preparations from the regenerated plantlets showed them to be haploid (n = 13).

*2. Yellow Flame Tree (*Peltophorum pterocarpum *L.)*

Rao and De (1987) reported the regeneration of haploid plantlets from anther culture of yellow flame tree (n = 28).

TABLE 4
Medium details for induction of embryogenesis in the arboreal legumes

	Peltophorum pterocarpum Rao and De, 1987	*Albizzia lebbeck* Gharyal *et al.*, 1983a
Macronutrients	MS modified with $KNO_3 = 2500$ mg l^{-1} and $NH_4NO_3 = 800$ mg l^{-1}	B5 modified with $KNO_3 = 2500$ mg l^{-1} and $(NH_4)_2SO_4 = 134$ mg l^{-1}
Micronutrients	MS	B5
Vitamins and Organics	Thiamine HCl = 10 mg l^{-1} Inositol = 1000 mg l^{-1} Glutamine = 800 mg l^{-1}	B5
Carbon	10% sucrose	Not detailed—assume 2% sucrose as per B5 medium
Hormones	NAA = 1.00 mg l^{-1} Kin = 1.00 mg l^{-1}	2,4-D = 0.50 mg l^{-1} Kin = 2.00 mg l^{-1}
Autoclaved	Yes	No information
Technique	Anther culture	Anther culture
Pretreatment	14°C for eight days	None
Osmolarity	434 mOsm	130 mOsm

Anthers containing microspores at the mid-uninucleate stage gave the best callus induction response when pretreated at 14°C for eight days and cultured on modified MS medium (Table 4). After 72 h, the majority of the microspores had enlarged and formed vacuolated and nonvacuolate cells, however only the nonvacuolate microspores continued to divide. In these microspores, the vegetative nucleus divided repeatedly to form multicellular pollen, which was released from the exine. Some of these multicellular structures developed into globular embryos of 50 to 70 cells, which continued development to an undifferentiated mass of cells rather than to direct embryo maturity and germination. In anther callus 20 to 30 days old, 60 to 70 percent of cells were haploid with the rest being either aneuploid or polyploid. After 50 days of culture the number of haploid cells was reduced to 20 to 30 percent and the majority became diploid (or doubled haploid). Shoots were regenerated from the callus and rooted. 12 percent of the regenerated plants were cytologically confirmed to be haploid.

3. Cassia *spp.*

Gharyal *et al.* (1983b) reported androgenic response in *Cassia siamea*, Lam., a member of the subfamily Caesalpinioideae, which has anthers at various stages of development within each bud. Anthers containing microspores at the late uninucleate to early bicellular stage were selected and cultured on B5-based medium (Table 4). After two weeks, pollen-derived callus was distinguished by its white color and haploid chromosome number, whereas somatic-derived callus was yellow. Symmetrical nuclear division was followed by cell enlargement and further divisions leading to the early rupturing of the exine and proliferation of callus, or more organized growth leading to the development of pollen embryogenesis (6 to 8 celled within the exine, then released) with some indication of bipolarity. No further development was reported. Within the same genus Bajaj and Dhanju (1983) reported pollen embryogenesis from *Cassia fistula*. Microscopic study revealed the occurrence of multinucleate and multicellular pollen and pollen embryos at various stages of development. Cytological analysis showed 1.6 percent of all pollen was multinuclear or multicellular and 0.65 percent formed embryos. No plants were regenerated from these embryos.

IV. FUTURE STRATEGIES BASED ON SUCCESSFUL LEGUME HAPLOID PROTOCOLS

We have undertaken an analysis of the protocols used for successful haploid plant development in the Phaseoleae and the arboreal legumes. In doing so, we have drawn general conclusions about suitable approaches for developing protocols for the species within these groups. We have also identified broad similarities across the groups which will assist researchers working with leguminous species.

There are a number of steps in the development of an *in vitro* androgenesis protocol. Responsive genotypes and appropriate growing conditions for the production of healthy pollen donor plants must be identified. These genotypes can then be used to optimize the conditions for the protocol, prior to widening its application to other genotypes. A trigger needs to be developed for switching microspores from the gametic to the sporophytic developmental pathway. Triggers are usually stress factors such as cold pretreatment of immature flower buds, carbon starvation pretreatment, or heat pretreatment of immature buds or isolated microspores (Touraev *et al.*, 1997). Finally, microspore culture conditions require optimization. Embryo induction, maturation and plantlet regeneration are primarily determined by the medium composition especially the nitrate:ammonium ion ratio, the osmolarity and the choice of plant growth regulator and relative auxin:cytokinin ratio.

Plant cells take up nitrogen more effectively and grow more rapidly on nutrient solutions containing both nitrate and ammonium ions. Generally, plant tissue will not grow on medium with nitrate as the only nitrogen source (George, 1993). However, before nitrate can be used biosynthetically it must be reduced, usually to the ammonium ion. Since high concentrations of ammonium ions are toxic to plant tissue and the pH of the medium needs to be controlled, nitrate is used as the principle nitrogen source. The ratio of nitrate to ammonium is therefore a significant factor in the selection of basal nutrient medium for plant tissue culture.

A. Phaseoleae

True haploid plants have been regenerated in four Phaseoleae species: soybean, winged bean, cowpea and pigeonpea. We have excluded winged bean from this comparison due to insufficient protocol details (Trinh *et al.*, 1986) (Table 3). A cold pretreatment of the buds prior to culture was inductive in pigeonpea microspore culture and soybean anther culture, whereas no pretreatment was necessary for cowpea.

Murashige and Skoog (1962) macro salts with an inorganic nitrate to ammonium ratio of 66:34 was reported to be appropriate for soybean, winged bean, and cowpea. For microspore culture of pigeonpea, the macronutrients were halved in concentration and modified with the micronutrients of Nitsch and Nitsch (1969), which was specifically developed for haploid culture.

We prepared the media as reported for each of the three species and measured the osmolarity (Table 3). As expected, the carbon concentration was the major determinant of osmolarity, reflected in the highest reading being for soybean medium (508 mOsm) followed by cowpea medium (300 mOsm) and pigeonpea medium (240 mOsm). The autoclaving of the medium in cowpea and pigeonpea and potentially soybean (no information available) may also have resulted in cleavage of sucrose at high temperatures to form fructose and glucose. The concentration and combination of the auxin and cytokinin also varied considerably in these media (Table 3). Pigeonpea had low concentrations of both auxin and cytokinin whereas soybean and cowpea had a higher auxin to cytokinin ratio. The auxin NAA and the cytokinin BAP were common across all three protocols. In soybean a low level of the highly active cytokinin TDZ was found to promote embryogenesis. This has also been found to promote somatic embryogenesis in field pea (Sanago *et al.*, 1996).

We can thus find similarities between the protocols for the three species of the Phaseoleae primarily in the following areas: the application of a 4°C cold pretreatment to immature flowers prior to culture; the use of MS basal medium and thus a similar nitrate:ammonium ratio (depending on organics used); the use of sucrose as a carbon source; and the requirement for both auxin and cytokinin in the induction medium. Given the promising results reported for species of this tribe, we are very surprised that further work has not been reported. Lack of funding in the more obscure crops such as cowpea and winged bean may be the reason for this lack of information, but this is not the case for soybean.

B. Arboreal Legumes

Confirmed haploid plants have been produced from anther culture of the arboreal legumes *Peltophorum pterocarpum* and *Albizzia lebbeck*. The arboreal legumes had an inorganic nitrate:ammonium ratio of 96:4 for *Peltophorum* and 76.5:23.5 for *Albizzia* (Table 4). The amount of organic nitrate available to the *Albizzia* cultures was increased by the 800 mg l^{-1} glutamine added to the medium. A supply of reduced nitrogen such as glutamine, in addition to inorganic nitrate, can benefit cell wall formation and growth regulator activity (George, 1993). The high level of 96% nitrate for *Peltophorum* perhaps reflects a tendency for this species to respond poorly to the presence of the ammonium ion in the culture medium. The promotive effect of high nitrate and low ammonium concentration in haploid callus induction cultures has also been recognized in nonlegume species such as Hevea (*Hevea brasiliensis*) and coconut (*Cocos nucifera* L.) (Chen *et al.*, 1982; Thanh-Tuyen and De Guzman, 1983).

The osmolarity for the *Albizzia* medium detailed in Table 4 is possibly incorrect as no information was provided in the paper. We have assumed for the purposes of this review the carbon source was 2% sucrose based on the composition of the B5 medium (Gamborg *et al.*, 1968). This has lead to a low osmolarity of 130 mOsm compared with the *Peltophorum* medium (434 mOsm). In *Peltophorum* a higher sucrose concentration was reported to be essential to prevent proliferation of somatic tissues. Rao and De (1987) suggest that sucrose at a high concentration acts primarily as an osmoticum rather than as a triggering agent for embryo development.

Both woody legumes were responsive to combinations of cytokinin and auxin but at varying concentrations. An approximately equal ratio of auxin:cytokinin was optimum for *Peltophorum*, whereas for *Albizzia* a higher concentration of cytokinin:auxin was more effective for induction of embryogenesis. The cytokinin kinetin was common to both culture media. It appears that a relatively high cytokinin level promotes the induction of haploid embryogenesis in these species. There are conflicting views regarding the role of growth regulators for androgenesis. In plants such as tobacco, a simple salt medium with sucrose and no plant growth regulators is sufficient to trigger microspore division (Reinert *et al.*, 1975). However in other species, such as *Peltophorum*, there is no response without the addition of growth regulators.

C. General Protocol Similarities for Haploid Production in Fabaceae

Across the species anther culture appears to be the technique of choice for androgenesis. This is also the case in

nonleguminous species where initial studies have focused on developing anther culture and then progressed to isolated microspore culture when protocols were more established. In the Fabaceae, a cold pretreatment appears to be useful for haploid embryo induction. This has generally been a two- to eight-day 4°C treatment applied to the immature flower buds.

A B5 or MS basal medium meets the nutritional requirements of the developing microspores of Fabaceae; however this may require optimization of the nitrate:ammonium ratio. Sucrose seems to be an appropriate carbon source, however its action as an osmo-regulator means that it is necessary to optimize the concentration for each species. The addition of vitamins (myoinositol, thiamine, nicotinic acid, and pyridoxine) is potentially promotive to embryo induction, as is the amino acid glutamine. The growth regulator concentration and combination of auxin and cytokinin varies considerably, however all authors found some form of growth regulator to be required for haploid embryo induction. We recommend undertaking quantitative experiments with the major growth regulators used in the above studies *viz*. NAA, 2,4-D and kinetin or BAP to optimize conditions for the target legume species under study.

V. CONCLUSION

The recurring theme of this review is the recalcitrant nature of leguminous species to doubled haploidy and to *in vitro* manipulation of morphogenesis. Difficulty with rooting and the genotype-specific nature of the *in vitro* response in some species will continue to provide challenges for doubled haploid researchers. However, there has also been significant progress with reported haploid plant regeneration from pigeonpea, soybean, cowpea, winged bean, and the arboreal legumes *A. lebbek* and *Peltophorum pterocarpum*. Positive steps towards androgenesis have also been achieved in peanut, lupin, chickpea, field pea, lucerne, *Lotus*, and *Cassia*.

The massive research effort that was required to develop reliable haploid production protocols in species such as barley, canola, and wheat has not yet been directed towards any legume species, with the possible exception of soybean. It is to be expected that the increasing importance of leguminous species in sustainable agricultural systems will be reflected in higher investment levels in the future and, we predict, in concomitant breakthroughs in all technologies associated with these species including doubled haploidy. Given the scarce resource allocated to leguminous species, coordinated international and national collaborations will be necessary in order to make rapid progress.

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