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Landmark research for pulses improvement

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Abstract

This chapter reports that pulse crops (rich in protein) and cereals (rich source of carbohydrate) are an excellent combination for balanced human diet. Crop rotation of legumes with cereals enriches the soil because legumes fix nitrogen in soil in a symbiotic association with *Rhyzobium* species. Pulse breeders have been confined to the primary gene pool (GP-1). Exploitation of secondary (GP-2) and tertiary (GP-3) gene pools for crop improvement is hampered because of pre and post-zygotic barriers. National and international research institutes have conducted extensive plant exploration to collect primitive cultivars, land races, and wild relatives before the spread of high yielding varieties and environmental destruction eliminates them. These invaluable materials are being deposited in medium and long-term storage of gene banks. Breeders have achieved substantial gain in the yield of pulse crops by conventional breeding by producing varieties that are resistant to abiotic and biotic stresses. Breeders have produced varieties with high protein content but without antinutritional elements. Roundup Ready 2 Yield® soybean, produced through genetic transformation, has revolutionized the soybean production particularly in the United States. An example to exploit tertiary gene pool of soybean is shown. Such approach should be conducted for other legumes to broaden their genetic base.

Key words: Grain legume, gene pools, conventional breeding, nutrition, wild species

Introduction

Pulses and cereals co-evolved in a symbiotic way. They are complementary components of agricultural systems worldwide**;** common bean and maize in South America, lentil, chickpea, wheat, and barley in the Middle East, rice and soybean in China. In Africa, cowpea grows in rotation with pearl millet and sorghum. Pulse crops are member of the family Fabaceae. Cereals belong to family Poaceae. The major primary dietary pulse crops are common bean (*Phaseolus vulgaris* L.), cowpea [*Vigna unguiculata* (L.) Walp.], pigeonpea [*Cajanus cajan* (L.) Millsp.], pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), faba bean (*Vicia faba* L.), lentil (*Lens culinaris* Medik.), mung bean [*Vigna radiata* (L.) Wilczek], azuki bean [*Vigna angularis* (L.) Ohwi & Ohashi] and soybean [*Glycine max* (L.) Merr.]. The hallmark trait of legume species is high protein content (Table 1). Lupin is used primarily for animal feed in Australia and as a forage crop in Europe, not included in the chapter.

The characteristic feature of legumes is the presence of root nodules containing the bacterium *Rhizobium* and related genera that help nitrogen fixation in the soil maintaining a symbiotic relationship while such bacterial association is absent in the cereals. Pulses are rich (20-50%) in protein while cereals are an excellent source of carbohydrates. The combination of cereals and pulses enriches the human diet specially supplementing the protein requirement. Furthermore, pulses are an important source of protein in countries where the majority of population is vegetarian.

Although pulses are an extremely valuable source of protein for both human and animals, research effort for producing high yielding cultivars lags far behind of cereals. The pulses are second only to the cereals in their dietary importance to human and animals (Graham and Vance 2003). Poor yield of pulse crops may be due to growing of inherently unproductive cultivars, which are not tolerant to abiotic and biotic stresses. These crops are often cultivated as subsistence crop in small holdings and often for home

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Table 1. Common name, scientific name, 2n chromosome number, origin and gene pools and protein (%) of major pulses

Common name	Scientific name	Chr. No. 2n	Origin	Gene pool (GP)			Protein (%)	Reference
				GP-1	GP-2	GP-3		
Common bean	*Phaseolus vulgaris*	22	Mexico, Middle America and Andean South America	Domesticated cultigens, wild populations	*P. coccineus* *P. polyanthus* *P. costaricensis*	*P. acutifolius* *P. parvifolius*	22	Singh, S. P. 2005
Pea	*Pisum sativum*	14	Fertile Crescent, the Mediterranean and Centra Asia	Domestic cultigens wild relative subsp. *P. pumilio*, *P. elatius* *P. abyssinicum*	*P. fulvum*	None	26	Redden et al. 2005
Pigeonpea	*Cajanus cajan*	22	India	Cultivated land races	*C. acutifolius*[@]	*C. cinereus*[#]	20-22	Saxena 2005
Cowpea	*Vigna unguiculata*	22	Africa	Four culti-groups, land races, and subsp. *tenuis*, *denkindtiana*, *stenophylla*	Subsp. *pubescence*	*V. vexillata* *V. radiata*	20-26	Singh, B. B. 2005
Faba bean	*Vicia faba*	12	Near East	Domestic cultigens	None	None	25-33	Cubero and Nadal 2005
Chickpea	*Cicer arietinum*	16	Southern Caucasus, northern Persia, and south-eastern Turkey	*C. arietinum* *C. echinospermum* *C. reticulatum*	*C. bijugum* *C. judaicum* *C. pinnatifidum*	*C. chorassanicum* *C. cuneatum* *C. yamashitae*	23	Ahmad et al. 2005
						All perennial *Cicer* spp.	34	
Lentil	*Lens culinaris*	14	Near East arc and Asia Minor	subsp. *culinaris* subsp. *odemensis* subsp. *orientalis*	*L. ervoides* *L. nigricans*	*L. lamottei* *L. tomentosus*	26	Muehlbauer and McPhee 2005
Mungbean	*Vigna radiata*	22	India	*V. radiata* var. *radiata* *V. radiata* var. *sublobata*	*V. mungo* *V. subramaniana*	Section *Aconitifoliae* Section *Angulares* *V. stipulacea* *V. grandiflora*	22.9	Tomooka et al. 2005
Azuki bean	*Vigna angularis*	22	Asia	*V. angularis* var. *angularis*[$] .	*V. umbellata*	Section *Ceratotropis* *V. trinervia*	21.1	Vaughan et al. 2005
Soybean	*Glycine max*	40	China, Korea	*G. soja*	None	*G. albicans**	40	Chung and Singh 2008

[@] *C. albicans, C. cajanifolius, C. confertifolius, C. lanceolatus, C. lineatus, C. reticulatus, C. scarsbseoides, C. sericeus, C. trinervius;* [#] *C. confertiflorus, C. crassus, C. goensis, C. latisepalus, C. mollis, C. platycarpus, C. rugosus, Dunbaria* spp., *Rhynchosia* spp; [$] *V. angularis* var. *nipponensis, V. hirtella, V. minima, V. nakashimae, V. nepalensis, V. riukiuensis, V. tenuicaulis*; ***G. aphyonota, G. arenaria, G. argyrea, G. canescens, G. clandestina, G. curvata, G. cyrtoloba, G. pullenii, G. falcata, G. hirticaulis, G. gracei, G. lactovirens, G. latifolia, G. latrobeana, G. microphylla, G. montis-douglas, G. peratosa, G. pescadrensis, G. pindanica, G. rubiginosa, G. stenophita, G. syndetika, G. dolichocarpa, G. tabacina, G. tomentella* consumption so called “kitchen gardening”. Research on pulse crops worldwide has been largely neglected in developing countries when compared to cereals. In this chapter, major landmark research to enhance yield in pulse crops is summarized briefly.

Establishement of international and national programs

FAO STAT (http://faostat.fao.org/site/535/default.aspx#ancor) lists soybean, dry bean, broad beans, horse bean, chickpea, cowpea, lentil, pea, pigeonpea, vetches, lupins, and Bambara beans. Mungbean and faba bean have not been included (Fig. 1). Among all the major pulses, soybean is used for oil, and feed while remaining crops don’t contain oil and protein content ranges from 20 to 26% (Singh, R. J. 2005). By contrast, soybean contains an average of 40% protein.

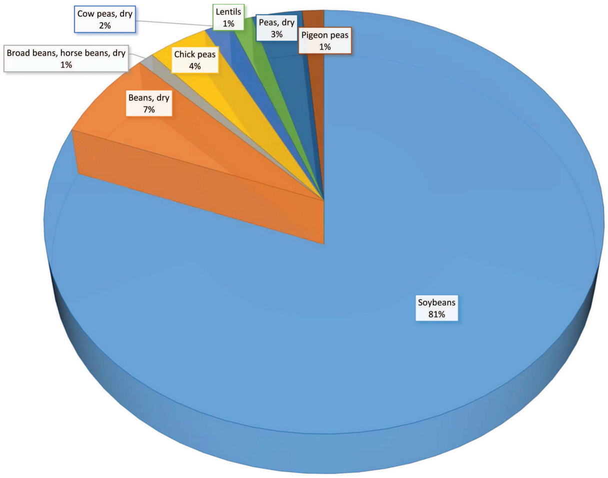

Fig. 1. Yield of major legumes based on data published by FAO (2015) (http://faostat.fao.org/site/535/default.aspx#ancor)

The following international and national centers have been established for major grain legume research:

Centro Internacional de Agricultura Tropical (CIAT) Cali, Columbia: Common bean is a mandate crop and conserves 36,000 *Phaseolus* accessions to date, corresponding to 44 taxa from 110 countries (http://isa.ciat.cgiar.org/urg/beancollection.do).

International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India: This organization (http://www.icrisat.org/) maintains 20,602 chickpea accessions and 13,778 accessions of pigeonpea (Hari D. Upadhyaya, personal communication, August 16, 2016).

International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria: Cowpea is a mandate crop. IITA keeps 15,122 accessions of cowpea from 88 countries representing 70% of the African cultivars and nearly half of the global diversity (http://www.iita. org/cowpea).

International Center for Agricultural Research in the Dry Areas (ICARDA), Beirut, Lebanon (http://www.icarda.cgiar.org/): This organization (Athanasios Tsivelikas; personal communi-cation, October 21, 2016) maintains the following accessions of pulses:

Pulses	Taxon	Number of accessions
Chickpea	*Cicer arietinum*	15,195
Wild *Cicer*	*Cicer* spp.	547
Lentil	*Lens culinaris*	13,907
Wild lentil	*Lens* spp.	603
Pea	*Pisum* spp.	6,115

Asian Vegetable Research and Development Center (AVRDC), Taiwan (http://avrdc.org/): This organization maintains 881 accessions of *Vigna angularis*, 9,247 accessions of *Vigna* species, and 278 accessions of *Vigna unguiculata*. AVRDC also conserves *Cajanus* (43), *Glycine* (3,213), *Phaseolus* (231) and *Pisum* (96).

National Programs: National (public) and private industries worldwide have pulse crops improvement programs. National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India (http://www.nbpgr.ernet.in/Research_Projects/Base_Collection_in_NGB.aspx) conserves the following legumes:

Pulses	Number of accessions
Chickpea	14,635
Pigeonpea	11,337
Mungbean	4,159
Pea	4,169
Cowpea	3,778
Frenchbean	3,815
Clusterbean	4,311
Horsegram	3,038
Ricebean	2,127
Soybean	4,005

A high number of legume accessions (13,842) have been listed as others; it seems that these accessions have not been characterized

Indian Council of Agricultural Research (ICAR; http://www.icar.org.in/en/node/325) has established Indian Institute of Pulses Research (http://www.iipr.res.in/) and mandate crops are: chickpea, pigeonpea, urd bean, mungbean, lentil, field pea, common bean and lathyrus. This institute maintains more than 10,000 accessions in medium term cold room (4°C with relative humidity 40%). For soybean, ICAR has established Indian Institute of Soybean Research (http://www.nrcsoya.nic.in/).

China holds the largest collection of soybean germplasm, representing 14% of the world's accessions (http://map.seedmap.org/solutions/conservation/seed-banks/chinas-institute-of-crop-germplasm-resources/).

USDA, ARS in Urbana, Illinois, U.S.A. maintains 19,956 accessions of *G. max*, 1,181 accessions of *G. soja*, and accessions of 19 wild perennial *Glycine* species (https://npgsweb.ars-grin.gov/gringlobal/site.aspx?id=24).

Redden et al. (2005) listed *Pisum* spp. collections in 16 countries (Table 2). However, number of accessions increased during one decade (Table 3). For example, pea collection in 2005 was 4,384 accessions in the United States but now number of accessions is 6,147 (Table 3).

Gene pools for pulse crops

The gene pool concept developed by Harlan and de Wet (1971) has played a pivotal role in utilization of germplasm resources for producing high yielding cultivars without antinutritional chemicals either by conventional method or by transformation methodology. They proposed three gene pool concepts based on the results of hybridization among species. These are primary (GP-1), secondary (GP-2) and tertiary (GP-3) (Fig. 2).

Primary gene pool of pulse crops

The primary gene pool of grain legumes consists of biological species, and crossing within this gene pool is easy. Hybrids are vigorous, exhibit normal meiotic chromosome pairing and possess total fertility. The gene segregation in F_1 is normal and gene exchange is generally easy. Primary gene pool (GP-1) A includes cultivated races, land races. B includes species/subspecies, wild and weedy relatives (Fig. 2). Table 1 lists primary gene pool of important pulses. Faba bean does not have GP-1 relatives.

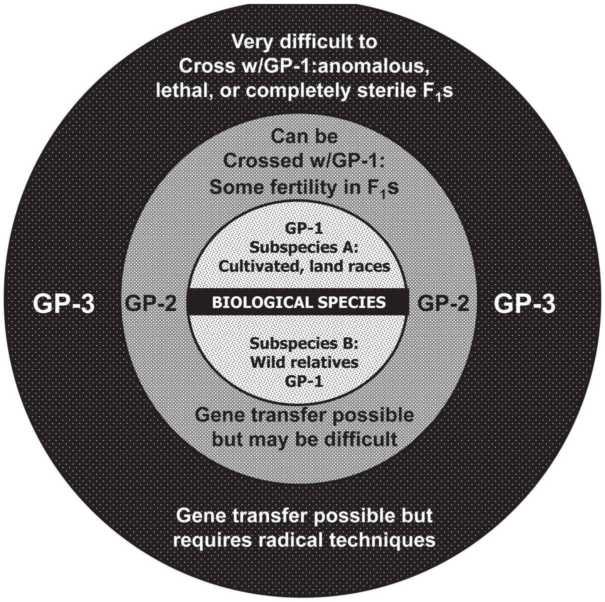

Fig. 2. Gene pool concept in plants established based on hybridization (Redrawn from Harlan, J.R. and de Wet, J.M.J., *Taxon*, 20, 509-517, 1971)

Secondary gene pool of pulse crops

The secondary gene pool (GP-2) includes all species that can be crossed with GP-1 with at least some fertility in F_1 (Fig. 2). The gene transfer is possible but may be difficult. In this regard GP-2 for common bean, pigeonpea, chickpea, lentil, mungbean, and azuki bean is available and can be used in varietal improvement. Soybean and faba bean do not have a GP-2 (Table 1).

Tertiary gene pool of pulse crops

Tertiary gene pool (GP-3) is the extreme outer limit of potential genetic resource (Fig. 2). Table 1 lists GP-3 for common bean, pea, pigeonpea, cowpea, faba bean, chickpea, lentil, mungbean, azuki bean and soybean which are rich reservoir for useful genes of economic importance. Prezygotic and postzygotic barriers may cause partial or complete failure of hybridization and introgression between GP-1 and GP-3. The exploitation of wild relatives of pulses is often hampered because of poor crossability, early embryo abortion, hybrid inviability, hybrid seedling lethality, and hybrid sterility due to low chromosome pairing. Technology to exploit GP-3 for broadening the genetic base of pulse crops yet to be developed.

Germplasm resources for pulses

All international and national institutes for pulses (common bean, pea, pigeonpea, cowpea, faba bean,

Table 2. Genetic resources collections of pea (Redden et al. 2005)

Country	Gene bank	Accessions
ICARDA Syria	ICARDA germplasm unit	5,935
USA	USDA, Washington	4,384
Italy	Institut del Germplasma, Bari	4,558
Russia	N.I.Vavilov All Russian Science Research Institute of Plant Industry, St Petersburg	6,790
Sweden	Nordic Gene Bank	3,921
United Kingdom	John Innes Institute	3,030
Germany	ZentralInstitutGenetikKulterplanzen, Braunschweig/Institute of Plant Genetic Resources and Crop Plant, (PGRDEU), Research, Gaterslaben	3,184
Poland	Plant breeding Institute, Wiatrowo	2,899
China	Institute Crop Germplasm Research (ICGR), Beijing	2,292
Czech Republic	The Research Institute of Crop Production, department of gene bank, Olomouc.	2,364
Australia	Australian Temperate Field Crops Collection (ATFCC), Horsham	2,283
Hungary	Institut for Agrobotany,Tapioszde	2,282
France	Laboratoire des legumineuses, URGAP-INRA, Dijon	1,850
Bulgaria	Institut for Introduction and Plant Genetic Resources”k.Malkovo”, Plodiv	1,490
Slovakia	Genebank of Slovak Republic, Piestany	1,105
Netherlands	Centre Genetic Resources, Wageningen	1,008

Table 3. Genetic resources of pea

Country	Gene bank	Accessions
ICARDA	https://www.icarda.org/research-sub/biodiversity-and-its-utilization	6,113
USA	https://npgsweb.ars-grin.gov/gringlobal/site.aspx?id=26	6,147
Italy	http://ibbr.cnr.it/mgd/?action=page&id=aims	4,400
Sweden	http://www.nordgen.org/index.php/en/content/view/full/344	3,763
Russia	http://www.vir.nw.ru/	7,400
United Kingdom	https://www.seedstor.ac.uk/search-browseaccessions.php	3,007
Germany	http://pgrdeu.genres.de/suche/list/page/12/sucheBuchst_P/P/genus/Pisum/species/sativum	3,471
Poland	http://www.igr.poznan.pl/uploads/resources/Linki%20WS/Pisum.pdf	2,896
Czech Republic	http://genbank.vurv.cz/genetic/resources/documents/20_let_NPGZ_final_EN.pdf	1,375
Netherlands	http://www.wur.nl/	1,561

chickpea, lentil, lupin, mungbean, azuki bean, and soybean) collect, maintain, disseminate and develop breeding lines with resistance to abiotic and biotic stresses. Plant exploration of wild relatives of common bean, faba bean, lentil, chickpea and cowpea is extensive. National Institute of Agrobiological Sciences in Japan has a very active research program for the Asian *Vigna* that includes mungbean and azuki bean.

Wild relatives of major pulses are being characterized based on classical taxonomy, cytogenetics, and molecular methods. The combination of the genus *Atylosia* with the genus *Cajanus* is a classic example (van der Maesen 1986). Cytogenetics of pulse crops has not progressed as rapidly as it has been for the cereals although foundation of genetics was laid from Mendel’s pea experiments in (1865, 1866). Cytogenetic maps of most of the pulse crops are lacking and so are the cytogenetic stocks although molecular maps are being developed and genome sequencing is at a rapid pace. Pulses are not considered model crops for cytogenetic investigations.

The impact of somaclonal variation is limited for producing better pulses. However, GM (genetically modified) soybean, resistant to glyphosate herbicide has been released for commercial production in the United States and is known as roundup ready soybean.

Some high yielding cultivars are eroding the natural habitat of the allied species (progenitor) and genera and these invaluable germplasm resources should be collected before they become extinct. International and national institutes are preserving indigenous varieties, land races, wild progenitors, and wild relatives in medium and long-term storages. Their viability is checked routinely.

Germplasm enhancement for pulses

The genetic base of pulses is rather narrow because breeders have been confined to GP-1 (primitive cultivated forms, land races, and wild progenitors). Although GP-2 has been used to improve common bean, it is beyond reach for improving lentil. GP-3 has not been exploited to introgress traits of economic importance in cultivated pulses.

A large number of exotic accessions are stored in seed banks worldwide (Tanksley and McCouch 1997). However, only a fraction of valuable genes has been tapped for improving pulses. Conventional breeding (selection from land races and primitive cultivars, pedigree, bulk, backcross, or single-seed descent methods of selection), mutation breeding, exploitation of somaclonal variability, and genetic transformation have helped breeders to select superior cultivars of pulses. Commercial hybrid production using cytoplasmic male sterility (CMS) is a success story for pigeonpea, where hybrids produced a 4 to 52% increase in yield over the parents. This is feasible because the natural out-crossing in pigeonpea ranges from 20 to 40% (Saxena 2005). Faba bean is also a partially (34%) allogamous crop and cross-pollination ranges from 4% (practically a selfer) to 84% (practically an out crosser) (Cubero and Nadal 2005). The major obstacle in producing hybrid legumes is the structure of the flower, which ensures a 99% chance of self-pollination in most pulses. Lentil contains small cleistogamous flowers, making it virtually 100% self-pollinating (Muehlbauer and McPhee 2005). Outcrossing in mungbean is only 0.5 to 3% (Tomooka et al. 2005).

Breeding for plant type

Breeders have developed determinate semi-dwarf and dwarf plant types with uniform maturity for common bean, pea, cowpea, faba bean, pigeonpea, lupin, mungbean, and azuki bean by conventional breeding. Semi dwarf varieties with determinate plant type are resistant to lodging and therefore adapted to mechanical harvesting. Early maturing (less than 98 days), high-yielding common bean varieties with upright growth habit can be machine harvested, which is cost effective for common bean growers (Singh, S. P. 2005). A major breakthrough in pea came about when breeders combined reduced crop height (e.g., *lg* gene) and conversion of leaflets to tendrils (e.g., *af* gene). An artificially induced mutant in the faba bean for determinate growth resulted in a cultivar (Fig. 3) that provided an easy mechanical harvesting (Cubero and Nadal, 2005). Dwarfing genes in pigeonpea have been identified and are being used in developing dwarf pigeonpea. Most pigeonpea varieties at reproductive stage achieve the height of 2-3 meters. The dwarf-inbred lines range in height between 70 to 80cm and produce reasonable yields (Saxena, 2005).

Fig. 3. Clustered pods of determinate faba bean induced by x-ray (Cubero and Nadal 2005)

Breeding for high yield

Substantial gain in the yield of all pulses has been achieved through innovative conventional breeding methods but it is still far behind those of cereals. Conventional breeding methods have produced high yielding cultivars as they contain genes for resistance to biotic (fungal diseases, viruses, and pests) and abiotic (tolerance to cold, heat, drought, adverse soil nutrition, and resistant to lodging) stresses. High yielding pigeonpea varieties have been produced by mutation breeding (Saxena 2005). A somaclonal variant in pigeonpea produces white seeds, with 25% increase in seed size and 30% advantage in yield (Saxena 2005). Transformation technique has been developed in several national and international laboratories to incorporate genes for resistance to pests and pathogens in grain legumes. Roundup Ready 2 Yield® soybeans provide farmers the same proven benefits of Roundup Ready® soybeans – combined with the only soybean trait that has the primary benefit of additional yield opportunity. And higher yields can mean higher profits for farmers (https://www.genuity.com/research/Pages/Roundup-Ready-2-Yield.aspx).

Breeding for canning quality

Major advances in breeding of some pulse crops are for producing varieties for canning. This has revolutionized the breeding programs and the canning industries. Major market classes of common bean include bayo, great northern, 'ojo de cabra' (creamed-striped), pinto, pink, and red Mexican beans. Breeding of dark red kidney bean cultivar Montcalm with excellent canning quality has been developed by conventional breeding technique (Singh, S. P. 2005). Green seeds are canned for local consumption and export in Puerto Rico and Dominican Republic.

Immature faba bean seeds are canned (usually precooked) and frozen.The canned and lightly precooked "baby" types (less than 12 mm in length) have a very high price in Spain (José Cubero, personal communication, May 26, 2004).

Breeding for high protein content

All pulses are a rich source of protein. Legume species contain protein as high as 48%. They are used for feeding animals as well for human consumption as *dhal* or soup. Protein content in pigeonpea ranges 20 to 22%. Its wild relatives have protein content of up to 32% and lines with high protein content have been developed from these materials (Saxena 2005). It has been suggested that 3 to 4 genes control protein content trait and action of these genes is additive and complementary. Soybean is rich source of protein (40%). Breeding for high protein reduces the amount of oil and *vice versa*.

Breeding for vegetable type

Vegetable type of pulses particularly common bean, pea, cowpea, pigeonpea, faba bean, and chickpea command high market prices. Snap bean cultivars with green-pods are known as French, garden, green, or stringless beans. Snap bean with flat or cylindrical pods, yellow (waxy type), green, or purple colors, and long or short pods are all used for fresh, frozen and canning purposes (Singh, S. P. 2005). Cowpeas with edible pods are widely grown in various Asian and Pacific countries and IITA has developed vegetable type cowpea varieties (Singh, B. B. 2005). ICRISAT has released several vegetable type pigeonpea varieties. One such variety (ICPL 87079) is highly popular in India, Africa, and China. Green pigeonpea is an important vegetable in the Caribbean, Africa and a few areas of India (Saxena 2005). Immature type peas are used to produce canned or quick frozen product (Redden et al. 2005).

Breeding for antinutritional elements

Pulses contain numerous antinutritional elements. Their presence contribute to a 7-10% reduction in protein digestibility (Norton et al., 1985). These reduce biological value of protein and are harmful to humans and animals if these legumes are consumed raw. For example, faba bean contains tannins, vicine, convicine, and two glycosides related to favism (Cubero and Nadal 2005). Favism causes strong stomach hemorrhaging. Faba bean is rich in L-3, 4-dihydroxiphenylalanine (L-DOPA), used in the treatment of Parkinson's disease, has a potential use in pharmaceuticals. Mechanical processing and breeding methods have reduced these antinutritional elements from faba bean (Cubero and Nadal 2005). Cowpea, soybean and pigeonpea contain trypsin, chymotrypsin inhibitors and tannins. These elements can be eliminated by heat treatment and by varietal improvement. However, cowpea is a rich source of high amount of calcium, iron and zinc, desirable from nutritional stand/point. These elements may increase the seed hardness and cooking time. Soaking of the seeds before cooking reduces the cooking time (Singh, B. B. 2005). A large fraction of (50% of total) seed protein in common bean constitutes storage protein phaseolin and by the components of lectin-related protein family. Phytohemagglutinin and lectin- related proteins in been seeds are toxic to monogastric animals. Common bean without phytohemagglutinin has been produced by conventional backcrossing method (Bollini et al. 1999). Consumption of common bean in diet reduces cholesterol and cancer risk (Singh, S. P. 2005). Soybean is rich source of molybdenum, vitamin K1, folate, copper, manganese, phosphorus, thiamin, isoflavones, phytic acid, saponins. It also prevents breast cancer, menopause, and osteoporosis. While relatives of cultigens often do not contain antinutritional factors and these traits could introgress into cultigens once wide hybridization technique is developed. Transformation may play a key role in producing grain legumes without antinutritional elements. Several laboratories worldwide are engaged in producing transgenic legumes.

Wide hybridization

Distant relatives of legumes have not been exploited than those in the cereals. The classical example to reach tertiary gene pool of soybean has been recently achieved (Singh and Nelson 2014, 2015). The genetic resources of the ca. 26 species (Table 1) of the genus *Glycine* subgenus *Glycine* have not been exploited to broaden the genetic base of soybean (*Glycine max*; $2n = 40$). This study has developed methods for producing F_1, amphidiploid, BC_1, BC_2, BC_3, and fertile soybean plants from crosses of soybean and the genus *Glycine* subgenus *Glycine* species (Singh 2010). In order to utilize the subgenus *Glycine* germplasm in soybean breeding, soybean cultivars were hybridized with six accessions of 78-chromosome *G. tomentella* as well as one accession each of 40-chromosome *G. tomentella*, *G. argyrea* and *G. latifolia*. They were chosen because they exhibit resistance to soybean rust. Successful in producing fertile soybean from soybean cv. 'Dwight' and 78-chromosome *G. tomentella* accession PI 441001 is shown in Fig. 4, while other hybrids were discontinued either at F_1 or amphidiploid stage. The F_1 seeds aborted prior to reaching maturity, so developing seeds from 19 to 21 days old pods were cultured aseptically in various media formulations. Seed maturation and multiple embryo generation media were developed (Singh 2010). F_1 plants with shoots and roots ($2n = 59$) were transplanted to pots in greenhouse. Amphidiploid ($2n = 118$) plants were backcrossed to 'Dwight'. BC_1 ($2n = 79$) plants were propagated through in vitro and 43 mature BC_2F_1 seeds were harvested. Fifteen surviving BC_2F_1 plants were morphologically distinct, sterile, and had chromosome numbers ranging from $2n = 56$-59. Chromosome numbers of the BC_3F_1 plants ranged $2n = 40$-49. Derived fertile soybeans were first planted in the field in 2008 and are being evaluated for yield; shown in the schematic photo flow chart. These lines are distributed to soybean scientists through material transfer agreement (MTA).

By using the above methodology, Singh and Nelson (2014) produced alloplasmic soybeans, currently not available to soybean breeders by *G. tomentella* PI 441001 (2n=78) x *G. max* cv. Dwight (2n=40). F_1 plants were rescued by immature seeds rescue procedure (Fig. 5 a, b, c, d). True nature of hybridity was confirmed by mitotic metaphase chromosome count (Fig.5 e). Meiotic metaphase of F_1 sporocytes showed rod and ring bivalents and univalent were already reached to the poles (Fig. 5f). Pachynema chromosomes occasionally paired end to end with clearly defined kinetochore (Fig. 5g, h; arrow). Amphidiploid (2n=118) was vigorous, and flowered profusely (Fig. 6a) and confirmed by mitotic metaphase chromosome count (Fig. 6b). An oddity that was observed with this amphidiploid plant was that simple contact with the leaves caused a skin irritation that was not observed with the amphidiploid when Dwight was the maternal parent.

Amphidiploid (2n=118) x "Dwight" (2n=40) produced mature black (Fig. 7a) and black seeds (Fig. 7b) immature pods and seeds; this was not observed in the reciprocal cross. Compared to the BC_1 from Dwight x PI 441001, BC_1 plants with PI 441001 cytoplasm were non-viney, and the stems were woody with few branches. Leaves were dark-green, thick, rugose and curved downward at the tips, and had short petioles (Fig. 7c). Plants produced only a few pinkish flowers that were chasmogamous, with deformed corollas and carpels. Pinkish flowers were not expected because both parents have purple flowers. Occasionally, bicarpel flowers were observed. Plants produced few flowers. However, BC_1 x "Dwight" produced mature 1-2-seeded pods (Fig.7d) and tan seeds with black hilum but were much smaller than Dwight seeds (Fig. 7e).

Chromosome numbers in BC_2F_1 plants ranged from 2n=41-50. From BC_2 F_2 to BC_3F_1, the number of plants in parentheses with 2n=40 (275), 2n=41 (208), 2n=42 (80), 2n=43 (27), 2n=44 (12), and 2n=45 (3) were identified. Figure 7f shows a plants with 2n=44 chromosomes expressing dark-green and curved leaves and produced mostly one-seeded pods (insert top right). This chromosome segregation was observed in BC_3F_1 in "Dwight" cytoplasm. This suggests preferential chromosome elimination in PI 441001 cytoplasm. Morphologically distinct MAALs were isolated in BC_3F_1 populations (Fig. 8a-d). MAAL of plant 13ST500-8 contained 3 satellite chromosomes

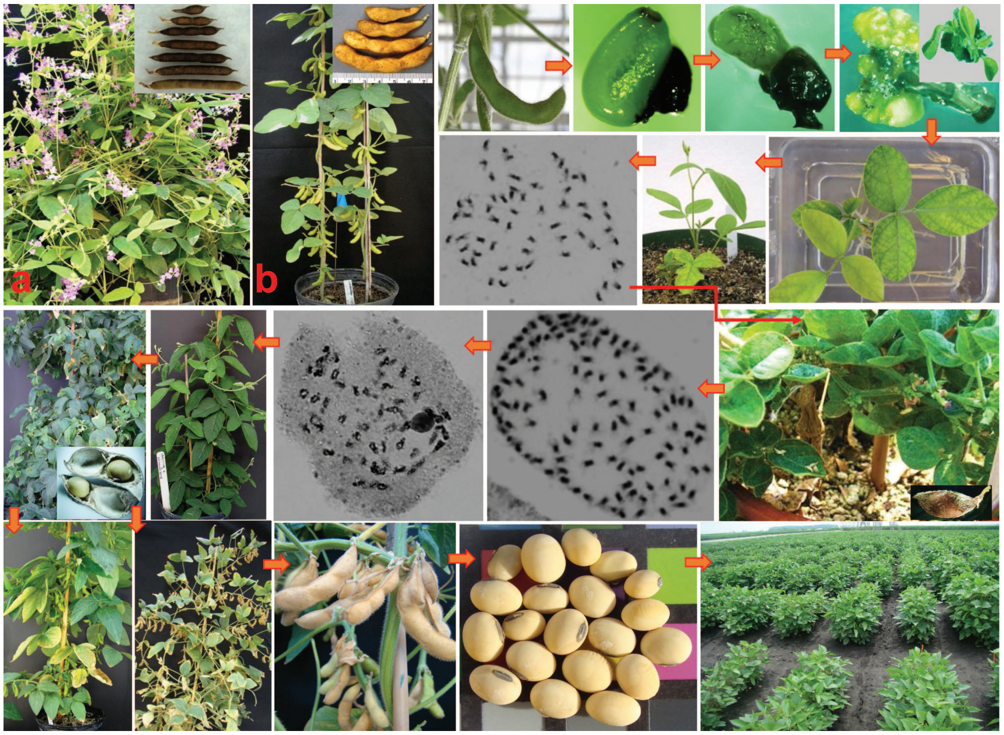

Fig. 4. **Wide hybridization in soybean. *Glycine tomentella* (PI 441001), growing in greenhouse, plant is a perennial and wild with 2n=78 from Brampton Island Queensland, Australia, mature pods insert right top (a): *Glycine max* cv. Dwight with 2n=40, mature pods insert top right, (b): Diagrammatic flow showing from crossing (19 days post pollinated Dwight pod), maturation of seeds, multiple shoot generation, a seedling with roots and shoot, F_1 plant in a pot in the greenhouse, expected 2n=59, amphidiploid plant with mature pod (insert bottom right), a mitotic cell with 2n=118, a diakinesis cell, BC_1 plant (2n=79), a BC_2 plant with 2n=58 (a mature pod with two greenish grey seeds, bottom right), BC_3F_1 plants with 2n=41, a BC_3F_2 plant with mature pods (2n=40), mature seeds, and plants growing in the field (from crossing to field required 5 years)**

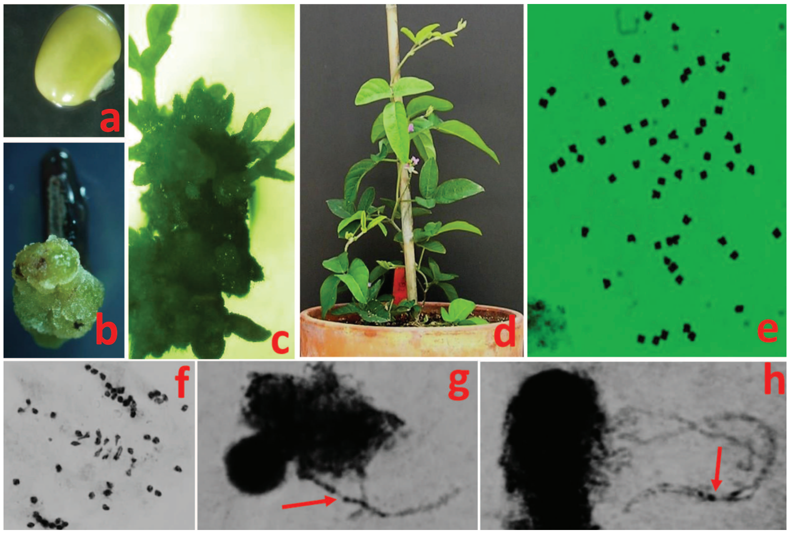

Fig. 5. **Production of fertile plants from *Glycine tomentella* PI 441001 (2n=78) x *G. max* cv. Dwight (2n = 40). a: immature 21 day-old seed in culture; b: embryogenic callus extruded leaving behind black seed coat; c, multiple shoot generation from an embryogenic callus; d: the F_1 hybrid plant growing in greenhouse; e: mitotic metaphase cell with 2n=59 chromosomes from the F_1 plant; f: metaphase I cell showing 5 rod shaped bivalents and few univalents remained at the equatorial plate; g: pachynema cell showing a bivalent synapsed end-to-end with differentiated euchromatin and heterochromatin (arrow shows kinetochore); h: pachynema cell showing a bivalent synapsed end- to-end with only the kinetochore flanked by heterochromatin (arrow)**

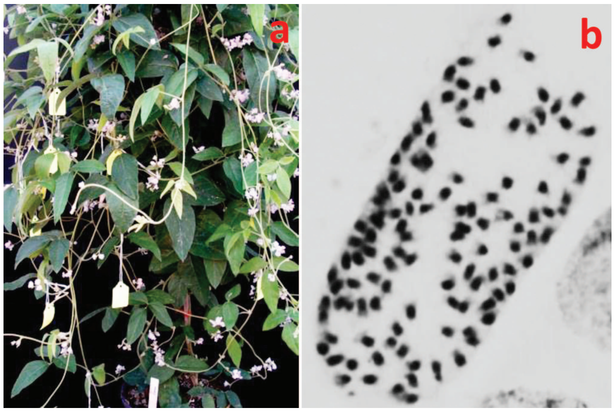

Fig. 6. Amphidiploid plant growing in greenhouse; plant has twinning habit like PI 441001; b: mitotic metaphase cell from Fig. 3a plant, showing 2n=118 chromosomes

and during meiotic metaphase sporocytes showed 20II + 1I. Derived fertile lines moved to field in 2012. This is the first report of the successful development of new alloplasmic soybean lines with cytoplasm from *G. tomentella*.

Exploitation of GP-3 in pulses is very challenging field of research and has a potential to significantly change the future of pulse breeding. Wide hybridization in pulse crops calls for perseverance, commitment, dedication, patience, and skill.

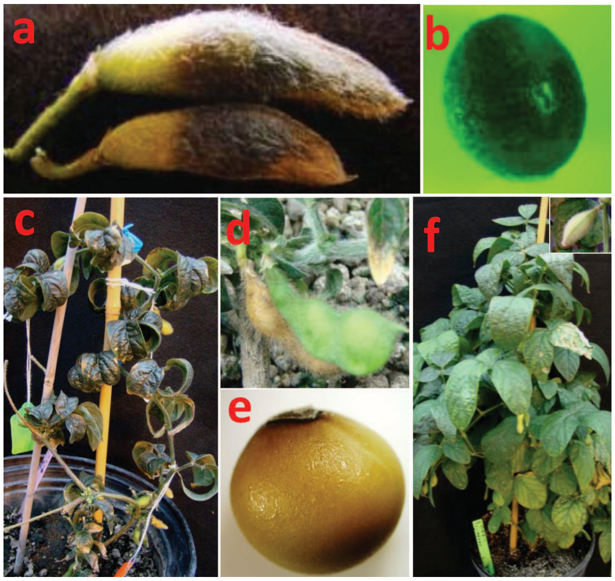

Fig. 7. a: two mature BC_1 pods each containing one seed; b: mature BC_1 seed showing black seed coat like PI 441001; c: BC_1 plant with 2n=79 chromosomes growing in the greenhouse. Plant is not a climber, and has dark-green thick curved leaves; d: two pods at a node of BC1 plant, one pod (tan) is close to maturity and second pod is still green; e: mature BC_2F_1 seed showing tan seed coat and black hilum; f: BC_2F_1 plant 10H270-1 (2n=44), showing dwarf growth habit, dark-green curved leaves, flowers on node (few) and pods hidden under the leaves, with mostly single-seeded pods (insert top right)

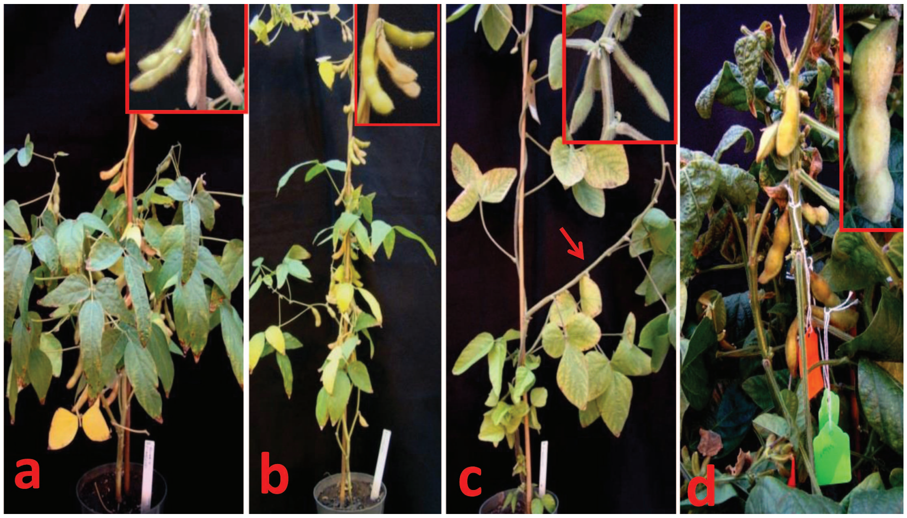

Fig. 8. Isolation and identification of four morphologically distinct MAALs (2n=41) derived from 9 seeds of a BC_3F_1 (13ST500; 11H1-4, 2n=47 x Dwight). a: Plant 13ST500-1 (2n=40), with mature pods despite green leaves; b: Plant 13ST500-2 (2n=41), with normal fertility but irregular pods at maturity; c: Plant 13ST500-3 (2n=41), showing one main branch (arrow), woody morphology, and setting a few flat single-seeded pods; d: Plant 13ST500-8 (2n=41), which was slow in vegetative growthwith dark-green, thick curved leaves, and produced only few large pods from self-pollination. This plant contained 3 satellite chromosomes; top right insert is pods size and shape

Declaration

The authors declare no conflict of interest.

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