UNIVERSITY OF SOUTHAMPTON

DEVELOPMENT OF TECHNIQUES FOR WIDE HYBRIDISATION OF THE GENUS *LUPINUS* L.

A Thesis submitted for the Degree of Doctor of Philosophy

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ABSTRACT

FACULTY OF ENGINEERING AND APPLIED SCIENCES

Doctor of Philosophy

DEVELOPMENT OF TECHNIQUES FOR WIDE HYBRIDISATION IN THE GENUS *LUPINUS* L.

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In the genus *Lupinus*, *L. mutabilis* has been recognised as potential breeding material to improve the quality and quantity of widely grown *L. albus*, through interspecific hybridisation. These species remain under-utilised due to the presence of certain undesirable characteristics such as alkaloids, indeterminate growth habit and susceptibility to diseases and pathogens. However, introgression of useful traits between the two species is difficult due to incompatibility barriers at the post-fertilisation level. The objective of this work was therefore, to develop appropriate technique(s) for hybridisation between *L. albus* and *L. mutabilis* species.

Interspecific compatibility between *L. albus* and *L. mutabilis* was investigated using a wide range of parental genotypes with equal number of somatic chromosomes (2n = 48). Comparative analysis of species on the basis of their morphological characteristics was carried out. The pollen viability of both species was also investigated. Following self and interspecific pollination it was observed that germination of pollen grains on the stigma and pollen tube growth in the styles were not inhibited. However, isolating mechanisms due to post-fertilisation barriers resulted in zygotic failure and thus hybrid embryo abortion at an early stage of development, which subsequently caused pod abortion within two weeks of pollination. Pod/seed development studies were carried out in the species and their reciprocal crosses and the time of embryo abortion was determined. In interspecific crosses it was observed that the period between 4^{th} and 5^{th} day after pollination was crucial for both pod and seed development. The normal development of hybrid pods and seeds for the first three days after pollination indicated fertilisation and early development of the hybrid embryos. The possible reason of embryo degeneration may be due to disharmonious interaction between embryo, suspensor, endosperm and maternal tissues. Growth regulators were applied to enhance pod development and to prevent early embryo abortion. The growth regulators were found to be not as effective in encouraging hybrid embryo development as in pod growth. Regardless of the genotype and growth regulator application, interspecific crosses resulted in pod abortion.

In this study the embryos and immature seeds of the selfed crosses of *L. albus* and *L. mutabilis* were cultured successfully and the plantlets were established in the soil. The reciprocal hybrid embryos however, produced callus and shoot initiation was observed only in limited cases. Following the failure of interspecific crosses and poor performance of hybrid embryos/immature seeds *in vitro* culture; a second technique of *in vitro* fertilisation was employed in order to achieve the genetic recombination of the two species. Both male and female gametes of both species *L. albus* and *L. mutabilis* were isolated successfully. *In vitro* fusion of isolated gametes was achieved by manipulating the calcium concentration and pH level in the fusion medium. The success rate of isolation and fusion was very low. This study indicates that the techniques need to be improved for free transfer of genes between the two species.

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ABBREVIATIONS

BA	Benzylaminopurine
DAP	Days after pollination
DNA	De-oxyribonucleic acid
2, 4-D	
F1	First Fellilial generation
FAO	Food and Agriculture Organisation of the United Nations
FCR	Fleuorochromatic reaction
FDA	Fleuorescein diacetate
GA_3	Gibberellic acid
IAA	Indole acetic acid
IBA	Indole butyric acid
L.	*Lupinus*
LA	*Lupinus albus*
LM	*Lupinus mutabilis*
MS	Murashige and Skoog
M	Molar
N	Normal
NAA	α-naphthaleneacetic acid
PGR	Plant growth regulators
TEMED	Tetramethylethylenediamine

Chapter One

General Introduction and Literature Review

1.1 The role of grain legumes

Grain legumes are known for their value, as a protein source in the diets of millions of people. These are particularly important in areas where animal protein sources are scarce or where poverty, religious or ethnic preferences inhibit the consumption of meat (Norton *et al.*, 1985).

The leguminous plants are found throughout the world with more than 600 genera and 18,000 species (Zohary & Hopf, 1993). Currently the most important of these belong to the tribe *Phaseoleae*. They comprise of soybean (*Glycine max*), pigeon pea (*Cajanus cajan*), common bean (*Phaseolous vulgaris*), lima bean (*Phaseolous lunatus*), mungbean (*Vigna mungo*), cowpea (*Vigna ungiculata*) and the winged bean (*Psophocarpus tetragonolobus*). Groundnut (*Arachis hypogea*) and chickpea (*Cicer arietinum*) belong to the *Aeschynomineae* and *Cicereae* tribes respectively. Pea (*Pisum sativum*) and faba bean (*Vicia faba*) of the *Vicieae* tribe are also important (Summerfield & Roberts, 1985).

Although, lupins, members of the family *Fabaceae* are known to have been cultivated 3,000 - 4,000 years ago, they were only brought into modern cultivation two centuries ago (Dunn, 1984; Gladstones, 1998). The high protein (30 to 45 %) and oil (13-23 %) contents in the seeds of these species have a remedial importance in human and animal protein deficiency situations (Petterson & Mackintosh, 1994). Lupins occupy a special niche among the grain legumes in farming systems because of their ability to tolerate acidic soils with low nutrients and organic matter. In addition, they help in the management of fertility of poor degraded soils by fixing atmospheric nitrogen. Therefore, it is desirable that the place of grain legumes in the current farming systems of the developing countries should be secured in order to maintain the nutritional balance of human beings and also of the soil.

1.2 Origin and distribution of lupins

Lupins are among the world's first recorded crops and their origin is referred to in the earliest Mediterranean and Andean civilisations (Lopez-Bellido & Fuentes, 1986; Gladstones, 1998). There are over 300 species in the genus *Lupinus* (Dunn, 1984) which are spread all over the world. There are four main domesticated species (*L. albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis*) of the genus *Lupinus*. *L. albus* and *L. mutabilis* were first cultivated in the Old World and in the New World respectively (Dunn, 1984). Both Greeks and Romans have written about the ability of lupins to grow in the poor soil and also their utilisation for soil improvement. The recent evidence from DNA analysis of lupins (Kass & Wink, 1994) has revealed that the genus *Lupinus* belongs to the tribe *Genisteae* of the *Leguminoseae*. In 1998 Gladstones carried out a comprehensive review of literature on origin, history, taxonomy, importance, and breeding of lupins.

The genus *Lupinus* (family *Leguminosae,* subfamily *Papilionoideae*) is comprised of herbaceous annuals and herbaceous to shrubby perennials. The greatest number of *Lupinus* species are found in the coastal and mountain regions of Western North America, Mexico, in the Andean Highlands of Peru and neighbouring regions of Brazil, Uruguay and Argentina (Gladstones, 1998).

All wild species of the genus contain toxic compounds, which constitute a vital part of the chemical defence system (Wink, 1984). However, this is an undesirable characteristic when the plant is domesticated for human and animal consumption. This is why the de-bittering process of seeds has been used since ancient times. Lopez-Bellido and Fuentes (1986) reviewed that Galeno, the Greek-born Roman physician (200-130 B.C) described methods which were used by the Romans to de-bitter lupins. The method involved cooking followed by soaking for some time in water. It confirms that the Romans were well aware of the toxic properties of lupins used for human and animal consumption. The Romans who used it as green manure for vineyards introduced the crop into France and Spain. The Spaniard, Alonso de Herrera in his publication "Agricultural General" (1513) reported cultivation of lupin in Spain in detail. The author referred to a series of cropping practices such as early sowing, soil preparation, and preference of the plant for loose

sandy soil and the advantage of shallow sowing. He also described the medicinal and insecticidal uses of the lupin plant.

In the New World, the references to *L. mutabilis* are also very old (Lopez-Belloido & Fuentes, 1986). In Peru "Tarwi" seeds over 2000 years old have been found in Nazca, which were used as offerings for the dead in the tombs. "Las Realciones Georgraphicas de las Indias" cited consumption of lupins in the form of grains in pre-Colombian times. The Andean people have always recognised the basic nutritional and medicinal properties of "Tarwi" (the Andean Lupin). They associated its consumption with religious rites and festivals. They also referred to its fertility properties and the curative role of its alkaloids in cardiac diseases, rheumatism, malaria and infections of human and livestock by parasites. With Spanish domination, the indigenous population adopted Spanish food habits, as the invaders disregarded the Inca dietetics simply because they could not understand them. This resulted in endemic malnutrition, which has lasted practically up to the present day.

In Europe in 1783, on the orders of Frederick II of Prussia, lupin was sown in many regions of Germany. However, the late maturing lupins imported from Italy did not mature, and were used only as green manure for the poor sandy soil of east Germany as well as for sheep forage (Hanlet, 1960). Borchard in 1881 (cited by Chaudhary, 1993) carried out trials on *L. luteus* to select short duration cultivars and obtained excellent results in grain production. Within a short time the success of *L. luteus* resulted in the replacement of *L. albus,* and it quickly spread all over Europe (Lopez-Belloido & Fuentes, 1986). Some cultivation of *L. angustifolius* was also seen in the coastal region of north Germany at about the same time. By the end of 19th century interest in lupin as grain crop had diminished due to the toxic alkaloids, which made the utilisation of its grains difficult. However, its cultivation as green manure crop continued. With the First World War, interest revived and research work on selection began in Holland, Sweden, Poland, South Africa, the USA, Australia, New Zealand and Germany. In Germany a non-shattering line of *L. luteus* was found for the first time. Later, one mutant with low alkaloid content was also developed and propagated under the name of "Weiko". This was first commercially distributed in 1943 under the name "Weiko II" (Belteky *et al.,* 1983). The three species of Mediterranean-African origin *(L. albus, L. luteus* and *L.*

angustifolius) and the one from South America (*L. mutabilis*) are widely used as a crop, while *L. cosentinnii* has the necessary characteristics for consideration to be a crop plant.

The wild species of lupins are distributed in two large areas, the Mediterranean and Western Hemisphere. The Mediterranean zone ranges from southern Europe to the highlands of northern and eastern Africa. The range in the Americas includes the western parts of North and South America and excludes the tropical lowlands and the Amazon basin (Gladstones, 1998). According to Gladstones (1984) and Plitmann & Heyen (1984) only 12 species are acknowledged as native to the Old World, among which the cultivated species are *L. albus*, *L. luteus* and *L. angustifolius*. The rest are native to the New World, although the numbers are not precise. It is estimated that there are between 300 to 400 species (Planchuello, 1984).

The South American species are distributed in two separate geographical regions, the Atlantic and Andean (Planchuello, 1984). In the Atlantic region (Eastern Brazil, Uruguay, Paraguay, central and eastern Argentina) the perennial species with simple or compound leaves are grown in abundance in open areas, mountainsides and river banks. The Andean region covers the mountains and mountainsides of the Andes of Venezuela, Colombia, Ecuador, Peru, Bolivia, Chile and northwestern Argentina to the plains of Patagonia. These species include perennial and annual shrubs with the latter forming a heterogeneous group. *L. mutabilis* is the only cultivated species amongst wild Andean lupins.

1.3 Taxonomy

Tournefort in 1694 used the name *Lupinus* for the genus (Planchuelo, 1982). Later in 1753 Linnaeus included the name of the genus in his book *Species Plantarum*, and described six species (*L. perennis*, *L. albus*, *L. varius*, *L. hirsutus*, *L. angustifolius* and *L. luteus*).

The number of known species increased rapidly during the eighteenth and nineteenth centuries by new findings in North, Centre and South America (Planchuelo, 1984). Gladstones (1974) revised parts of the genus especially for the Mediterranean species.

Dunn (1984) and Planchaelo (1985) revised American species. Plitman and Hayn (1984) revised the whole genus on the basis of biological isolation from one another. The distribution, chromosome numbers and common names of lupins are given in table 1.1.

1.3.1 The Old World lupins

Nowacki and Prus-Gloacki (1971) separated the Old World species in five groups or sections (Albus, Angustifolius, Luteus, Micranthus and Rough seeded) based on serological grounds, morphological characters, and crossability. Plitmann and Heyn (1984) classified the species according to the seed coat texture into two distinct sub-generic groups: Section *Lupinus* and Section *Scabrispermae*, which correspond to the rough seeded-group. Plitmann and Heyn (1984) reported that the principal taxonomic grouping of lupins should be based on biological basis with low genetic polymorphism in general. However, several characters, particularly vegetative ones, display continuous variability within species and populations. This is due to polygenic characters, phenotypic plastivity, or disruptive differentiation, which may be used in breeding.

Gladstones (1984) revised the taxonomy of Old World lupins starting from the five native sections in the Mediterranean/African region. The albus section comprises only one species; the described species *L. graecus*, *L. jugoslavicus* and *L. vavilovi* are wild ancestors of *L. albus*. Crossing experiments by Kazimierski (1960, 1961, 1963, 1964) showed a complete lack of genetic barriers between these and *L. albus* and also showed that only a few genes govern the main differences that arose from domestication. Such differences do not indicate speciation or even sub-speciation in the true genetic or botanical sense; therefore the wild forms have been integrated as var. *graecus*. Another section Angustifolius also contains only one species although a number of synonyms in both old and recent literature have given rise to taxonomic confusion. However, Gladstones (1984) affirmed after exhaustive research and observation that neither the lack of genetic barriers nor any of the morphological and physiological differences forms a sufficient basis for taxonomic distinctions.

The *luteus* section contains two species, *L. luteus* and *L Hispanics*, and both have equal number of chromosomes and their main centre of distribution is the Iberian Peninsula.

Kazimierski (1982), contrary to Gladstones (1984), maintained that *L. rothmaleri* is a species distinct from *L. hispanicus*. The species *L. luteus* is confined to high-rainfall areas of Portugal, Northwest Spain and in coastal areas of Israel. The lupin population found in Israel differs morphologically, bio-chemically, and cytologically from those of the western Mediterranean, producing partial sterility when crossed (Kazmierski, 1982). Thus the Israeli form is described as a separate subspecies, spp. *orientallis* instead of spp. *Luteus*. The species *L. hispanicus* is very common in western Spain and northern Portugal but its intra-specific taxonomy is more controversial. Gladstones (1984) maintained that the two distinct forms of this species, which occur in the Iberian Peninsula, are two separate subspecies: ssp. *hispanicus* and ssp. *bicolor*. Although difficult *L. luteus* does cross with ssp. *bicolor* and there is enough fertility to allow artificial introgression of genes from one species to another. The same occurs with ssp. *hispanicus*. Swiecicki (1985) has obtained a fertile hybrid of *L. hispanicus* ssp. *hispanicus* X *L. luteus* named *Lupinus eurohybridus* Sweic.

The Micranthus section is little known and comprises only one species, *L. micranthus*, whose chromosome number, after much uncertainty has been established as 2n = 52 (Pazy *et al.*, 1977). This chromosome number, together with growth habit and seed form, suggests a possible affinity with *L. luteus*; however, the serological evidence shows that it should continue to be regarded as separate species.

Despite the diversity in chromosome number, the grouping of the rough-seeded section is based on the serological and morphological characteristics. Among these species a new species *L. atlanticus* is found in Southern Morocco (Gladstones, 1974). It appears to be both cytologically and morphologically an intermediate species between *L. cosentinii* and *L. pilosus*, and therefore could be used as a genetic bridge between them. The other species of the group worth mentioning because of their interesting agricultural potential are *L. varius* and *L. digitatus*. Although morphological differences exist due to distinct habitats (Iberian Peninsula and Morocco) the species is essentially homogeneous, which implies that both populations have been genetically isolated for some time (Gladstones, 1974). In summary, limited information on rough-seeded lupins along with their morphological similarities indicates a close relationship between them. Gladstones (1984) concluded that characteristics and distribution of these species indicate that they

are relics of a possibly much larger species group, centred in North Africa, which evolved when the climate was much wetter than now.

1.3.2 The New World lupins

C. P. Smith carried out the first complete review of the New World lupins in his series Species lupinorum published in 1938 - 1945 (as cited by Planchuelo, 1984). Hundreds of new lupin species were described on the basis of their superficial morphological characteristics, and many of taxons are now considered as synonyms. Recently Dunn and collaborators have partially resolved the chaotic nomenclature state of many American species, using more precise criteria of bio-systematic chemotaxonomic and cytogenetic studies, (Dunn, 1980; Dunn; 1984; Planchuelo, 1984).

The number of chromosomes of the American lupins, distributed from the Andes to the Arctic, is 24, 36, 48 and 96, constituting an allopolyploid series of multiple levels, triploid, tetraploid and octoploid, with a basic number X = 6 (Dunn, 1984). This has given rise, from an ecological standpoint, to a wide genetic flexibility that has enabled these species to inhabit not only the high Andes and the mountains of western America but also coastal and desert regions. According to Nowacki & Prus-Glowacki (1971) the American lupin species have a closer cytogenetic relationship and are more similar to one another than to the species from the Old World.

Lupinus mutabilis or Andean lupin (2n = 48) is practically the only cultivated species in the New World, domesticated in the pre-Columbian age. Sweet in 1825 made the first description, but at present, confusion exists with respect to its exact taxonomic definition (Gross, 1982a). One of the causes of this confusion is that until now no wild form of the species has been found, although Blanco (1984) cited finds of wild material in Peru (in the area bordering Ecuador and Bolivia), where the largest variabilities are found and where the situates are the centre of domestication.

According to Gross (1982a) there are two subspecies of *L. mutabilis* in Peru, separated geographically along the 10.5° latitude south. In the north of Peru the species presents a greater vegetative development and late maturity, the leaves are narrower, and the seed is

almost round. In the south the plants are smaller and mature early, the leaves are wider, and the seeds are flatter and more oval with a greater variety of colours. Curiously, the genetic difference of the two subspecies corresponds to a linguistic difference, namely "chocho" in the north and "tarhui" in the south. There are different hypotheses over the nomenclature of *L. mutabilis* due to large genetic variety of the species, suggesting a need for a more precise taxonomic definition of this plant (Gross 1982a).

1.4 Utilisation of lupins

Lupin seeds have been used for human and animal consumption for thousands of years. The earliest references to utilisation of lupins as food are found in Hippocrates (430 – 377 BC) as cited by Lopez-Bellido and Fuentes (1986). The use of lupins as human food and animal feed is reviewed in the following sections.

1.4.1 Human nutrition

Lupin grains of various species have been used as food for more than 3000 years around the Mediterranean (Gladstones, 1970) and more than 6000 years in South America (Uauy *et al.,* 1995). Lupins are known to have been used probably, first in Greece and subsequently in Egypt and Italy from where its use spread to France, Spain, Portugal, and Russia (Petterson, 1998). However, the utilisation of lupin seeds in modern food production is still limited due to their high alkaloid contents. Previously, lupin grains used as food had to be processed to remove the bitter seed component through a process of debittering. Plant breeders have however succeeded in producing lupin varieties with low alkaloids. Lupin seed flour, like other legume flours, can be used as a supplement to improve the dietary protein quality of local foods. Processed alkaloid-free lupin isolate contains up to 62 % protein and is suitable for incorporation into many prepared foods (Feldheim, 1990). Metabolic studies on human alimentation have been carried out in the last few years, mainly in South America. This has revealed that the nutritional characteristics of lupins are similar to those of other legumes used in human food (Lopez de Romana *et al*, 1983). The nutritional protein value of lupins is significantly improved when supplemented by synthetic methionine at a level of 2.0 % of total protein (Lopez de

Romana *et al*, 1983). Investigations have shown that lupins, which are low cost source of protein, are best used in combination with other foods.

The use of de-bittered lupin seeds in South America and Europe for thousands of years indicates that lupins are safe as food, provided the alkaloids are mostly removed. Gross (1988) reported results of an allergy test performed on Peruvian school children for three years. He did not find any indication that the children were more liable to suffer from allergy to lupin products than to other foodstuffs.

Lupin flour has been used in bread making, subsidising 10 – 20 % wheat flour (Haq, 1993). The bread obtained had a good volume and texture, pleasant colour, high protein content, improved amino acid composition and pleasant aspect from the consumer point of view. Lupin flour is also used in biscuit and cake making, pastas, lactic substitutes, pre-cooked foods, snacks and burgers. It has also been incorporated into the preparation of baby foods (Haq, 1993).

Studies carried out by Camacho *et al.,* (1988) have shown that spray drying at 17 °C was the best thermal treatment to obtain a nutritive and well-accepted powder lupin milk. Its tin-can packing has extended the shelf life to a minimum period of six months at normal conditions. Additionally it was reported that the installation of a powder milk plant in Chile is technically and economically feasible and could contribute to an increase in the income of farmers of that region (Camacho *et al.,* 1988). The oil of *L. mutabilis* has good nutritive qualities with a composition similar to that of other vegetable oils. Lopez de Romana (1982) demonstrated that its oil is more digestible than mixtures of Soya and cotton oil. Gross (1988) has visualised that the introduction of lupin products for human consumption is not only a matter of research in food chemistry and technology but also a challenge to food and agricultural policy.

1.4.2 Animal nutrition

Lupins have been used for animal feed for centuries. The high crude protein content of their seed, high productivity and great adaptability to various agro-climatic conditions make lupins a valuable resource for efficient livestock production (Edwards *et al.,* 1998).

The seed is fed as a grain, whole or ground, to all types and ages of livestock. Their low levels of starch and high levels of fermentable carbohydrate make them a highly desirable ruminant feed due to the low incidence of acidosis when they are fed and the fact that they are an excellent microbial fermentation substrate.

The entire vegetative part of the sweet lupin can be used as green fodder for ruminants, horses and pigs. The plants are cut at flowering or when the first pods are formed. The green feed is good forage for cows and can be included in the daily diet at a rate of 20 – 25 kg/head (Ballester *et al.,* 1984a).

European workers have evaluated lupin as a substitute for soybean (*Glycine max*) meal in lamb rations. Perez Cuesta *et al.,* (1980) reported that Merino lambs fed on *L. albus* cv. Neuland gained more weight (14.2 kg compared with 13.7 kg) at a higher feed conversion efficiency ratio (4.00 compared with 4.22) than soya-fed animals. In France, Lacassagne, (1984) found that there were no significant differences between soybean and lupin based rations for lamb feeding. Vallable and Seroux (1984) also noted little difference in live weight gain of lambs fed either whole or ground lupin seeds as a supplement to maize or barley. Cazes *et al.,* (1982) compared Kalina (*L. albus*) with soya meal, field beans (*Vicia faba*) and peas (*Pisum sativum*) as a supplement to barley-based ration. They found no significant differences in any measured parameter among the four protein sources. Lambs fed on lupins gained 264 g/day and had a final carcass weight gain of 6.7 kg compared with soy-fed controls which gained 227 g/day for a final carcass weight gain of 6.8 kg. Arnold *et al.,* (1976) compared lupins, peas and vetches (*Vicia sativa*) feeds and found that there was little difference in live weight gain of Merino weaners on lupins and peas. Vetches gave considerably lower live weight gains. Although live weight gain at 140 g/day was the same on lupins and peas more lambs (73 %) reached slaughter weight on lupins than on peas (60 %).

Besides its effect on body weight, lupin seed can have major effects on wool production. Carbon *et al.,* (1972) reported that animals, which grazed standing lupins for 100 days, produced significantly more wool (weanners, 12.6 g/day; wethers, 17.5 g/day) than animals on lupin stubble (weanners, 8.7 g/day; wethers, 14.8 g/day). Hill (1988) noted an

increased tendency of wool and meat production when animals were fed on *L. angustifolius*, *L. albus*, *L. cosentinii* and *L. luteus*.

Emile *et al.*, (1988) studied feeding of dairy cows and young bull on sweet lupin seeds. It was found that sweet lupin seed, either ground or full, in comparison with maize silage and flattened wheat diet for fattening bulls, were well accepted and well used by the animals. Growth and carcass production was as good as those of animals eating classical concentrates. Numerous digestibility experiments have been carried on young calves, milking cows, lambs, rabbits, broiler and egg-laying hens and even fish to observe its advantages when used to replace corn and soya (Emile *et al.*, 1988 and Hill, 1988).

1.5 The status of lupins among other legumes

The status of lupins among the grain legumes has been reviewed by various authors (Siamasonta, 1996; Gladstones, 1998). In comparison with most other grain legumes, the lupins have not up to the present entered the World agricultural systems, and their minority status still reflects problems in their agronomy and ecological adaptation. Atkins *et al.*, (1998) have reviwed the factors, which negate the high potential of lupins as a grain legume. Lupins have declined in popularity in most countries, mainly due to disase. However, in the late quarter of the last century outstanding performance of lupnis was observed in Australia and Chile. Australian exports of lupin in 1985 amounted to 14 percent of the World trade in pulses. On a global scale, grain legumes are the third largest group of food crops and in which lupins occupy the bottom of the publishing league. Lupins contribute only 0.5 % of the grain harvest compared to soybean, groundnut, and other pulses which contribute 58 %, 10 % and 31.5 % respectively from under 1 % of the 130 million hectares devoted to the grain legumes group (Gladstones, 1998).

1.6 Lupin improvement (breeding)

Cowling *et al.*, (1998) has made a comprehensive review of lupin breeding. Although *L. albus* and *L. mutabilis* were partially domesticated around the first millennium BC in the Mediterranean region and South America respectively. Von Sengbusch laid the foundation for modern lupin breeding in the late 1920s. He selected the first low alkaloid

cultivars of *L. luteus* and *L. angustifolius*. In 1928, Von Sengbusch found a natural mutant of *L. luteus* with permeable seed coats, low alkaloids, and non-shattering pods (von Sengbusch, 1942, as cited by Lopez-Bellido & Fuentes, 1986).

In 1960, Gladstones discovered two recessive mutants (*lentus* and *tardus*) of *L. angustifolius* with non-shattering pods. These mutants were crossed into white flowered and white-seeded sweet types and the first variety of sweet narrow-leafed lupins (cv. "Uniwhite") was released in 1967 (Cowling *et al.*, 1998).

Von Sengbusch also found the first low alkaloid forms of *L. mutabilis* in the 1930s (Von Sengbusch, 1942 and Hondelmann, 1984). According to Cowling *et al*, (1998) genes essential for domestication such as low alkaloids, non-shattering pods and permeable seed coats have been found in *L. albus, L. mutabilis, L. angustifolius* and *L. luteus*. These genes are being incorporated in different species according to the breeding objectives.

1.6.1 *Lupinus albus* L.

Lupinus albus known for its tolerance to frost and resistance to *Pleiocheata* has large seeds, indehiscent pods and permeable seeds but high alkaloid content (Haq, 1993). However, the main problem associated with this species is slow rate of seed development and maturation, which, combined with a strongly indeterminate growth habit, results in late, and uncertain harvest (Kasten & Kunert, 1991). In *L. albus* the inefficient use of photosynthates for thickening the pod and seed wall also reduces the grain yield. For instance, seed coat in *L. albus* is 18 % of seed dry weight as compared to 13 % in *L. mutabilis*. Efforts made through selection have resulted in the isolation of lupin lines with less vigorous lateral branches, a more rapid sequence of open flowers and determinate types with short duration of flowering, allowing the plants to produce all seeds in a single pod and to mature earlier. However, quantitative characters such as seed yield, oil and protein contents need to be improved (Cowling *et al.*, 1998).

1.6.2 *Lupinus mutabilis* L.

The Andean lupin is not only an important source of protein (42.2 % in the dry grain, 20 % in cooked grain and 44.5 % in the flour), but also of fat, which is 16 % to 23 % in the dry grain and in the flour respectively (Petterson, 1998). Moreover, these species tolerate considerable soil acidification and are more resistant to frost and drought than that of Old World species. It is used for human consumption after the bitter taste has been removed. Sweet mutants of *L. mutabilis* were found by von Sengbusch in 1930s but apparently were lost due to problems in maturity (Romer, 1986,1996; Cowling *et al.*, 1998). Recently a sweet cultivar of *L. mutabilis* (cv. Inti) is reported from Chile (von Baer and von Baer 1988), which marks the full domestication of this species.

Cultivation of the Andean lupin, like other crops of Andean origin, is limited by the lack of continued support for research and promotion. The main limitation is the alkaloid content of the seed and plant itself. Alkaloids give them a sharp, bitter taste and have to be removed by way of various laborious processes. The traditional and best-known method is cooking, followed by rinsing for several days. Although, at present, there are ecotypes with a low alkaloid content and one variety which is free of them, these still show adaptation difficulties, low resistance to pests and diseases, a long vegetative period and little growth vigour. Its nutritional value and forms of use are not widely known, which is why its consumption is not more widespread among the population. Through selection and crossing, there is potential for the development of varieties that are free of alkaloids and have desirable agronomic and productive characteristics.

In terms of nutritional value and potential for food processing, lupins offer an exact substitute for soybean (Haq, 1993). Therefore plant breeders are currently interested in exploiting this plant as a source of plant protein. The successful cultivation of this plant would reduce the dependence on soy cakes for animal feed as well as for human consumption. However, increased cultivation of this plant will depend on the development of suitable cultivars with improved harvest index, early maturation and high levels of disease and pest resistance. Therefore, the genetic variability that exists in this species should be exploited.

1.7 Wide hybridisation in the genus *Lupinus*

Gene introgression from one species to the other is a way of improving crop plants. Wide hybridisation, which includes inter-specific and inter-generic crosses, is considered as one of the tools for the transfer of desirable genes from one species to the other. By crossing of different lupin species it is possible to introduce new sources of resistance, new morphological characteristics or special quality features into the lupin species and varieties commonly used.

A brief search through literature reveals a paucity of references to interspecific crosses or the production of hybrids of *Lupinus* species. There are several reasons for such paucity. Firstly, the number of researchers and breeders working on this genus is limited because it is an under-utilised species. Secondly, the practical difficulties faced even in intraspecific crosses. However, despite these difficulties, interspecific hybridisation of the genus *Lupinus* has been attempted sporadically over the past century or so (Fruwith, 1910; Gollmick, 1937; Jaranowski, 1962; Williams *et al*, 1980; Pszyborowski *et al.*, 1996 and Siamasonta & Calligari, 1998) and has met with some success (Roy & Gladstones, 1985; Buirchell, 1994).

Some of the early works are reported by Fruwith, (1910); Gollmick (1937); Jaranowski (1962b); Kazimieriska (1965). Interspecific crosses were attempted, between different Mediterranean species (Gollmick 1937; Jaranowski 1962b; Kazimierski, Kazimieriska 1965; Kazimierski, Kazimieriska, 1975), between different African species, (Roy 1985; Gladstones, 1988; Roy, 1988), and between South American species (Schafer-Menhur 1988; Kasten *et al.* 1991). Partly fertile hybrids of *L. atlanticus*, *L. pilosus* and *L. cosentini* were obtained (Roy and Gladstones, 1988). Recently Gupta *et al.,* (1996) has reported the possibility of gene transfer among *L. cosentinii*, *L. digitatus* and *L. atlanticus*. They also studied the nature of reproductive barriers and meiotic behaviour of chromosomes in interspecific crosses among six rough-seeded *Lupinus* species. These examples of success are a useful signal for what might be possible and the advantages to be gained in this domain.

Interspecific hybrids involving Old World (*L. mutabilis*) and New World (*L. albus*) lupins could provide a valuable combination of characters listed by Atkins *et al.*, (1998) and Kasten & Kunert, (1991). Andean lupin (*L. mutabilis*) for example, has a wide genetic variability in plant form such as vegetative growth, susceptibility to frost and disease; protein, oil and alkaloids content (Romer, 1995). On the other hand the Old World lupins (*L. albus*) are lower in oil and protein content than the New World lupins (*L. mutabilis*). *L. albus* is also susceptible to mildew; root rot, thrips and aphids. The main problem with *L. albus* is slow growth of seed, late maturity, and indeterminate growth habit resulting in low and uncertain harvest. Plant breeders have however succeeded in isolating strains of *L. albus* with determinate branches, short duration of flowering, allowing the plants to mature earlier. However, *L. albus* is resistant against Bean Yellow Mosaic Virus (BYMV) and Cucumber Mosaic Virus (CMV) and is better adapted as a crop plant. However, quantitative characters such as seed yield, oil and protein contents also need to be improved. It is believed that genes of high oil and protein contents of *L. mutabilis* can be used to improve Old World lupins which are well adapted as crop plants (Haq, 1993, Gladstones, 1998).

Interspecific crosses between New World species and Old World species have also been attempted (Gollmick 1937; Williams *et al.,* 1980; Vuillaume & Hoff, 1986; Schafer-Menhur *et al.,* 1988; Siamasonta and Calligari, 1998). Results of the interspecific crosses attempted between two such species are not satisfactory as most of the crosses failed or embryos died within few days after pollination. The failure of seed development in interspecific crosses is believed to be due to abnormalities in the endosperm development (Ramasay & Pickersgill, 1986) or due to abnormalities in suspensor cells (Taheri, 2000). Although success in interspecific hybridisation of New World lupin species has been reported, the species involved in these crosses were of American origin and with equal number of chromosomes (Schafer-Menhur *et al.,* 1988; Kasten *et al*, 1991). However, attempts to hybridise Old World and New World species are still unsuccessful (Atkins *et al.,* 1998; Faluyi & Williams, 1981; Siamasonta, 1996).

Barriers to inter-specific hybridisation occur as a result of either sexual incompatibility or hybrid breakdown. In nature there are strong mechanisms acting before or after fertilisation, which can prevent the free combination of hereditary material of different

species. The phenomena are called as pro-gamic and post-gamic incompatibility (Kasten & Kunert, 1991). In the genus *Lupinus* only a few papers are known concerning pro-gamic incompatibility or pre fertilisation barriers in the genus *Lupinus* (Williams *et al.,* 1980; Faluyi & Williams, 1981; and Busmann-Loock, 1990- as cited by Kasten *et al.*, 1991).

Contrary to pre-fertilisation (pro-gamic) incompatibility, post-fertilisation (pre-gamic) incompatibility prevents, in most inter-specific crosses especially in legumes (Taheri, 2000), the formation of normal seeds through nutritional disturbances of the developing immature embryo. Consequently, hybrid embryo abortion takes place at an early stage of development (Vuillaume & Hoff, 1986; Siamasonta, 1996).

The second reason of failure of the interspecific crosses between these two species may be due to different chromosome numbers of the parents. The dissimilarity in chromosome number results in changes in gametes, fertilised ovules, and endosperm. Using parents with the same chromosome number may be useful in sustaining the development of the interspecific hybrid as it has been demonstrated experimentally by Schafer-Menhur *et al.,* (1988), and Kasten *et al.*, (1991).

1.7.1 Selection of compatible genotypes

Selection of more compatible parental genomes and making reciprocal crosses can help in obtaining more success in interspecific hybridisation. Since there is variation in the extent of physiological compatibility among different genotypes, attempts to produce interspecific hybrids should be preceded by screening the most compatible parental combinations. For example, Smartt (1990) reported that the wild species of *Phaseolus coccineus* was much more readily cross compatible with *P. vulgaris* than were modern cultivars. Considerable differences have also been seen in the rate of successful hybridisation using different genotypes of other legumes such as *Arachis*, *Vigna* and *Cicer* (Smartt, 1980; Chen *et al.,* 1983; Rashid *et al.,* 1989; Verma *et al.,* 1990). On the other hand, a cross, which yields abortive seed in one direction, may yield viable seed in the reciprocal direction. Examples have been reported in interspecific crosses of *Secale cereale* X *S. africanum* (Price & Shutz, 1974) and *Phaseolus vulgaris* (♀) x *P. coccineus*

(♂) (Haq *et al.,* 1973). In such crosses the abnormal endosperm development, failure of suspensor cell development, cytoplasmic incompatibilities or genomic imbalance result in failure of hybrid production (Hadley & Openshaw, 1980). For normal development of the endosperm in interspecific crosses a balance between paternal and maternal genomes is necessary.

1.7.2 The number of chromosomes and their role in interspecific crosses of the genus *Lupinus*.

The role of chromosomes in interspecific crosses is well documented (Barar and Khush, 1986; Atkins *et al*, 1998). Manipulation of the ploidy level of parents in interspecific crosses helps to overcome the disharmonious interactions and neutralising lethal factors. However, interspecific gene transfer can be difficult in a number of instances between species even with the same chromosome number and a fair degree of chromosome homology for example *Lycopersicon* species. The apparent causes of this are genetic or cytoplasmic interactions, genetic incompatibility and sterility, and undesirable linkages (Hadley & Openshaw, 1980).

In *Lupinus* species the cytological studies are not well advanced, as compared to other crop species. The reason may be due to the fact that lupin species have relatively small chromosomes (about 1 – 4 μm) and large in numbers (2n = 32 – 52) as a consequence, considerable difficulty has been experienced in determining the number of all species (Gladstones, 1998). However, the chromosome number of various *Lupinus* species has been reported in the literature (Pazy *et al.,* 1977; Plitmann and Pazy, 1984; Carstairs *et al.,* 1992; Gupta *et al.,* 1996). Gladstones (1984) indicated the exact chromosome number of at least nine species from the cyto-taxonomic studies published by Kazimierski, (1960). Pazy *et al* (1977) confirmed the chromosome number of seven species. The chromosome numbers (2n) of *L. albus* and *L. mutabilis* have now been established as 30 – 50 (2n) and 42 – 48 respectively (Lopez – Bellido and Fuentes, 1986). The basic chromosome number (x) for *Lupinus* species is established as x = 6 (Gupta *et al.,* 1996).

However, information on cytogenetics of major lupin species is meagre because of smaller size of the chromosome and the practical difficulties experienced in preparing high quality chromosome spreads. The arm lengths of chromosomes in *Lupinus* species are indistinguishable except in case of *L. princei* and a few of the chromosomes of *L. cosentinii*, *L. digitatus* and *L. atlanticus* (Carstairs *et al.*, 1992). The chromosomes of *L. princei* have been found to be the largest among all the species (1.77 – 4.40 μm).

Studies on the similarity of chromosomes among the rough-seeded (group of seven species) lupin group were conducted for the first time by Gupta *et al.*, (1996). They reported meiotic behaviour of the chromosomes in eight successful interspecific F1 hybrids. The behaviour of chromosomes in reciprocal interspecific crosses of *L. cosentinii* and *L. digitatus* indicated that on average 12 – 14 of the possible 17 pairs of chromosomes formed bivalents in every cell and were homologous or homoeologous. A few chromosomes appeared to have structural rearrangements (inversions and translocations) as they form multivalents in low frequency. The rest of the chromosomes are non-homologous.

Kazmierski (1980) studied meiotic behaviour of chromosomes in the *lutei* section by crossing *L. luteus* and *L. hispanicus*. Similar to the rough-seeded group, chromosome configurations that include bivalents, univalents and tetravalents were found. A partial homology of chromosomes in this section of the genus was also reported. Information on chromosomal number and behaviour in the interspecific crosses of *L. albus* and *L. mutabilis* is lacking in the literature.

1.7.3 Pollen tube growth

Several researchers have studied pollen tube growth following self, intra and inter-specific pollination of *Lupinus* species (Faluyi & Williams, 1981; Williams *et al.*, 1980, Przyborowski *et al.*, 1996 and Siamasonta, 1996). Unilateral incompatibility has also been reported in lupins (Williams *et al.*, 1980; Busmann-Loock *et al.*, 1992). In such cases the pollen grows normally on the stigma when the cross is made in one direction but failure is observed when pollen and female parents are reversed. Siamasonta (1996) investigated pollen tube using fluorescence microscopy in reciprocal crosses of *L. albus* and *L. mutabilis* and reported no barriers in fertilisation. Previously this combination was

reported as unilaterally incompatible (Williams *et al.,* 1980; Busmann-Loock *et al.,* 1992).

Microscopic observations on the pollen grain germination and pollen tube growth after reciprocal intra- and interspecific pollination of *Lupinus albus, L. angustifolius* and *L. mutabilis* Sweet also enabled Przyborowski *et al.,* (1996) to report the non-existence of pre-fertilisation barriers between these species. Pollen tube growth is slower in interspecific crosses as compared to the selfed crosses (Williams *et al.,* 1980; Siamasonta, 1996). There are reports of reduced growth rates of pollen tubes with certain combinations of species (Jaranowski, 1962; Williams *et al.,* 1980) but there is no clear evidence for these problems in the literature.

1.7.4 Exogenous plant growth hormones

Application of exogenous plant growth regulators, often as a mixture, has been reported to increase the number of hybrid embryos by enhancing pod retention and embryo growth to a stage that it can be rescued by use of the embryo culture. Application of growth regulators to increase pod set and hybrid embryo growth *in vivo* has been reported in many interspecific crosses such as *Phaseolus*, *Cajanus*, *Vigna*, *Trifolium*, *Cicer* and *Lens* (Al-Yasiri & Coyne, 1964; Kumar *et al.,* 1985; Rashid, 1988; Sailan, 1991; Pandey *et al.,* 1987; Verma *et al.,* 1990; Ahmed *et al.,* 1995).

1.7.5 Embryo development

Investigations have revealed that in certain interspecific crosses the hybrid embryo growth comes to a standstill after a particular stage (Singh *et al.,* 1990). The normal development of a hybrid embryo can adversely be affected by several factors, ranging from the action of a single deleterious gene to the action of several genes affecting development, organisation and differentiation of the hybrid embryo besides the differences in ploidy level of the two parents.

Pod and embryo/seed development studies following self and interspecific crosses of various leguminous species such as *Vicia*, *Phaseolus*, and *Cicer* have been reported in

the literature (Ramasay & Pickersgill, 1986; Taheri, 2000). In the genus *Vicia*, seeds of interspecific crosses between *Vicia faba* and *V. johannis* grew more slowly as compared to the intraspecific ones. Growth did not maintain a logarithmic rate but slowed down after five days. Interspecific embryos did not survive longer than 21 days after pollination (Ramasay and Pickersgill, 1986). In the genus *Cicer,* Taheri (2000) reported pod abortion within two weeks after pollination due to very early degeneration of hybrid embryos in selfed and interspecific crosses of three *Cicer* species and concluded that barriers to interspecific hybridisation existed at or after fertilisation.

Although various researchers have investigated pod and seed development in self and interspecific crosses of the genus *Lupinus* (Pszyborowski & Packa, 1997; Siamasonta & Calligari, 1998) information on pod/seed development of *L. albus* and *L. mutabilis* and their crosses is limited.

In reciprocal crosses of *L. albus*, *L. angustifolius*, and *L. luteus* Przyborowski & Packa, (1997) observed a slower growth rate of hybrid pods in comparison to selfed ones. They also observed a higher number of degenerated seeds in interspecific crosses after the 5th day of pollination. In the reciprocal crosses of *L. albus*, *L. angustifolius* and *L. luteus* Williams *et al* (1980) reported a slower growth rate of hybrid pods in comparison to selfed ones. In interspecific crosses of *L. albus* and *L. mutabilis* species, Siamasonta & Calligari (1998) reported a slower growth of pod and embryos as compared to selfed ones. They also reported that fertilisation in interspecific crosses between *L. albus* and *L. mutabilis* occurred but embryos did not reach a size sufficient enough to be excised. Busmann-Loock *et al.*, (1992) reported similar observations. However, the studies could not provide information on suitable time for *in vitro* embryo culture and evidence for fertilisation.

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Interspecific crosses between *L. albus* and *L. mutabilis* studied here showed pod abortion at different stages of embryo development or resulted in production of mature pods containing shrivelled or deformed seeds (chapter 2). The use of growth hormones to retain pods and enhance seed development *in vivo* may help in obtaining successful interspecific crosses. The role of growth regulators on pod and seed development of different species has been discussed in (section 1.7.4) and their effect on self and

interspecific crosses between *L. albus* and *L. mutabilis* species has been presented in section 1.8.1).

Researchers have given different reasons for embryo degeneration such as disturbances in the development of the pro-embryo, suspensor and endosperm. The process of degeneration of embryos also depends on the direction of the cross (Busmann –Loock *et al.,* 1992). However, there are no reports on the genetic composition of the developing embryos, to confirm the presence of both male and female DNA in the developing embryo.

1.8 Embryo rescue techniques

Sharma *et al.,* (1996) have made a comprehensive review of embryo culture in plants. The earliest record of embryo rescue dates back to the work of Charles Bonnet in the 18th century, who excised embryos of *Phaseolus* and *Fagopyrum* and planted them in soil. These embryos resulted in the formation of dwarfed plants. Nutrient solutions for rearing embryos were used around 1890 by Brown and Morris. More systematic steps in embryo culture, however, started from the beginning of the last century when Hanning (1904) cultured embryos of *Cruciferae* excised from a few taxa such as *Raphanus* species and *Chochlearia damica* in a medium containing mineral salts and sugar under aseptic conditions and succeeded in obtaining plants. This study opened up a field for basic investigations in plant embryology. The main emphasis was laid on understanding physiological and developmental aspects and effects of growth substances. Brown (1906) studied the relative efficiency of various organic nitrogen compounds on growth and development of excised barley embryos cultured on a medium containing mineral salts and sugar. These studies provided basis for further investigations, mainly on the role of different embryonal parts (Sharma *et al*, 1996).

Ragahavan (1980) stated in his review on embryo culture that there are heterotrophic and autotrophic stages in embryo development. The embryo in the heterotrophic stage of development is smaller (termed as pro-embryo) than in the autotrophic stage and usually requires the presence of growth regulators to allow for its proper development

(Raghavan, 1994). In the autotrophic stage, development of an embryo does not depend on exogenous sources of growth regulators.

The embryos usually have to be rescued from being aborted when they show inherent nutritional deficiency or if they are the result of distant hybridisation. In such situations the embryos usually abort without germination but can, in most cases, be rescued following a proper *in vitro* procedure.

1.8.1 Use of exogenous growth regulators

Exogenous application of growth regulators is thought to be helpful in preventing flower abscission or in pod and seed retention since the normal production or accumulation of hormones may be restricted by alien pollination. On the other hand in interspecific crosses resulting in embryo abortion, application of growth regulators may help increase the size of hybrid embryos *in vivo* sufficiently enough to be excised from ovules and grown *in vitro*. Gibberellins, cytokinins and auxins individually or as a mixture have been used for such purposes in many crops.

Lang & Gorz (1960) found that application of IAA and NAA as a solution at a concentration of 10 and 100 mg/l to pods of the cross *Melilotus pfficinalis* x *M. alba* was effective in delaying abscission of the hybrid pods. Abscission usually occurred 12 to 20 DAP while treated pods remained on the plant up to 40 days after pollination.

In an interspecific cross of *Phaseolus vulgaris* x *Ph. acutifolius*, Al-Yasiri & Coyne (1964) used NAA in a mixture with potassium gibberellate acetamide in lanolin paste. They found a remarkable effect in slowing down the rate of flower drop as well as delaying pod abscission, which resulted in larger embryos.

Payan & Martin (1975) applied growth substances containing GA_3, NAA and IBA at a concentration of 1 % in lanolin paste to the base of the ovary to prevent abscission in interspecific crosses in the genus *Passiflora*. The action of hormones appeared to be a delay in abscission of the flowers. Similarly, Pandey *et al.*, (1987) smeared 1% IAA in

lanolin paste to the pedicels to prevent early degeneration of flowers in crosses of *Trifolium repens* and *T. uniflorum.*

Kumar *et al.,* (1985) reported that in the interspecific crosses of *Cajanus* and *Atylosia* the rate of success could be increased with hormone treatments. This treatment increased the percentage of pod set, pod length and number of seeds per pod in the cross combinations. Moreover, the onset of flower drop among unsuccessful crosses was delayed following hormone treatments. They also observed that higher concentration of GA_3 and GA_3 + kinetin reduced the rate of successful crosses, but prompted pod length and number of seeds per pod. The increased pod set associated with the hormone treatment was most likely to be due to enhanced post-fertilisation development of both ovary and ovules, since treatments commenced one day after pollination when fertilisation would have been completed.

Altman (1988) applied a mixture of GA3 and NAA solution to the surface of boll bracts after pollination in interspecific crosses in the genus *Gossypium.* He claimed that it resulted in higher boll retention and more embryos for subsequent embryo culture or seed. He stated that, in general, exogenous hormone application was superior to the *in vitro* culture method in obtaining the hybrid seeds.

Gosal & Bajaj (1983) used a mixture of GA_3, NAA, and kinetin to prevent hybrid abortion in interspecific crosses of *Vigna mungo* and *V. radiata.* They noticed that hormone application considerably helped to retain a large number of pods longer *in vivo* whereas, in the control fewer pods were retained after 15 days. Furthermore, the number of developed seeds was higher in the hormone treated pods. A similar attempt was made by Rashid *et al.,* (1989) in interspecific crosses of the genus *Vigna.* They mentioned that pod retention was enhanced but the pods contained shrivelled seeds at maturity. Nevertheless, Rashid (1988) and Sailan (1991) were able by applying growth regulators to retain hybrid embryos in the genus *Vigna* to a size where they could be excised and regenerated plants were obtained through *in vitro* culture. Singh *et al.,* (1993) and Haq (1994) used similar rescue techniques in interspecific hybridisation of the genus *Cajanus.*

Ahmed *et al.,* (1995) observed the effect of GA_3 application for producing interspecific hybrids in the genus *Lens.* They suggested that the artificial supplement of GA_3 is needed after fertilisation for the normal growth of the hybrid embryo possibly as the natural GA_3 production is restricted by alien pollination. This was noticed in both F1 and backcross hybrids. Similar results were obtained when GA_3 was used with NAA and kinetin to enhance pod retention in interspecific hybrids of *Glycine* spp (Singh *et al.,* 1990).

Application of growth hormones has also been attempted to prevent the abscission of the flowers and to give more time for the pollen tubes to grow and to get a faster and longer pollen tube growth for successful fertilisation in interspecific hybrids of *Cicer.* Swamy & Khanna (1990) used a mixture of 40 ppm IAA and GA_3 in solution in interspecific crosses of *Cicer.* The solution was applied at the base of the pistil with the help of a needle and brush. The cotton around the pistil was then moistened with the hormone solution for 3 days. In general, they noticed more seed set in crosses as compared to the control. Badami *et al.,* (1997) and Mallikarjuna (1999) also reported sustained pod growth and more growth of hybrid embryos in interspecific crosses between *Cicer arietinum* and *C. pinnatifedum.*

1.8.2 Embryo/ovule culture

The hybrid embryo of wide crosses usually degenerates in the early stages due to poor/abnormal development of endosperm or overgrowth of the maternal tissues into the embryo sac. Embryo and ovule culture techniques have been used to overcome such an incompatibility.

Embryo rescue has been successfully used to produce hybrids involving interspecific and inter-generic crosses in various crop plants (Sharma *et al,* 1996). Interspecific hybrids of various leguminous crop plants have also been obtained making use of the embryo rescue technique in leguminous crops namely: *Phaseolus* (Ben-Rejeb & Benbadis, 1989; Kuboyama *et al.,* 1991), *Vigna* (Rashid, 1988; Sailan, 1991; Pellegrineschi *et al.,* 1997), *Glycine* (Tilton & Russell, 1984, Bodanese-Zanettini *et al.,* 1996), *Cicer* (Mallikarjuna, 1999), *Cajanus* (Singh *et al.,* 1993; Haq, 1994; Mallikarjuna & Moss, 1995) and forage legumes such as *Trifolium* (Ferguson *et al.,* 1990; Przywara *et al.,* 1996).

When the hybrid embryo aborts at a young stage it is difficult to excise without injuring it. Whole ovules or ovaries may be cultured in such cases. However, *in ovulo* embryo culture is not likely to be useful in those instances where maternal tissue or the endosperm exerts an inhibitory action on the development of the embryo. In these cases the culture of isolated embryos would be advantageous (Collins *et al.,* 1984). In some instances, nutrient exchange may be improved by culturing only the embryo-containing sectors of the cut ovules. Examples involving the use of ovule culture to generate desirable hybrids are interspecific hybrids in *Gossypium* (Thengane *et al.,* 1986), *Lens* (Cohen *et al.,* 1984), *Medicago* (McCoy & Smith, 1986), *Arachis* (Stalker & Eweda, 1988) and *Trifolium* (Przywara *et al.,* 1996).

A number of protocols for organogenesis and regeneration of plants have been developed for various species of the genus *Lupinus* (Scahfer-Menhur, 1989; Rahim & Caligari, 1998). Several attempts have been made to regenerate plants from aborting hybrid embryos of the genus *Lupinus* (Sator, 1985; Vuillaume & Hoff 1986b; Schafer-Menhur *et al.,* 1988; Kasten & Kunert, 1991; Kasten *et al.,* 1991). However, information on securing a hybrid plant through *in vitro* embryo/ovule culture of interspecific crosses involving *L. albus* and *L. mutabilis* is limited.

In 1988, Schafer-Menhur and co-workers were the first to secure hybrid plants of *Lupinus* spp. through embryo culture. Plants were regenerated from young hybrid embryos of the cross between *L. mutabilis* (♀) and *L. hartwegii* (♂) species. Both *L. mutabilis* and *L. hartwegii* originated from the Andean region of South America and each had a complement set of chromosomes (2n = 48). They concluded that the success rate was generally better when hybrid pods were left on the mother plant for at least 18 days prior to culture on B5 medium solidified with T39 agar and supplemented with growth hormones (NAA and BA).

Kasten & Kunert (1991) developed an embryo culture system for five lupin species viz. *Lupins luteus*, *L. albus*, *L. angustifolius*, *L. mutabilis* and *L. polyphyllus*. Using this method Kasten *et al* (1991) managed to obtain a plant by culturing hybrid embryos of the cross *L. mutabilis* (♀) x *L. hartwegii* (♂) at late heart and torpedo stages of development

in vitro. The species involved in this cross were also of South American origin and contain an equal number of chromosomes (n = 24). They used the B5 basic medium solidified in 0.7 % agar containing gibberallic acid (0.525 mg/l), indole butyric acid (1.5 mg/l), and casmino acids (725 mg/l).

1.9 *In vitro* isolation and fertilisation of gametes

One of the major problems in wide hybridisation through conventional crossing methods is sexual incompatibility due to various barriers operating at different levels as explained earlier (section 1.3). In the genus *Lupinus* it is now apparent that these barriers operate at post-fertilisation level and result in early abortion of hybrid embryos due to degeneration of endosperm (Siamasonta, 1996; Atkins *et al.*, 1998). Attempts have been made to rescue the aborting embryos but apart from the limited success in Germany (Schafer-Menhur *et al.*, 1988; Kasten & Kunert, 1991), most of the attempts resulted in failure (Atkins *et al*, 1998). Even having managed to get an embryo developed through embryo rescue does not guarantee a mature and viable plant as hybrid sterility; hybrid break down in F1 generation are common in interspecific crosses.

One of the methods, such as *in vitro* isolation and fertilisation of gametes, can be used to circumvent the difficulties in interspecific crosses of related species. This technique not only helps to solve the complex problems of incompatibility but also, provide new ways of achieving genetic recombination between different plant species, which is difficult to achieve otherwise (Dumas *et al.*, 1998).

1.9.1 Isolation of male gametes

In angiosperm plants, the male gametophyte (the pollen grain and tube) produces two sperm cells, which are transported to the female gametophyte (the embryo sac). Several attempts were made to isolate sperm cells of different species during mid eighties of the last century. The first significant results were obtained with *Plumbago* (Russell, 1986) and maize (Dupuis *et al.*, 1987); where the sperm cells of the two species were isolated successfully. They also verified the viability and integrity of the isolated sperm cells.

Since then several protocols have been developed to isolate and examine sperm cells in a wide range of species (Southworth, 1992; Chaboud & Perez, 1992).

Three-dimensional reconstruction of pollen from several species have shown that in three-celled pollen species, the two sperm cells and the vegetative nucleus are physically associated. This association between the sperm cell plasma membrane and the nuclear envelope of the vegetative cell was termed the 'male germ unit' (MGU) because of its putative importance in relation to the phenomenon of double fertilisation (Dumas *et al.*, 1998). Thus all the hereditary DNA of, both cytoplasmic and nuclear, is held together as a single unit. The validity of this concept was partly demonstrated by isolating this physical unit or MGU (Matthys-Rochon *et al.*, 1987), and second by demonstrating that such an association also exists in the two-celled pollen grain.

All sperm cells have some common features: they are small, ranging from 1– 10 μm in diameter. After isolation sperm cells become spherical, like vegetative protoplasts, and contain a large nucleus and a small volume of cytoplasm (Dumas *et al.*, 1998). These sperm cells have no cell wall indicating that they are prepared for fusing with their female cell target (Dumas *et al*, 1998). The quality of sperm cells has been examined using numerous techniques, including the Fleuorochromatic reaction (FCR) test (Dupuius *et al.*, 1987), Evans blue staining, ATP measurement (Roeckel – Drevet *et al.*, 1995), or cell sorting (Zhang *et al.*, 1992). Clearly the most absolute assay would be to improve their ability to fuse with a female gamete and to form a true zygote.

1.9.2 Isolation of female gametes

In angiosperms the female gametophyte or embryo sac develops in a structure called an ovule. One or two integuments filled with nucellar tissue and attached to the placental region of the ovary by a funicle surround the ovule. The embryo sac and the gametes are deeply immersed in the diploid female organ (Wagner *et al.*, 1990) A cellulosic wall sometimes complete with callose surrounds the embryo sac (Matthys-Rochon *et al.*, 1992). The most common type of embryo sacs is the polygonum type (monosporic, eight-nucleate) (Haig, 1990; Reiser & Fischer, 1993). At the micropylar end there are two synergids on each side of the egg cell, although there are exceptions, for instance in

Plumbago zeylanica there are no synergids (Russell, 1992). One of the two synergids will be penetrated by the pollen tube for male gamete (two sperm cells) deposition. The true female gamete, the egg cell, will fuse with one of the two sperm cells and the resulting zygote will give rise to a nutritive tissue: the endosperm. At the chalazal end, the antipodal cells (basic number: 3) which are active embryo sac appears to be a major plant structure, a functional unit (Jensen, 1972), in which the original double fertilisation occurs, and is the recipient when the new plant starts its development.

Zhou and Yang (1985) and Hu *et al.* (1985) succeeded in developing enzymatic maceration techniques to isolate viable embryo sacs of several angiosperms. Using this technique viable embryo sacs and egg cells of various species such as *Lolium* (van der Mass *et al*, 1993), maize (Kranz & Lorz, 1993; Leduc *et al.* 1996), and *Nicotiana* (Tian and Russell, 1997) have been isolated. Characterisation of female gametes has also been carried out in many species using electron, light, and fluorescence microscopy (Heslop-Harrison, 1984; Hause, 1991; Huang *et al.,* 1992; Mathys-Rochon *et al.,* 1992; Kranz *et al.,* 1998).

1.9.3 Fusion of male and female gametes

Double fertilisation in angiosperms, during which one sperm nucleus fuses with the egg nucleus (the origin of the zygotic embryo) while the second sperm nucleus fertilises the two polar nuclei of the embryo sac or female gametophyte, leading to the formation of the endosperm, was first reported by the Sergius Nawaschin (1898) and Leon Guignard (1899). Since then a considerable development has been made in this domain.

Kanta *et al.,* (1962) were able, for the first time, to accomplish artificial fertilisation of opium poppy by pollination of excised ovules with mature pollen. Successful *in vitro* fertilisation was reported by Kranz *et al.,* (1991a, 1991b) ten years ago and by some other research workers including Kranz & Lorz (1994), Kranz *et al.,* (1995) and Faure *et al.,* (1994). Recently, Kranz *et al.* (1998) were successful in the isolation and fusion of central and sperm cells of maize plants. Development of endosperm *in vitro* was also reported.

Two main strategies for *in vitro* fertilisation have been developed in angiosperms during the past few years. One strategy consists of injecting isolated male gametes or male nuclei into isolated embryo sacs (Keijzer *et al.,* 1988; Mathys-Rochon *et al.,* 1994) but the rate of success is very low. In addition, the microinjection of sperm cells into egg cells or central cells completely isolated from surrounding cells has not yet been achieved because of the technical difficulty of the procedure. Such methods of intracytoplasmic sperm injection are already practised in humans (Alikani *et al.,* 1995).

The second strategy consists of fusion of gametes, completely isolated from the surrounding tissues, under *in vitro* conditions (Kranz *et al.,* 1991; Kranz & Lorz, 1993, 1994; Faure *et al.,* 1994; Kovacs *et al.,* 1995). This method consisted of aligning pairs of gametes by di-electrophoresis and then fusing them by the use of one or more electric pulses. This method was used successfully for maize (Kranz *et al.,* 1991; Kranz & Lorz, 1993) and wheat (Kovacs *et al.,* 1995). An alternative to electro-fusion involves bringing the male and female cells into contact using micro-needles in media containing 1- 10 mM calcium (Faure *et al.,* 1994) or 50 mM calcium (Kranz & Lorz, 1994). The gametes adhere for a few minutes and then fuse. Unlike electro-fusion, this second *in vitro* system retains gamete specificity (Faure *et al.,* 1994).

Comparison of physiological state of gametes *in vitro* and *in vivo* is important for fertilisation experiments. Gametes must be put into close contact to allow *in vitro* fusion. This can be achieved either by di-electrophoresis (Kranz *et al.*, 1991; Kranz & Lorz, 1993) or by using micro-needles and allowing the gametes to adhere to each other (Faure *et al.,* 1994; Kranz & Lorz, 1994). Under *in vivo* conditions, cytoskeletol elements in the embryo sac might be responsible for this apposition of the male gametes to the egg and central cells.

In embryo sacs of *Plumbago zeylanica* and *Nicotiana tabacum* actin distribution is induced by pollen tube (Huang *et al.,* 1993; Russell, 1993; Huang and Russell, 1994). One or two-actin carona(s) appear close to the presumed sites of gamete fusion. The involvement of these structures in pulling the male gametes towards their targets still needs to be investigated and demonstrated experimentally. The molecules involved in the later adhesion of the gametes are unknown. Several studies have led to the identification

of such molecules in animal species (Almeida *et al.,* 1995) as well as in algae, e.g. *Fucus* (Wright *et al.,* 1995a). The molecules involved in the adhesion of angiosperm gametes may be ubiquitous. Indeed, male gametes can also adhere to each other and to mesophyll protoplasts although their fusion is infrequent (Faure *et al.,* 1994). *In vitro* fertilisation without electrical pulses could be used as a bioassay to identify molecules involved in the adhesion of gametes.

The physiological conditions during *in vivo* gamete fusion are unknown. The osmolarity of the environment surrounding the gametes, the composition, its pH as well as the temperature at which fusion occurs, have not so far been measured. The only information we have are from *in vitro* experiments that the highest fusion rate is obtained in 5 mM calcium with fewer fusions being obtained at lower concentrations (Faure *et al.,* 1994). Therefore, it seems that calcium in the millimolar range is essential for gamete fusion as it is in other organisms such as mammals (Yanagimachi, 1994). Additional observations suggest that calcium might be essential for gamete survival and fusion. The concentration of bound calcium is high in the synergids of grass species (Chaubal and Reger, 1992) and after synergid degeneration around the egg cell (Huang & Russell, 1992). It is also reported that calcium concentration is important for egg cell membrane stability in barley (Holm *et al.,* 1994) and for sperm cell viability in maize (Zhang *et al.,* 1995).

The process of gametic fusion itself is very rapid (Faure *et al.,* 1994; Kranz & Lorz, 1994). Indeed, the fusion of the gametes has rarely been observed by electron microscopy indicating that this is a rapid event (Russell, 1992). The fusion of gametes in 5 mM calcium is frequent and about 80 % of male gamete-egg cell pairs fuse quickly. This suggests the absence of preferential fertilisation, i.e. a higher probability of one male gamete fusing with the egg cell is the same as for fusion with the central cell. These observations contradict data obtained for *Plumbago zeylanica.* In this species, the two male gametes from one pollen grain are dimorphic: one is plastid-rich and fuses more frequently with the central cell (Russell, 1985) Another aspect of gamete recognition is whether a barrier to interspecific crosses exists at the gamete plasma membrane level. So far, electrofusion of maize egg cells with coix, sorghum, *Triticum Hordeum* and *Brassica* male gametes have been reported (Dumas *et al.*, 1998). As electric pulses forced fusion in these examples, this does not address the question of interspecific crossability.

Corresponding *in vitro* fertilisations without electro-fusion are therefore required. Potential implications of this *in vitro* fertilisation are important. Because the fusion parameters can be controlled and the timing is precise, the study of gamete adhesion, fusion and signalling should now be feasible. In addition, *in vitro* protocols can be combined with new molecular tools to achieve the hybrids of various incompatible crosses. Despite the fact that a wide diversity of techniques has been developed for other species very little is reported in the literature for other angiosperms especially legumes regarding *in vitro* fertilisation.

1.9.4 Plant regeneration from fused gametes.

Zygotes and endosperm are able to self-organise in culture independently from maternal tissues. In combination with tissue culture methods, which are adapted for the culture of single cells, micromanipulation techniques allow individual development of zygotes and endosperm *in vitro* (Kranz & Lorz, 1998). The development of fertile plants from zygotes both produced *in vitro* and zygotes isolated after *in vivo* pollination has been reported (Kranz & Lorz, 1994). In 1993, Kranz & Lorz reported *in vitro* regeneration of fertile maize plants from fused gametes. They used regeneration medium containing 2, 4-D (0.1 or 0.5 mg/l), NAA (1.0 mg/l) and, (benzyladenine (1.0 mg/l) during the first phase and for later stages they used MS medium without hormones supplemented with 40 - 60 mg/l sucrose. This technical development not only establishes an *in vitro* system for examining fertilisation events and embryo development of higher plants, but also provides new methods to regenerate plants from fused gametes with desirable genotype.

1.10 Summary

In summary this literature survey indicates the tremendous potential of lupins as grain legume (especially *L. mutabilis*). The diversity of Andean lupin can be exploited to improve the agronomic as well as qualitative characteristics of other species of the genus *Lupinus*. However, previous attempts to combine useful traits of Andean lupins through interspecific hybridisation were not successful. The crosses failed due to embryo abortion at an early stage of development. Although it has been reported that in such crosses

fertilisation occurred the available studies have failed to provide the evidence for it. Moreover, in successful interspecific crosses studies have not been able to confirm the hybrid nature of the plantlets obtained. The reason may be due to the scarcity of the hybrid material obtained. In most of the cases the number of plantlets obtained was only one or two as they were rescued through *in vitro* embryo/ovule culture. Furthermore, the plantlets developed did not survive long and died shortly after their transfer to green house conditions. The literature further suggests the possibility of obtaining hybrids of the genus by using genotypes of both the species with equal number of chromosomes.

In angiosperms the isolation and *in vitro* fertilisation of gametes is now possible. This technique can also be used to avoid the barriers in interspecific hybridisation of the genus *Lupinus*. The ability of zygotes to organise and develop into plants may be used for genetic recombination of incompatible lupin species in particular, and in other grain legumes in general. For each species a precise concentration of enzymes, pH level, incubation temperature and incubation time are required to isolate the embryo sacs/egg cells in a living state (Zhou and Yang, 1985).

1.11 Aims and Objectives

The potential of lupins as grain legume and the difficulties encountered by earlier lupin breeders in hybridising different *Lupinus* species have encouraged us to carry out this project. *Lupinus albus* has lower protein and oil content than *L. mutabilis* but it is better adapted as a crop plant in diverse range of agro-climatic conditions. *L. mutabilis* has a wide genetic base for vegetative growth, susceptibility to disease and frost, good yield and yield stability, high oil and protein content, resistance to root rot, drought tolerant and early maturing. In contrast *L. albus* is cold tolerant, particularly at the seedling stage and has a favourable growth habit, but it is lower in oil and protein contents than *L. mutabilis*. It is also susceptible to root rot, thrips and aphids and is late maturing. Therefore, transfer of important traits between the two species may help to develop an appropriate breeding strategy. The primary aim of the project was to develop appropriate technique(s) to transfer desirable genes between *Lupinus albus* L. (*L. albus*) and *Lupinus mutabilis* L. (*L. mutabilis*) species. The objectives of the project were:

- Selection of genotypes of the two species for interspecific crosses on the basis of their morphological characteristics including somatic chromosome numbers.
- To identify the possible barriers to hybridisation between the two species of the Old World (*L. albus*) and the New World (*L. mutabilis*) by comparison of pollen grain germination, pollen tube growth, and pod and seed development in self and interspecific crosses.
- To rescue the interspecific hybrids through:
    1. Use of exogenous growth hormones
    2. Embryo/ovule culture (*in vitro*)
- To develop methods for *in vitro* fertilisation of *L. albus* and *L. mutabilis* thorugh:
    1. Isolation of male and female gametes and their fusion
    2. Plant regenration from fused gametes

Table1.1: Distribution, chromosome numbers and common names of lupins.

SPECIES	Synonyms	Chromosome no. (2n)	Distribution and common names
I. Old World, smooth-seeded			
1. Lupinus albus L.			
Var. albus	L. termis Forsk	50	Mediterranean etc., cult. 'European white lupin', 'albus lupin'
Var. graecus (Boiss. And Sprun.) Gladst.	L. graecus Boiss. And Sprun L. jugoslavicus Kazim. And Now L. vavilovi Atab. And Maiss	50	NE Mediterranean, native
2. L. angustifolius L.	L. varius L., sensu Sp. Pl. edn 1 (1753) L. linifolius Roth L. reticulatus Desv. L. opsianthus Atab. And Maiss.	40	Pan-Mediterranean, native and semi-cult.; Australia naturalised and cult.; elsewhere, cult. 'Narrow-leafed lupin'; ;blue lupine' (USA)
3. L. micranthus Guss.	L. hirsutus L., sensu Sp. Pl. ed 2 (1763)	52	Pan-Mediterranean, native. 'Lesser hairy blue lupin'
4. L. luteus L.		52	W iberia, scattered pan-Mediterranean, native or semi-cult.; Elsewhere, cult. 'Yellow lupin'
5. L. hispanicus Boiss. And Reut subsp. Hispanicus subsp. Bicolor (Merino) Gladst.	 L. rothmaleri Klink	 52 52	 C and S Spain; Algeria, Greece, Turkey; native C and NW Spain, Portugal, ? Greece; native
Old World, rough-seeded			
1. L. pilosus Murr.	L. hirsutus L., sensu Sp. Pl. edn 1(1753) L. anatolicus Sweic.	42	NE Mediterranean, native and ? semi-cult. 'Greater hairy blue lupin
2. L. cosentinii Guss.		32	W Mediterranean, Morroco, native; Australia, naturalised 'Sandplain lupin'
3. L. digitatus Forsk.	L. semiverticillatus Desr. L. tassilicus Maire	36	Sahara, native
4. L. atlanticus Gladst.		38	S Morroco, native. 'Moroccan lupin'
5. L. princei Harms		38	E Africa, native
6. L. somaliensis Baker		Not known	E Africa, native
II. NEW WORLD			
L. mutabilis Sweet		42, 48	South America: Andean Highlands, native and cult. "Tarwi"; pearl lupin

Source: Lopez - Bellido and Fuentes, 1986; Smartt, 1990; Gladstones, 1998.
C = Cultivated W = Wild

Chapter Two

Interspecific hybridisation

2.1 Introduction

Plant breeding techniques have been used for generations to improve crop plants through enlargement of genetic variability of cultivated species. Interspecific hybridisation is one of the most widely used plant breeding tools to transfer genes of desirable traits from one species to the other. Interspecific crosses help to introduce new sources of resistance, new morphological-anatomical characteristics or special quality features into the plant species and varieties (Hadley and Openshaw, 1980; Kasten & Kunert, 1991).

Despite recent progress in lupin breeding chapter 1 (section 1.7), breeders are still trying to improve the qualities of this crop through gene introgression from one species to the other. By and large, interspecific hybrids and even intergenric hybrids are formed far more rapidly in *Gramineae* than in *Leguminoseae*. Thus in grain legumes, interspecific hybridisation is usually restricted (Smartt, 1979). Interspecific hybridisation between Euoropean and South American lupin species result in embryo abortion in the early stages of development.

The aim of this work was to combine useful traits (Table 2.1) of *L. albus* and *L. mutabilis*. Chromosomes play an important role in interspecific hybridisation programmes (Brar & Khush, 1986; Singh *et al.*, 1990) therefore, information on cyto-logical aspects of *L. albus* and *L. mutabilis* species can help in evaluating their potential use in genetic improvement of lupins and the transfer of desirable traits from one species to another. A wide range of genotypes of *L. albus* and *L. mutabilis* was investigated for their useful traits including yield related characters, pollen viability, equal number of chromosome (2n = 48) to facilitate the free genetic recombination of the two species.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Initially, eight accessions of *L. albus* and *L. mutabilis* were selected from a wide range of accessions obtained from the Food and Agricultural Organisation of the United Nations Rome (Italy), Barani Agricultural Research Institute, Chakwal (Pakistan), University of Agriculture and Technology, Olsztyn (Poland), and collections held at the University of Southampton, United Kingdom. Selection of the genotypes was carried out on the basis of their crossing behaviour, important morphological characteristics and equal number of somatic chromosomes for interspecific hybridisation. A list of the genotypes and their origin used in this study is given in table 2.2.

The seeds of selected accessions with equal number of somatic chromosomes (2n=48) were sterilised in 15 % domestos (a commercial sodium hypochlorite solution) for 20 – 25 minutes to disinfect against fungal disease pathogens. Then, the seeds were sown in 3" pots containing F2 Levington compost (seed and modular compost with sand) soil and grown at 25/15 ± 2 °C day/night temperatures in a glasshouse of the University of Southampton, United Kingdom. High light-intensity sodium lamps provided the daylight (16 hours) and the humidity was maintained at 60 – 70 ± 5 %. After two weeks the seedlings were transferred to 7" pots containing John Innes No. 2 compost (blended mixture of sterilised Mendip loam, sand and peat (7:2:3). The growth rate of some of the accessions was very slow or they did not flower well under the glasshouse conditions of Southampton. Therefore, according to their performance in our glasshouse and their useful traits (mentioned in Table 2.1) a total of eight accessions, four from each *L. albus* and *L. mutabilis* were selected for making reciprocal interspecific crosses.

2.2.2 Somatic chromosomes of *Lupinus* species

Young root tips of about 1 cm. in length from different accessions of two species were collected between 0800 to 1000 hours. The root tips were washed in distilled water and pre-treated with saturated Para-dichlorobenzene (PDB) for 1 to 3.5 hours at 14 – 17 °C. These were then washed free of the PDB by rinsing in distilled water for half an hour on a

shaker with three changes of water. To stain and soften, the root tips were placed in few drops of acetic orcein stain (9:1, mixture of 1 % acetic orcein: 1Normal Hydro-chloric acid (HCl) in a watch glass and warmed over a spirit lamp. Warming was repeated three times at one-minute intervals. After warming, the material was left for 20 – 30 minutes. The meristematic portions of root tips were cut off using a scalpel and were then put in fresh acetic orcein on a glass slide. A cover slip was applied and pressing the tip of a wooden rod on the cover slip to disperse the cells of the squashed root tips. Heavier pressure was then applied after embedding the slide in a filter paper to spread the cells. The slides were then studied under a light microscope (Zeiss Axioskop, Germany).

2.2.3 Morphological Study

Some morphological and yield related characteristics of *L. albus* and *L. mutabilis* species were studied for comparison between species and hybrid plants. These characters were, plant growth habit, plant height, number of branches per plant, number of flowers per plant, number of pods per plant, leaf length, number of leaflets per leaf, pedicile length, pod length, number of seeds per pod, 100 seed weight and seed colour. Seed colour was determined using the Royal Horticultural Society Colour Chart.
Calculation of correlation between different plant characters is a proven statistical method in the varietal evaluation of crop species in plant breeding programs. The experimental data thus collected for the above-mentioned characters was statistically analysed and correlation coefficients were calculated using computer statistical package SPSS.

2.2.4 Pollination

Flowering in *L. albus* and *L. mutabilis* species starts approximately after 50 to 60 days and 65 to 70 days of germination respectively under the glasshouse conditions of Southampton University. To maintain continuous supply of flowers of both the species for crossing experiments, *L. mutabilis* accessions were planted two weeks before *L. albus* accessions. Pollination was carried out under glasshouse conditions. Flower buds, which were due to open the next day, were selected for emasculation. Buds were held between thumb and forefinger and then anthers (ten in number) were removed with the help of a

forceps through a small slit made in the keel (Townsend, 1980). Flowers were pollinated with fresh pollen grains obtained from fully opened flowers. Then, petals were closed tightly to prevent the stigma from desiccation. Emasculation and pollination was carried out mostly between 9.00 am – 11.00 am. However, in cooler months (September to May), sometimes pollination was carried out in the afternoon (till 4.00 p.m.). Initial investigation of all interspecific crosses was carried out through examination of all hybrid pods and ovules/seeds under a stereo-microscope. At the same time, self-pollinated pods and ovules/seeds were examined and measured as a control. Information obtained from self-pollinated pods, ovules/seeds was used to compare the rate of growth in hybrids. Accordingly, ovaries with no fertilised ovules were considered as failed at the flowering stage. Pods containing fertilised ovules were categorised as early aborted or late aborted according to the size of the pods and ovules at the time of abortion as compared to self-pollinated flowers. If pod abscission occurred within 5 days of pollination they were considered as abscised flowers. The pods, which were retained after 10 days of pollination, were considered as set pods. In mature pods, fertilised ovules, which aborted at an earlier seed developmental stage, were considered as shrivelled seeds. Those aborted at a later stage but not fully filled were regarded as deformed seeds. Fully developed and plump seeds were considered as normal. The pods retained 30 days after pollination were considered as successful crosses and the success of crosses was expressed in percentage and was calculated as:

$$\frac{Number\ of\ successful\ crosses\ in\ each\ cross\ combination}{Total\ number\ of\ crosses\ made\ for\ each\ cross\ combination} \times 100$$

2.2.5 Determination of fertilisation through pollen tube growth studies

An understanding of the process of pollen germination and pollen tube growth is useful in solving the complex problems of incompatibility. Furthermore, pollen germination and pollen tube growth studies provide a useful tool for determining the cross compatibility between species (Williams *et al.,* 1980). Pollen germination and pollen tube growth in self and interspecific crosses of *L. albus* and *L. mutabilis* species were investigated with an aim to understand the causes of hybrid pod abortion. Pollen germination and pollen tube growth following self-pollination was treated as control.

Pistils from selfed and interspecific crosses were removed at intervals of 6, 12, 24, 36 and 48 hours after pollination. Presence of pollen grains was checked with the help of a magnifying glass. Then the pistils were fixed in 1 formaline 1 acetic acid 8 alcohol (80%) for 24 hours. The pistils were rinsed with water and transferred to a glass tube containing water (previously boiled) and were kept in a water bath at 100 °C for 30 minutes. Then water was replaced with 4 Normal Sodium hydroxide (NaOH) for 70 minutes. Then again the pistils were rinsed with water and stained with 0.1 % analine blue (water soluble in 0.1 Normal potassium phosphate (K_3PO_4) according to the method of Adachi *et al.,* (1983). After staining the material was placed on a slide in a drop of glycerine and observed under a fluorescent microscope (Zeiss Axioskop, Germany). Five pistils per treatment were examined under the microscope. Length of pollen tube in each pistil was measured using stage and eyepiece micrometer and the mean of the pollen tube length was estimated.

2.2.6 Use of growth regulators

A high rate of pod abortion was observed in the interspecific crosses (Table 3.7 and 3.8). Different mixtures of growth regulators were applied in order to enhance pod retention and indirectly enhance the *in vivo* growth of abortive embryos. Three mixtures of growth regulator containing GA_3, NAA and Kinetin were used in this study. The composition of the mixtures is given below:

S1 = GA_3 80 mg/l, NAA 40 mg/l and Kin 4 mg/l

S2 = GA_3 100 mg/l, NAA 50 mg/l, Kin 5 mg/l

S3 = GA_3 120 mg/l, NAA 60 mg/l and Kin 6 mg/l

A drop of a mixture of growth regulators was applied immediately after pollination at the base of pedicels of cross-pollinated flowers through a scratch made by a needle (Haq, 1994). The growth mixtures were applied according to the following scheme:

a) Twice a day for 10 – 15 days after pollination (DAP)
b) Three times a day for 5 – 10 days after pollination (DAP)
c) Controls- interspecific crosses were made without the application of growth regulators.

2.2.7 Pollen viability /stainability studies

The aim of this study was to compare pollen viability of parents and hybrids. Anthers, from fully opened flowers of parental accessions, were collected and placed on a microscopic slide with a drop of 2 % aceto-carmine: glycerol (1: 1) mixture. Anthers were squashed gently to spread out the pollens evenly on the slide and studied after 24 hours or the same day by warming the slide for few seconds over a spirit lamp. Slides were studied under a microscope (Zeiss, Axioskop, Germany) at 10x objective magnification. Round or triangular solidly stained grains and empty aborted grains were counted in a field extending from edge to edge of the cover slip. The percentage of normal pollen grains was determined as pollen viability and calculated as below:

$$Pollen\ viability\ (\%) = \frac{Number\ of\ solidly\ \ stained\ \ pollen\ \ grains\ counted}{Total\ number\ of\ \ pollen\ grains\ \ counted} \times 100$$

2.3 Results

2.3.1 Somatic chromosomal studies

Results from somatic chromosome investigations of *L. mutabilis* and *L. albus* species are presented in plates 2.1 and 2.2. A wide range of *L. mutabilis* accessions were investigated for their somatic chromosome numbers (2n). All accessions studied showed 48 (2n) chromosomes (Plate 2.1). The results confirmed the earlier reports on chromosomes of *Lupinus mutabilis* (Pazy *et al.,* 1977; Plitmann & Heyn, 1984; Plitmann & Pazy, 1984; Lopez – Bellido & Fuentes, 1986 Carstairs *et al.,* 1992). The chromosomes varied in length (1 – 2 µm) and chromosome arms were not distinguishable as reported by earlier researchers (Carstairs *et al.,* 1992). Analysis of karyotype showed three types of chromosomes in terms of size: long (2 µm), medium (1.5 µm) and short (1 µm).

Investigations carried out on somatic chromosomes of *L. albus* accessions revealed variation and in most accessions the number of somatic chromosomes was counted as 2n = 48 while in two accessions the somatic chromosome number was established as 2n = 40. The length of somatic chromosomes of *L. albus* accessions ranged between 1 to 1.5

μm. Karyotype analysis revealed two types of chromosomes: medium and short. The chromosomes of *L. albus* accessions were shorter in size (1 μm) as compared to *L. mutabilis* chromosomes (Plate 2.2). The results from the present study of chromosomes have confirmed the previous reports on chromosomes of *L. albus* species (Plitmann & Pazy, 1984; Lopez – Bellido & Fuentes, 1986 and Carstairs *et al.,* 1992).

2.3.2 Morphological studies

Table 2.3 summarises the results of morphological characteristics of different accessions of *L. albus* and *L. mutabilis*. The South American species (*L. mutabilis*) differs from *L. albus* as being taller and having an erect growth habit, larger leaves, more leaflets per leaf, bigger size flowers and pods. Seeds of *L. albus* are bigger (8-14 mm) in size as compared to *L. mutabilis* (6-10 mm). The flower colour in *L. mutabilis* varies from purple, blue, pink and white, while flowers of *L. albus* are white with violet or blue tinge.

Results from correlation analysis of plant characteristics of *L. albus* and *L. mutabilis* are presented in tables 2.4 and 2.5, respectively. In *L. albus* a significant ($P < 0.05$) correlation between number of flowers per plant and 100 seed weight (0.6*), and between leaf length and number of leaflets per plant (0.66*) was observed. No significant correlation was observed between yield components and other morphological characters (Table 2.4). The results indicate that pod/seed development in *L. albus* species is somewhat independent of other characters.

In *L. mutabilis* accessions, a highly significant ($P < 0.01$) correlation between number of seeds per pod and pods per plant (0.74**), and pod length and number of seeds per pod (0.82**) was observed. A highly significant ($P < 0.01$) correlation between 100 seed weight and pedicile length (0.74**) was also observed. In *L. mutabilis* pod length was correlated significantly ($P < 0.05$) with plant height (0.59*) and with pods per plant (0.66*). This association of characters indicates that in *L. mutabilis*, pod and seed development is not an independent process but it is associated with other morphological traits of the plant.

* Denotes significance at 0.05 probablity level

** Denotes significance at 0.01 probablity level

2.3.3 Self-pollinated crosses (hand)

Table 2.6, figs 2.1 & 2.2 show the results of controlled (hand self-pollination) pollination of different accessions of *L. albus* and *L. mutabilis.* In selfed crosses involving accessions of *L. albus* (*L. albus* ♀ x *L. albus* ♂), flower abscission varied from 26.44 % (LA004) to 61.53 % (LA003), respectively. Maximum pod set (10 DAP) was recorded in self-pollinated crosses of accession LA004 (73.55 %) and among them 54.71 % reached maturity and produced seeds. The second highest pod set was recorded in the accession LA001 (71.90 %) after 10 days of pollination and among them 60.95 % reached maturity.

In self-pollinated crosses of *L. mutabilis* accessions (*L. mutabilis* ♀ x *L. mutabilis* ♂) the mean percentage of flower abscission, pod set and mature pods were recorded as 34.06 %, 65.45 % and 47.81 %, respectively. The highest percentage of pod set (85.92 %) was recorded in selfed crosses of LM003 (♀) x LM003 (♂), followed by LM001 (♀) x LM001 (♂) with 70.99 %, LM004 (♀) x LM004 (♂) with 38.46 % and LM002 (♀) x LM – 002 (♂) with lowest pod set 17.60 %.

2.3.4 Interspecific hybridisation

Interspecific crosses were made between different accessions of *L. albus* and *L. mutabilis.* The results are shown in tables 2.7, 2.8, fig 2.3, and plates 2.3 & 2.4. Because of the high rate of pod abortion, it was not possible to carry out crossing in all possible combinations.

2.3.4.1 Crosses between *L. albus* (♀) and *L. mutabilis* (♂)

Table 2.7, fig. 2.3, and plate 2.3 show the results of interspecific crosses between *L. albus* (♀) and *L. mutabilis* (♂) accessions. Four accessions of *L. albus* (LA001, LA002, LA003, and LA004) were crossed with four accessions of *L. mutabilis* in all possible combinations. A total of 1332 crosses were attempted when *L. albus* was used as female parent and *L. mutabilis* as pollen parent.

When *L. albus* accessions were used as female parents the percentage of flower abscission, pod set (10 DAP) and mature pods harvested, were 61.03 %, 36.86 %, and 0.30 %, respectively. Two mature pods were harvested from the cross LA004 (♀) x LM001 (♂) and contained one shrivelled and three deformed seeds. No normal seeds could be obtained from this cross. In the reciprocal cross of LA001 (♀) x LM003 (♂), 49.1 % of the total pollinated flowers abscised and the percentage of pod set recorded was 50.89 % after 10 days of pollination. However, most of the pods aborted at 10 to 15 days of pollination and only two (0.89 %) mature pods were harvested. The mature pods harvested contained 6 deformed seeds, which were empty and appeared to be non-viable.

When four accessions of *L. albus* (LA001 – LA004) were pollinated with pollen from LM001, the percentage of flower abscission and pod set was recorded as 60.86 % and 33.33 %, respectively. Only 0.41 % mature pods of the total crosses attempted were harvested and 38.73 % pods were aborted at 10 to 20 days after pollination. When accessions LM002 and LM004 were crossed with all four accessions of *L. albus* in all possible combinations no mature pod was achieved as most of the pods aborted within 10 to 15 days of pollination.

2.3.4.2 Crosses between *L. mutabilis* (♀) and *L. albus* (♂)

Results from crosses between *L. mutabilis* (♀) and *L. albus* (♂) are presented in table 2.8, fig 2.3 and plate 2.4. A total of 1456 crosses were attempted using *L. mutabilis* as female parent and *L. albus* as pollen parent. Using LM001 as seed parent when crossed with four male accessions of *L. albus* (LA001- LA004), the rate of flower abscission and pod set recorded was 65.66 % and 34.33 %, respectively. Only two mature pods (0.55 %) were obtained from the cross LM001 (♀) x LA001 (♂). Three seeds (two shrivelled and one deformed) were obtained. The seeds obtained were empty and appeared to be non-viable.

In interspecific crosses involving LM003 as female parent and four male accessions of *L. albus* (LA001- LA004), the rate of flower abscission and pod set was 46.9 % and 53.09 %, respectively. In the cross combination LM003 (♀) x LA001 (♂), the percentage of

pods that reached maturity was 0.43 % and contained three shrivelled and one deformed seeds.

A higher percentage of flower abscission (74.26 % and 72.25 %) was observed in interspecific crosses involving LM002 and LM004 as female parents. The rate of pod set was 25.73 % and 27.74 % and most of the set pods aborted after 10 to 15 days of pollination. No mature pod was obtained in these combinations.

2.3.5 Pollen viability in *L. albus* and *L. mutabilis* species

Results of pollen viability tests carried out on different accessions of *L. albus* and *L. mutabilis* are presented in table 2.9 and plates 2.5 & 2.6. The mean pollen viability in *L. albus* accessions was estimated as 96.54 %. The mean pollen viability in *L. mutabilis* accessions was estimated as 94.16 %.

2.3.6 Pollen tube growth

The results of pollen germination and pollen tube growth in *L. albus* and *L. mutabilis* following self and interspecific reciprocal crosses are given in table 2.10, fig 2.4, and plates 2.7 & 2.8. Pollen germination and pollen tube growth following self-pollination is given for comparison.

Pollen germination and pollen tube growth rate did not differ in self-pollinated pistils of *L. albus* and *L. mutabilis* species. Pollen tube growth rate in selfed crosses of *L. mutabilis* accessions was greater than *L. albus* accessions. A number of pollen tubes were observed in the styles after 6 hours of pollination but only one pollen tube traversed through the style and approached ovules (Plate 2.8).

Pollen of *L. mutabilis* germinated normally on the stigma of *L. albus*. Pollen of *L. albus* also germinated normally on the stigma of *L. mutabilis*. In the interspecific crosses pollen germination of *L. mutabilis* on the stigma of *L. albus* was not different from self-pollinated *L. albus*. Pollen tube penetration through stigma was normal in both interspecific crosses in both directions i.e., when *L. mutabilis* was kept as female parent

and *L. albus* as male parent and vice versa. Pollen tubes reached to respective ovaries, after 24 and 36 hours of self and interspecific pollination of *L. albus* and *L. mutabilis*, respectively. The rate of pollen tube growth was slower when *L. albus* was used as female parent than when it was used as male parent. Pollen tube growth in interspecific crosses involving *L. albus,* as female parent was similar to those of self-pollinated crosses of *L. albus* but the number of pollen tubes was less compared to selfed ones.

2.3.7 Interspecific crosses between *L. albus* and *L. mutabilis* with application of growth regulators (S1=GA_3 80 mg/l, NAA 40 mg/l and Kinetin 4 mg/l)

Two accessions of *L. albus* and *L. mutabilis* were each crossed in eight combinations. Table 2.11 and fig 2.5 show results after application of growth regulators (S1). In crosses where *L. albus* accessions were used as female parent the percentage of flower abscission varied from 42 to 64 %. The percentage of pods retained after 10 days of pollination varied between 41.42 to 60 %. The percentage of pods remained on the plants after 30 days of pollination was 2.02 % and out of them only 3 pods (1.21 %) reached maturity and produced seeds. The mature pods were obtained from the crosses of LA001 (♀) x LM003 (♂) and LA004 (♀) x LM001 (♂). These three mature pods contained 7 shrivelled and deformed seeds and were found to be empty when observed under stereo-microscope.

The percentage of abscised flowers and pods set was recorded as 44.69 % and 40.78 % respectively (Table 2.11) when the same concentration of growth regulators was applied to the crosses where *L. mutabilis* accessions were used as female parent. Two mature pods (1.11 %) were harvested from the crosses of LM003 (♀) x LA001 (♂) and LM003 (♀) x LA004 (♂). The mature pods obtained from the above crosses contained three shrivelled and one deformed seed.

2.3.8 Interspecific crosses between *L. albus* and *L. mutabilis* with application of growth regulators (S2=GA_3 100 mg/l, NAA 50 mg/l and Kinetin 5 mg/l)

Results from the reciprocal crosses of *L. mutabilis* and *L. albus* are summarised in table 2.12 and fig 2.6 show the results of the application of growth regulators (S2). When

growth regulators were applied to the interspecific crosses involving *L. albus* accessions as female parent, the rate of flower abscission and pod set recorded was 41.23 % and 47.93 %, respectively. Five pods (2.57 %) reached to maturity and contained 7 seeds (5 shrivelled and 2 deformed).

In the cross LA001 (♀) x LM003 (♂), the rate of flower abscission, pod set and number of mature pods was 38.18 %, 61.81 %, and 1.80 %, respectively. The seeds obtained from the cross were shrivelled, deformed and found to be empty when observed under the stereo-microscope. In another cross LA004 (♀) x LM003 (♂) the rate of fallen flowers and pod set was 40.81 % and 34.69 %, respectively. Only 8.16 % of pods reached maturity and produced seeds (3 shrivelled and 1 deformed).

When growth regulators were applied to crosses involving accessions of *L. mutabilis* as seed parent and accessions of *L. albus* as pollen parent, of the total crosses attempted (226) the percentage of flower abscission and pod set was 37.61 % and 44.69 %, respectively. Seven mature (3.09 %) pods were harvested from the crosses and contained 12 seeds altogether (9 shrivelled and 3 deformed).

In the cross LM001 (♀) x LA001 (♂) the percentage of fallen flowers and pod set was 36.53 % and 63.46 %, respectively after 10 DAP. In this cross 1 pod (1.92 %) reached maturity. Four mature pods (5.79 %) were harvested from the cross LM003 (♀) x LA001 (♂). The percentage of abscised flowers and pod set was recorded as 28.98 % and 56.52 %, respectively after 10 days of pollination. In the cross combination of LM003 (♀) x LA004 (♂) about 62 % of flowers abscised and percentage of pods set was 38 % after 10 DAP. The percentage of pods that reached maturity and produced shrivelled seeds in this cross was 4 %. All seeds obtained from the above crosses were either shrivelled or deformed and found to be empty when examined under microscope.

2.3.9 Interspecific crosses between *L. albus* and *L. mutabilis* with application of growth regulators (S3=GA_3 120 mg/l, NAA 60 mg/l and Kinetin 6 mg/l)

Table 2.13 and fig 2.7 show the results of the interspecific crosses of four accessions of *L. albus* and *L. mutabilis* in 16 combinations. The growth regulator mixture containing

120 mg/l GA_3 60 mg/l NAA and 6 mg/l kinetin (S3), was used for these interspecific crosses.

When *L. albus* accessions were used as female parents, 72.9 % flowers abscised and 24.32 % pods were set. Two pods (1.08 %) of the total crosses attempted (185) reached maturity. The mature pods were obtained from the cross combinations of LA001 (♀) x LM003 (♂) and LA004 (♀) x LM003 (♂). However, the pods contained shrivelled seeds and found to be empty when observed under the stereo-microscope.

The percentage of flower abscission was 57.60 % when *L. mutabilis* accessions were used as female parents. The percentage of pods retained after 10 days of pollination was 35.02 %. Five (2.26 %) pods reached maturity and produced seeds. One mature pod was obtained from the cross LM001 (♀) x LA004 (♂) and four mature pods were obtained from the cross LM003 (♀) x LA001 (♂). Seeds obtained from these crosses were shrivelled and found to be empty. The pods did not contain any fertilised ovules and were probably developed through parthenocarpic growth as a result of growth regulator applications.

2.4 Discussion

Studies on the interspecific hybrids of South American (*L. mutabilis*) and European lupin species (*L. albus*, *L. angustifolius* and *L. luteus*) were started in the first half of the last century and are still continuing (Williams *et al.* 1980; Faluyi & Williams 1981; Busmann Loock *et al.,* 1992; Siamasonta, 1996 and Przyborowski & Packa, 1997). These studies have concluded that incompatibility barriers operate at post-fertilisation level, which cause the failure of interspecific crosses between *L. albus* and *L. mutabilis* species.

It is apparent from the literature review that an important factor, such as the use of genotypes with equal number of chromosomes, was not taken into consideration in earlier attempts to produce interspecific hybrids between *L. albus* and *L. mutabilis*. In this study several parameters were used to select the genotypes of *L. albus* and *L. mutabilis* species for interspecific hybridisation. These parameters included, the crossing behaviour of genotypes, important morphological characteristics such as number of

flowers per plant, pods per plant, number of seeds per pod, and equal number of somatic chromosomes.

The information on the degree of association (correlation) between morphological characters was found to be useful in selecting the genotypes with desirable characters such as higher number of pods, seeds per pod that might consequently increase the chances of obtaining a hybrid of *L. albus* and *L. mutabilis* species. In addition, the study of morphological characteristics of species is also useful for comparison between the species and hybrids.

In *L. albus*, no significant association of plant characters such as plant height, pods per plant, and number of seeds per pod was observed in the accessions studied. However, in *L. mutabilis* a strong association of characters was observed in all accessions studied. For example plant height in *L. mutabilis* is negatively correlated with number of branches per plant. This indicates that by selecting short stature genotypes of *L. mutabilis* species we can change the growth habit of the species from indeterminate to determinate type. Similarly number of pods per plant in *L. mutabilis* is positively correlated with number of seeds per pod, which indicates that by selecting genotypes with higher number of pods per plant indirectly increases the number of seeds per pod and subsequently probability of achieving a hybrid seed is higher.

In self-pollinated crosses a high level of flower abscission was observed in *L. albus* as well as in *L. mutabilis* species. This may be due to several reasons such as the mechanical damage made in the course of emasculation and pollination as the *Lupinus* species have smaller and more delicate flowers than crop species. In addition the styles/stigmas are within the closed buds and covered up by petals at anthesis in lupins. Therefore, when buds are opened for hand-pollination there is a risk of the stigma drying out when exposed to the air and crosses failing. Furthermore, in the case of *L. albus* it was noticed that this species was more sensitive to high temperature as compared to *L. mutabilis*. High temperature (above 30° C) during hot summer days would also cause a higher percentage of flower abscission and lower seed set in both self-pollinated (either natural or by hand) flowers as well as interspecific crosses. Sensitivity of reproductive

processes to high temperature has also been observed in *Phaseolus* (Gross & Kigel, 1994).

In hand self-pollinated crosses of *L. albus* and *L. mutabilis* genotypes, genetic differences within the species were observed. The crossing behaviour of genotypes used from both species was different from each other in terms of flower abscission, pod set, number of mature pods harvested, and number of seeds per pod (Table 2.6 and Figs. 2.1 & 2.2). In general, the percentage of mature pods harvested was higher in *L. albus* (50 %) than *L. mutabilis* (48 %). The number of seeds set in selfed (both natural and hand self-pollinated) was very low which indicates the usual trend of lupins in seed setting (Vuillaume & Hoff, 1986; Atkins *et al*, 1998). In most of the selfed pods of *L. mutabilis* species only one developed seed per pod was observed while in selfed pods of *L. albus* contained two to three seeds per pod. This may be due to earlier domestication of *L. albus* and selection of genotypes over the years.

The presented results of interspecific crosses using genotypes of *L. albus* and *L. mutabilis* with equal number of chromosomes (2n=48) suggest that the number of chromosomes play little role in the success or failure of interspecific crosses of these species. Similar results were also obtained by Roy and Gladstones (1988). They were successful in producing partly fertile hybrids when they crossed *L. atlanticus* (2n = 38), *L. pilosus* (2n = 42) and *L. cosentini* (2n = 32) with each other.

Genotypic differences were also observed in reciprocal interspecific crosses of *L. albus* and *L. mutabilis* species. In reciprocal cross combinations there was not large difference in the percentage of pod set however, the percentage of pod set was higher (40.7 %) in the crosses where *L. mutabilis* genotypes were used as female parents. A lower parentage of pod set (36.86 %) and higher number of flower abscission (61.08 %) was seen when *L. albus* genotypes were used as female parent. This crossing behaviour of *L. mutabilis* may be due to plasmon/genotype interaction.

Cross-incompatibility may be due to the lack of pollen grain germination on the stigma, with inhibition of pollen tube growth on the stigma or with its gradual reduction, leading to cessation of growth at the pistil base (Przyborowski *et al*, 1996). Pollen grain viability

of accessions used for interspecific crosses between *L. albus* and *L. mutabilis* was estimated as 96.58 % and 94.16 %, respectively. The presented results of pollen germination and pollen tube growth investigations indicate that both species are cross compatible at pollen germination and pollen tube growth level. Pollen tube reached the ovary in both self and interspecific crosses of *L. albus* and *L. mutabilis*. However, a slower pollen tube growth was observed in reciprocal interspecific crosses of *L. albus* and *L. mutabilis* as compared to self-pollinated ones (table 2.10). Williams *et al.,* (1980) and Siamasonta (1996) who evaluated cross-compatibility among European and American lupin species reported similar results. In the present work although pollen tube growth was observed in the ovary of interspecific crosses it was not possible to determine whether fertilisation had actually occurred or not. However, further comparative studies on pod and ovule development in both selfed and interspecific crosses of *L. albus* and *L. mutabilis* species were carried out to determine the process of fertilisation (chapter 3).

Mature pods were harvested from reciprocal crosses of *L. albus* and *L. mutabilis*. In the cross combinations of *L. albus* (♀) and *L. mutabilis* (♂) the number of mature pods obtained was low as compared to the cross where *L. mutabilis* was used as female parent. Most of the seeds obtained from these crosses were either shrivelled or deformed and found to be empty when observed under the stereo-microscope. Production of shrivelled or inviable deformed seeds suggests that hybrid breakdown has occurred because of embryo abortion. This embryo abortion could be due to endosperm degeneration. This will result in embryo starvation since the endosperm is the main nourishing source for the young embryos. Disharmonies between developing embryo and its supporting tissues also act as an isolating barrier to gene exchange between two species.

It appeared from the results that hybrid embryo breakdown in interspecific crosses of *L. albus* and *L. mutabilis* species is mainly due to abortion of the embryo at an early developmental stage. This is evident from the fact that in most of the crosses, pods abscised during the first ten days after pollination (early stage) presumably as a result of embryo abortion due to hybrid lethality. Even late aborted pods did not survive more than two weeks after pollination. Furthermore, most of the mature pods contained shrivelled seeds, which indicated that the embryo had aborted at an early developmental stage (Table 2.7 and 2.8). There may be several reasons for the embryo abortion ranging

from deleterious effect of one or two genes to abnormalities between the embryo and its supporting tissues such as suspensor, endosperm, or the surrounding maternal (integumentary and nucellus) tissues leading to embryo starvation (Hadley & Openshaw, 1980; Brar & Khush, 1986).

It is considered that seeds are sites of hormone synthesis and/or accumulation and that these substances play a role in successful pod and seed retention and growth (Mosjidis *et al.,* 1993). In legumes, cytokinins, gibberellins, and auxins have been implicated in the attraction of nutrients to the pods since they create active sinks within plants (Atkins & Pigeaire, 1993). It has been shown in *L. albus* (Davey & Van Staden, 1978c) that a loss in the sink strength occurs within pods through conversion of cytokinins to glucosides. At this time the pod apparently reverses its role and an import-export transition takes place. According to Hocking & Pate (1977) the embryos take up most of the nutrient exported from the pod. When growth regulators are applied in interspecific crosses, they may first act as pod growth promoters to compensate for the restricted production or accumulation of hormones by the seed as a result of alien pollination. If hormones are available until the stage that pods are able to export nutrients to the embryos, then further embryo development may be maintained and mature seeds may be obtained.

In the present study the results of interspecific crosses made with the application of growth regulators revealed that in all cross combinations the degree of flower abscission decreased after growth regulator application as compared to the results from crosses attempted without application of growth regulators. The role of growth regulators in delaying flower abscission has also been reported in interspecific hybridisation of the genus *Passiflora* and *Trifolium* (Payan & Martin, 1975; Pandey *et al.,* 1987). Rashid (1988) and Tahiri (2000) have mentioned that the use of growth regulators helped pod retention in *Vigna* and *Cicer* interspecific crosses. Growth regulators had a positive effect on pod retention but only when applied at appropriate concentrations. In this work when the concentration of growth regulators was increased, the number of pods retained on the plants was reduced (Table 2.10 - 2.12). Despite the above explanations, seed formation appears to be independent of pod formation in relation to growth regulator application. Since growth regulator treatment induced parthenocarpic pod formation in a few cases (in the first two treatments, low concentrations). On the other hand, many of the mature

pods harvested contained shrivelled seeds, which had aborted at an early stage of development. Rashid *et al.*, (1988) and Taheri (2000) reported similar observations.

In this investigation, some of the accessions used in the interspecific crosses seem more promising for hybrid seed production than others. These accessions were: LA001, LA004 from *L. albus* and LM001 and LM003 from *L. mutabilis*. In this work *L. mutabilis* accessions (LM001 and LM003) were found to be the most effective seed parent when crossed with *L. albus* accessions. In the reciprocal interspecific crosses of the two species involving *L. albus* as seed parent, a higher flower abscission, lower pod set, and production of deformed seeds was noticed. This suggests a cytoplasmic control of *L. mutabilis* accessions, which may cause disharmonious interaction between parental genomes or between the genome and cytoplasm resulting in endosperm degeneration. Similar observations were reported by Williams *et al.*, (1980). From the results of interspecific crosses, it is evident that the number of somatic chromosomes had a lesser role in the success or failure of interspecific crosses. Selection of more compatible male accessions of *L. albus* may help in production of viable and fertile hybrid plants. Rashid *et al.*, (1989) and Taheri (2000) have also mentioned the use of a wide range of parental genotypes in order to gain successful interspecific hybrids of *Vigna* and *Cicer* species respectively.

Table 2.1: Useful traits existing in *L. albus* and *L. mutabilis* species.

Characteristics	*L. albus*	*L. mutabilis*	Reference
Growth habit	Dwarf, Determinate	Tall, Indeterminate	Haq, 1993
Protein (%)	36.10	44.74	Petterson, 1998
Oil (%)	14.5	23.1	Petterson, 1998
Alkaloids (%)	0.005 to 0.367	0.007 to 3.970	Haq, 1993
Seed yield (Kg/ha)	500 to 1500	300 to 2000	Gladstones, 1998
Maturity	Late maturing	Early maturing	Sawicka, 1998
Tolerance: 1. Lime and high pH 2. Drought 3. Frost	 Tolerant Susceptible Resistant	 Tolerant Tolerant Tolerant	Haq, 1993
Diseases: 1. Anthracnose (*Collettrichum gloeosporioides*) 2. Brown leaf spot (*Pleiochaeta setosa*) 3. Root rot (*Fusarium, Pythium*)	 XX X XXX	 XXX - X	Lopez-Bellido and Fuentes, 1986 Sawicka, 1998

XXX - High susceptibility; XX – Medium susceptibility; X – Low susceptibility; - No information

Table 2.2: Accessions of *L. albus* and *L. mutabilis* species used in interspecific crosses.

Species	Accession No.	Identity No.	Origin	Donor
Lupinus albus	LA001	*Lupinus albus* var. 1	Mediterranean	FAO
	LA002	*Lupinus albus* var. 3	Mediterranean	BARI (PK.)
	LA003	*Lupinus albus* var. 10	Mediterranean	BARI (PK.)
	LA004	*Lupinus albus* cv. watt	Mediterranean	Poland
Lupinus mutabilis	LM001	*Lupinus mutabilis*	South America	FAO
		Cogeshall selection		
		1990		
	LM002	*Lupinus mutabilis*	South America	FAO
		var. 8		
	LM003	*Lupinus mutabilis*	South America	BARI (PK.)
		var. 11		
	LM004	*Lupinus mutabilis*	South America	BARI (PK.)
		var.15		

BARI = Barani Agricultural Research Institute Chakwal, Pakistan.
FAO = Food and Agricultural Organisation of United Nations Rome (Italy).
LM = *Lupinus mutabilis*
LA = *Lupinus albus*

Table 2.3: Comparison of morphological and yield related characters of *L. albus* and *L. mutabilis*.

	Lupinus albus						***Lupinus mutabilis***					
Characters	R1*	R2*	R3*	Total	Mean	SD	R1*	R2*	R3*	Total	Mean	SD
Plant height (cm)	42.50	62	70	174.5	58.16	14.14	118.6	120.90	116.50	356	118.66	2.20
No of branches/plant	4.8	5.00	4.00	13.8	4.6	0.53	4.4	4.8	4.3	13.5	4.5	0.26
No flowers per plant	60	59	65	184	61.33	3.21	238	239	245	722	240.66	3.8
No of pods/plant	20	19	21	60	20	1.00	89	91	75	255	85	8.71
Leaf length (cm)	9.63	10.21	8.76	28.6	9.53	0.73	11.37	11.86	11.65	34.88	11.62	0.24
No of leaflets/leaf	6.9	7.00	5.90	19.8	6.6	0.60	8.8	8.6	8.4	25.80	8.6	0.28
Leaflet length (cm)	4.02	3.89	4.00	11.91	3.90	0.01	4.39	4.38	4.36	13.13	4.37	0.1
Leaflet width (cm)	1.40	1.29	1.52	4.21	1.40	0.01	1.24	1.68	1.19	4.11	1.37	0.26
Pedicile length (cm)	0.35	0.40	0.29	1.04	0.34	0.05	0.63	0.68	0.69	1.98	0.66	0.14
Pod length (cm)	9.16	10.10	8.29	27.55	9.18	0.90	7.92	7.85	7.97	23.74	7.91	0.30
Pod width (cm)	2.00	1.90	2.10	6.00	2.00	0.10	1.8	2.1	1.9	5.8	1.93	0.15
No of seeds/pod	5.5	5.3	5.6	16.40	5.46	0.15	4.5	4.3	4.6	13.4	4.46	0.15
100-Seed weight (g)	22.89	21.60	23.10	67.59	22.53	0.81	16.87	18.90	20.37	56.14	18.71	1.75
Seed colour	White tinged with violet or blue dull light yellow						Brownish black white or white with black or grey hilum					

R1= Replication 1
R2= Replication 2
R3= Replication 3
* Average of five observations
SD = Standard deviation

Table 2.4: Correlation coefficients between different plant characters of *L. albus*.

Plant character	Plant height	Branches/ plant	Flowers/ plant	Pods/ plant	Leaf length	Leaflets/ Leaf	Pedicile length	Pod length	Seeds/ pod	100-seed weight
Plant height		0.118	0.549	0.018	-0.268	-0.082	-0.133	0.187	0.255	0.426
Branches/plant			-0.83	0.248	0.181	0.183	-0.139	0.276	-0.147	0.065
Flowers/plant				0.25	-0.235	-0.01	-0.094	0.127	0.574	0.609*
Pods/plant					0.033	0.065	-0.516	0.516	0.128	-0.043
Leaf length						0.663*	-0.082	0.35	0.173	-0.54
Leaflets/leaf							-0.162	0.465	-0.115	-0.146
Pedicile length								-0.559	-0.197	0.232
Pod length									0.119	0.009
Seeds/pod										-0.027
100-seed weight										

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

Table 2.5: Correlation coefficients between different plant characters of *L. mutabilis*.

Plant character	Plant height	Branches/ plant	Flowers/ plant	Pods/ plant	Leaf length	Leaflets/ leaf	Pedicile length	Pod length	Seeds/ pod	100-seed weight
Plant height		-0.383	0.265	0.176	0.505	a	0.555	0.599*	0.368	0.492
Branches/plant			-0.264	0.371	-0.086	a	-0.11	0.282	0.495	-0.1
Flowers/plant				0.201	0.02	a	0.55	0.011	0.168	0.46
Pods/plant					0.021	a	0.248	0.665*	0.745**	0.152
Leaf length						a	0.039	0.322	0.298	-0.173
Leaflets/leaf							a	a	a	a
Pedicile length								0.234	0.203	0.747**
Pod length									0.824**	0.21
Seeds/pod										0.237
100-seed weight										

* Correlation is significant at the 0.05 level

** Correlation is significant at the 0.01 level

[a] Correlation could not be calculated as number of leaflets per leaf were constant

Table 2.6: Results of hand self-pollinated crosses of *L. albus* and *L. mutabilis* accessions.

Parents		Crosses	Fallen flowers		Pods retained after days of pollination						Mature pods		Seeds formed					
					10 days		20 days		30 days				Shrivelled		Deformed		Normal	
Female	Male	No	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
LA001	LA001	210.00	59.00	28.09	151.00	71.90	132.00	62.85	130.00	61.00	128.00	60.95	160.00	36.44	23.00	5.23	256.00	58.31
LA002	LA002	185.00	79.00	42.70	106.00	57.29	98.00	52.97	74.00	40.00	72.00	38.91	98.00	45.37	12.00	5.55	106.00	49.07
LA003	LA003	52.00	32.00	61.53	20.00	38.46	10.00	19.23	8.00	15.38	8.00	15.38	6.00	37.50	1.00	6.25	9.00	56.25
LA004	LA004	329.00	87.00	26.44	242.00	73.55	209.00	63.52	196.00	59.57	180.00	54.71	159.00	25.72	5.00	0.80	454.00	73.46
Total		**776.00**	**257.00**		**519.00**		**449.00**		**408.00**		**388.00**		**423.00**		**41.00**		**825.00**	
Mean %				**33.11**		**66.89**		**57.86**		**52.57**		**50.00**		**32.81**		**3.18**		**64.00**
LM001	LM001	131.00	34.00	25.95	93.00	70.99	78.00	59.54	77.00	58.77	70.00	53.43	94.00	38.52	19.00	7.78	131.00	53.68
LM002	LM002	46.00	38.00	82.60	8.00	17.60	6.00	13.00	5.00	10.86	5.00	10.86	3.00	30.00	2.00	20.00	5.00	50.00
LM003	LM003	398.00	56.00	14.07	342.00	85.92	261.00	65.57	252.00	63.31	249.00	62.56	102.00	20.48	25.00	5.02	371.00	74.49
LM004	LM004	247.00	152.00	61.53	95.00	38.46	83.00	33.60	71.00	28.74	69.00	27.93	51.00	36.95	14.00	10.14	73.00	52.89
Total		**822.00**	**280.00**		**538.00**		**248.00**		**405.00**		**393.00**		**250.00**		**60.00**		**580.00**	
Mean %				**34.06**		**65.45**		**30.17**		**49.27**		**47.81**		**30.41**		**7.30**		**70.56**

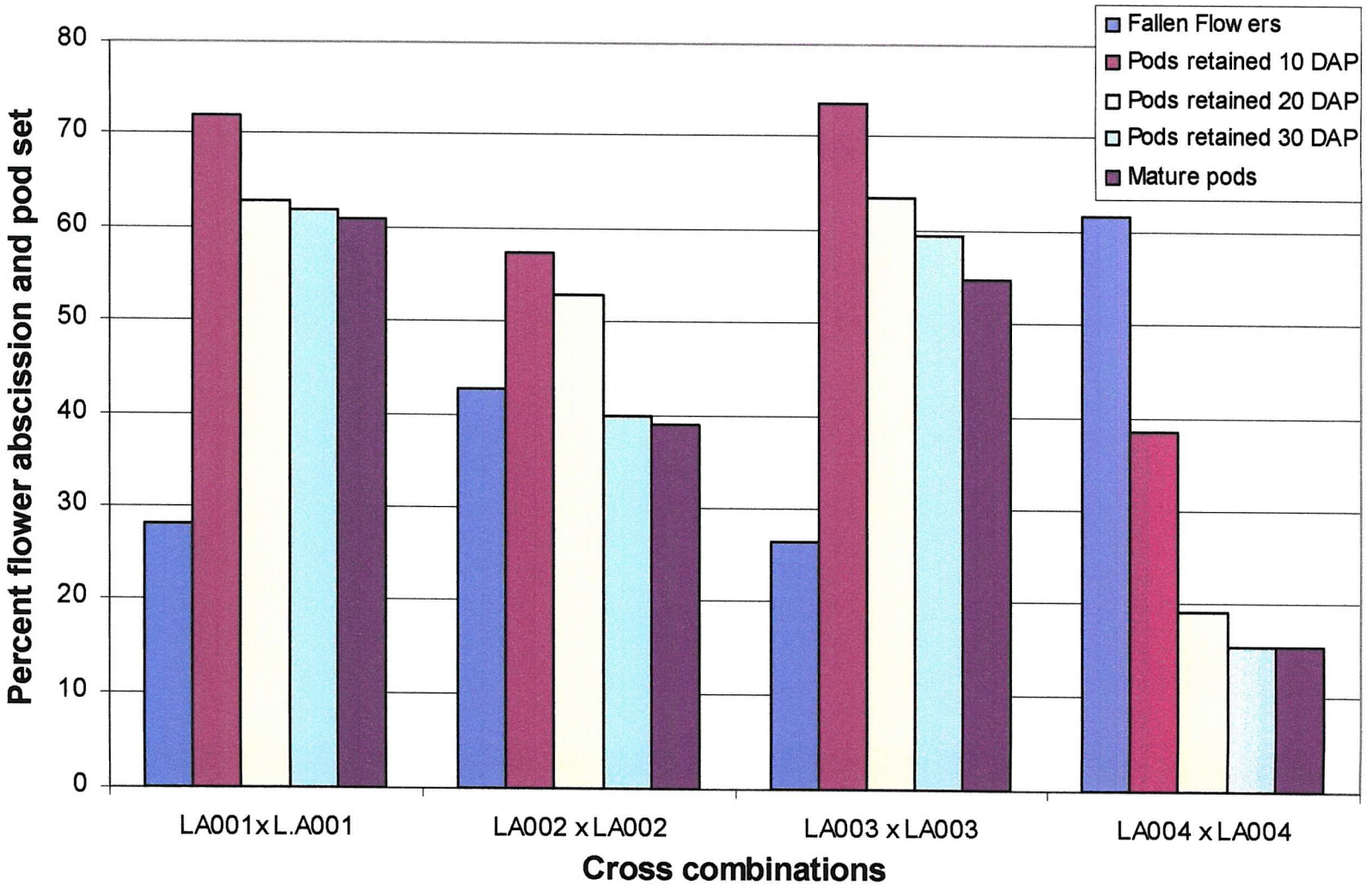


Fig 2.1: Results of self-pollinated (hand) crosses of *L. albus* accessions

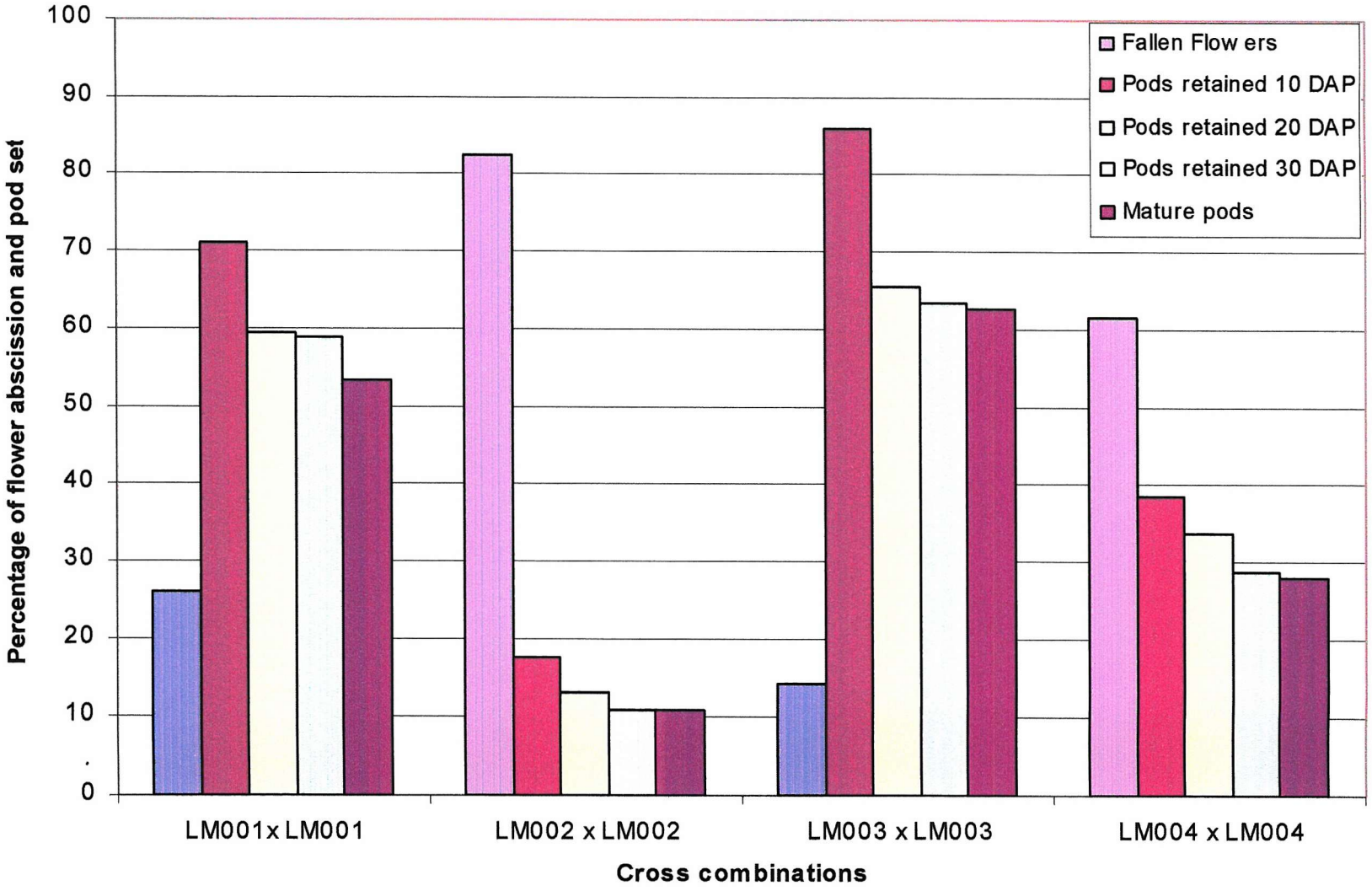


Fig 2.2: Results of self-pollinated (hand) crosses of *L. mutabilis* accessions.

Table 2.7: Results of interspecific crosses of *L. albus* (♀) and *L. mutabilis* (♂) without application of growth regulators

Parents		Crosses	Fallen flowers		Pods retained after days of pollination															
					10 days		15 days		20 days		30 days		Mature pods		Seeds formed					
Female	Male														Shrivelled		Deformed		Normal	
		No	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
L. albus	*L. mutabilis*																			
LA001	LM001	89	65	73.03	24	26.96	1	1.12	0	0	0	0	0	0	0	0	0	0	0	0
LA002	LM001	53	35	66.03	18	33.96	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA003	LM001	20	14	70	6	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA004	LM001	321	180	56.07	113	35.2	12	3.73	5	1.55	2	0.62	2	0.62	1	25	3	75	0	0
Mean %				**60.86**		**33.33**		**2.69**		**1.03**		**0.41**		**0.41**		**25**		**75**		**0**
L. albus	*L. mutabilis*																			
LA001	LM002	29	15	51.72	14	48.27	2	6.89	0	0	0	0	0	0	0	0	0	0	0	0
LA002	LM002	36	25	69.44	11	30.55	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA003	LM002	58	33	56.89	25	43.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA004	LM002	46	26	56.52	20	43.47	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean %				**58.57**		**41.42**		**1.18**		**0**		**0**		**0**		**0**		**0**		**0**
L. albus	*L. mutabilis*																			
LA001	LM003	224	110	49.1	114	50.89	48	21.42	7	3.12	4	1.78	2	0.89	0	0	6	100	0	0
LA002	LM003	25	17	68	8	32	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA003	LM003	119	117	98.31	2	1.68	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA004	LM003	79	51	64.55	28	35.44	5	6.32	0	0	0	0	0	0	0	0	0	0	0	0
Mean %				**65.99**		**34**		**1.85**		**1.56**		**0.89**		**0.44**	**0**	**0**		**100**		**0**
L. albus	*L. mutabilis*																			
LA001	LM004	74	32	43.24	42	56.75	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LA002	LM004	69	45	65.21	24	34.78	1	1.44	0	0	0	0	0	0	0	0	0	0	0	0
LA003	LM004	68	34	50	34	50	3	4.41	1	1.47	0	0	0	0	0	0	0	0	0	0
LA004	LM004	22	14	63.33	8	36.36	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean %				**53.64**		**46.35**		**1.71**		**0.42**		**0**		**0**		**0**		**0**		**0**

Table 2.8: Results of interspecific crosses of *L. mutabilis* (♀) and *L. albus* (♂) without application of growth regulators

		Crosses	Fallen flowers		Pods retained after days of pollination															
Parents					10 days		15 days		20 days		30 days		Mature pods		Seeds formed					
Female	Male														Shrivelled		Deformed		Normal	
		No	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
L. mutablis	*L. albus*																			
LM001	LA001	362	259	71.54	103	28.45	15	4.14	5	1.38	2	0.55	2	0.55	2	66.6	1	33.3	0	0
LM001	LA002	32	28	87.5	4	12.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM001	LA003	115	47	40.86	68	59.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM001	LA004	24	16	66.6	8	33.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		**533**	**350**	**65.66**	**183**	**34.33**	**15**	**2.81**	**5**	**0.93**	**2**	**0.37**	**2**	**0.37**	**2**	**66.6**	**1**	**33.3**	**0**	**0**
L. mutabilis	*L. albus*																			
LM002	LA001	39	31	79.48	8	20.51	3	7.69	0	0	0	0	0	0	0	0	0	0	0	0
LM002	LA002	48	37	77.08	11	22.91	1	2.08	0	0	0	0	0	0	0	0	0	0	0	0
LM002	LA003	30	19	63.33	11	36.66	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM002	LA004	19	14	84.21	5	26.31	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		**136**	**101**	**74.26**	**35**	**25.73**	**4**	**2.94**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
L. mutabilis	*L. albus*																			
LM003	LA001	466	185	39.69	281	60.3	21	4.5	7	1.5	2	0.43	2	0.43	3	75	1	25	0	0
LM003	LA002	24	13	54.16	11	45.83	1	4.16	0	0	0	0	0	0	0	0	0	0	0	0
LM003	LA003	75	60	80	15	20	7	9.33	3	4	0	0	0	0	0	0	0	0	0	0
LM003	LA004	49	30	61.22	19	38.77	1	2.04	0	0	0	0	0	0	0	0	0	0	0	0
Total		**614**	**288**	**46.9**	**326**	**53.09**	**30**	**4.88**	**10**	**1.62**	**2**	**0.32**	**2**	**0.32**	**3**	**75**	**1**	**25**	**0**	**0**
L.mutabilis	*L. albus*																			
LM004	LA001	55	48	87.27	7	12.72	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM004	LA002	38	26	68.42	12	31.57	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM004	LA003	64	39	60.93	25	39.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LM004	LA004	16	12	75	4	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total		**173**	**125**	**72.25**	**48**	**27.74**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Grand total		***1456***	***864***	***59.34***	***592***	***40.7***	***49***	***3.4***	***15***	***1.03***	***4***	***0.27***	***4***	***0.27***	***5***	***71.4***	***2***	***28.57***	***0***	***0***

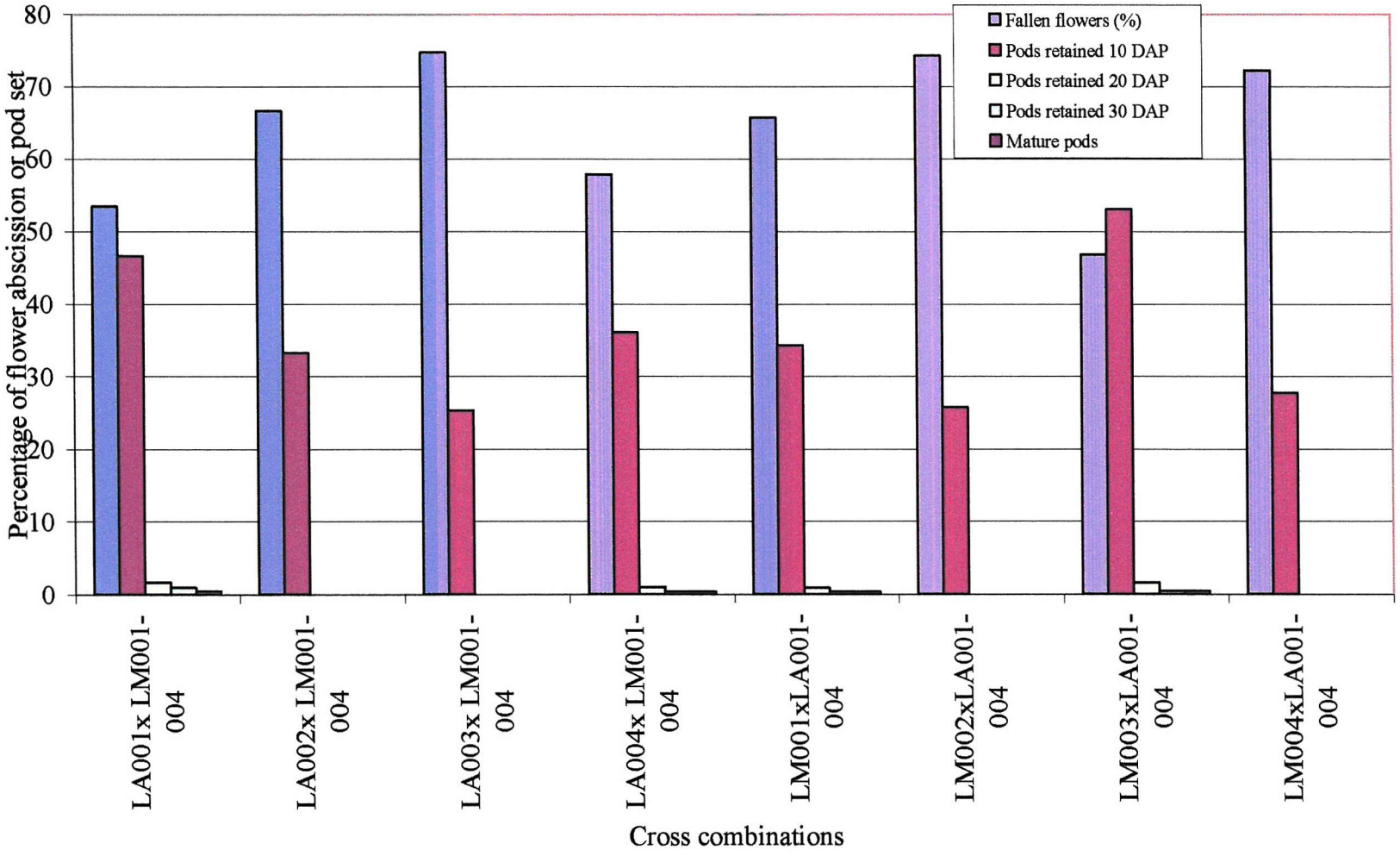


Fig 2.3: Results of interspecific crosses without application of growth regulators.

Table 2.9: Results of pollen viability in *L. albus* and *L. mutabilis* estimated by aceto carmine test

Species	Rep.	Viability		
L. albus		*No. of Pollen grains	Viable (No)	% age
	1	143	140	97.90
	2	257	253	98.44
	3	165	154	93.33
L. mutabilis				
	1	213	208	97.65
	2	382	372	97.38
	3	471	424	87.47

Rep. = Replication
* Total of five observations

Table 2.10: Mean pollen tube growth in self and interspecific crosses of *L. albus* and *L. mutabilis*

Combination	Penetration of pollen tubes (mm.) along the style				
	6 hours	12 hours	24 hours	36 hours	48 hours
L. albus (♀) x *L. albus* (♂)	0.6	3.53	5.92	6.37	7.74
L. mutabilis (♀) x *L. mutabilis* (♂)	0.59	4.73	7.43	8.23	8.83
L. albus (♀) x *L. mutabilis* (♂)	0.53	3.1	6.07	6.88	7.76
L. mutabilis (♀) x *L. albus* (♂)	0.52	4.48	6.95	7.74	8.18

* Average of five observations for each time point.

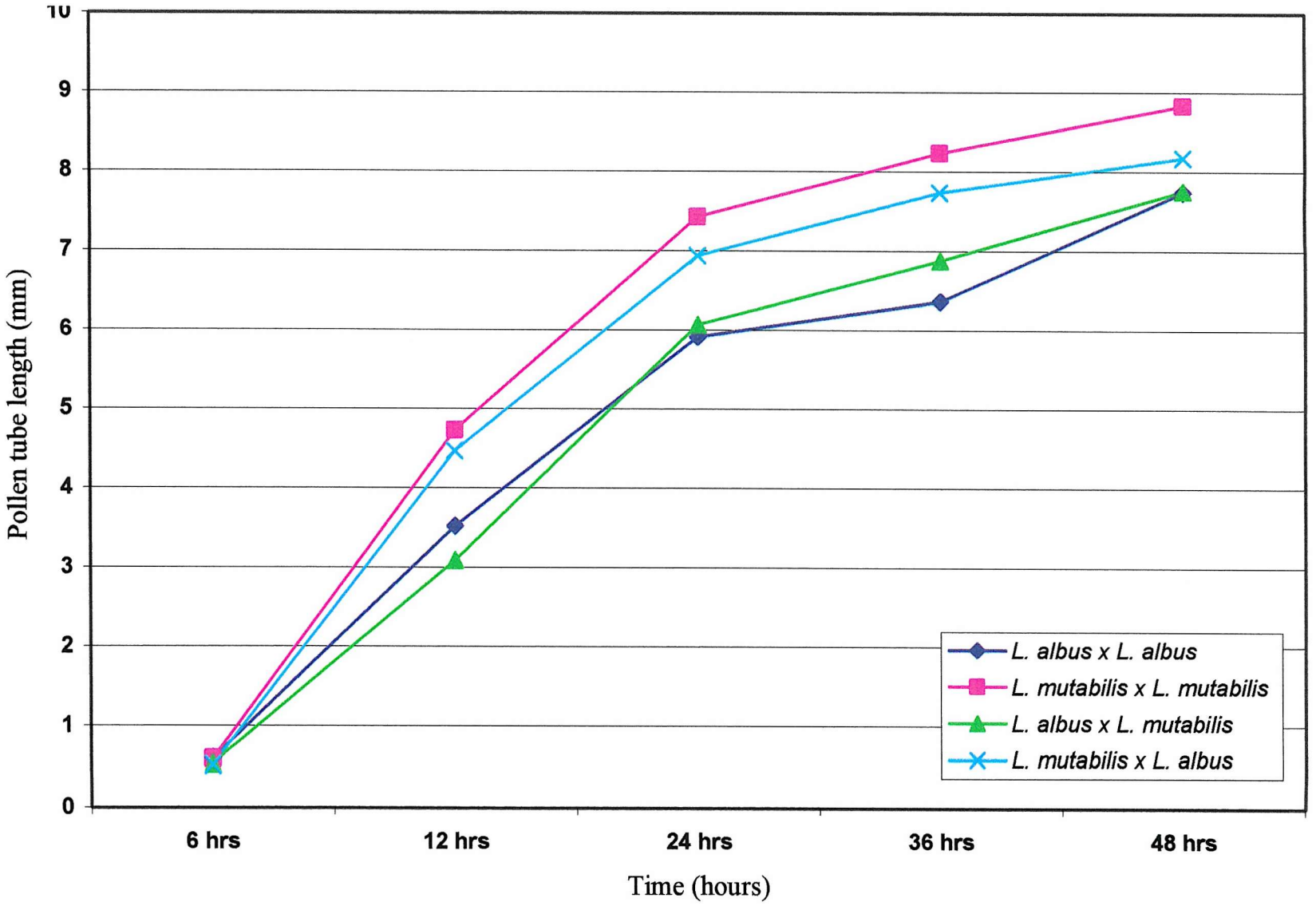


Fig. 2.4: Results of pollen tube growth in self and interspecific crosses of *L. albus* and *L. mutabilis* species.

Table 2.11: Results of interspecific crosses with application of growth regulators (S1 = Solution 1 = GA3 80 mg/l, NAA 40 mg/l, Kin 4 mg/l)

	Crosses		Fallen flowers		Pods retained after days of pollination													
Parents					10 days		20 days		30 days		Mature pods		Seeds formed					
Female	Male												Shrivelled		Deformed		Normal	
		No	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
L. albus	***L. mutabilis***																	
LA - 001	LM - 001	68.00	29.00	42.64	38.00	55.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LA - 001	LM - 003	70.00	30.00	42.85	29.00	41.42	3.00	4.28	2.00	2.85	1.00	1.42	2.00	66.6	1.00	33.3	0.00	0.00
LA - 004	LM - 001	59.00	19.00	32.20	31.00	52.54	4.00	6.77	3.00	5.08	2.00	3.38	3.00	75	1.00	25	0.00	0.00
LA - 004	LM - 003	50.00	32.00	64.00	30.00	60.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total		**247.00**	**110.00**		**158.00**		**7.00**		**5.00**		**3.00**		**5.00**		**2.00**		**0.00**	
Mean %				**44.53**		**63.96**		**2.83**		**2.02**		**1.21**		**71.4**		**28.6**		**00**
L. mutabilis	***L. albus***																	
LM001	LA001	36.00	23.00	63.33	13.00	36.11	2.00	5.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LM001	LA004	40.00	14.00	35.00	5.00	12.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LM003	LA001	60.00	24.00	40.00	31.00	51.66	4.00	6.66	1.00	1.66	1.00	1.66	2.00	100	0.00	0.00	0.00	0.00
LM003	LA004	43.00	19.00	44.18	24.00	55.81	1.00	2.32	1.00	2.32	1.00	2.32	1.00	50	1.00	50	0.00	0.00
Total		**179.00**	**80.00**		**73.00**		**7.00**		**2.00**		**2.00**		**3.00**		**1.00**		**0.00**	
Mean %				**44.69**		**40.78**		**3.91**		**1.12**		**1.12**		**75**		**25**		**0.00**

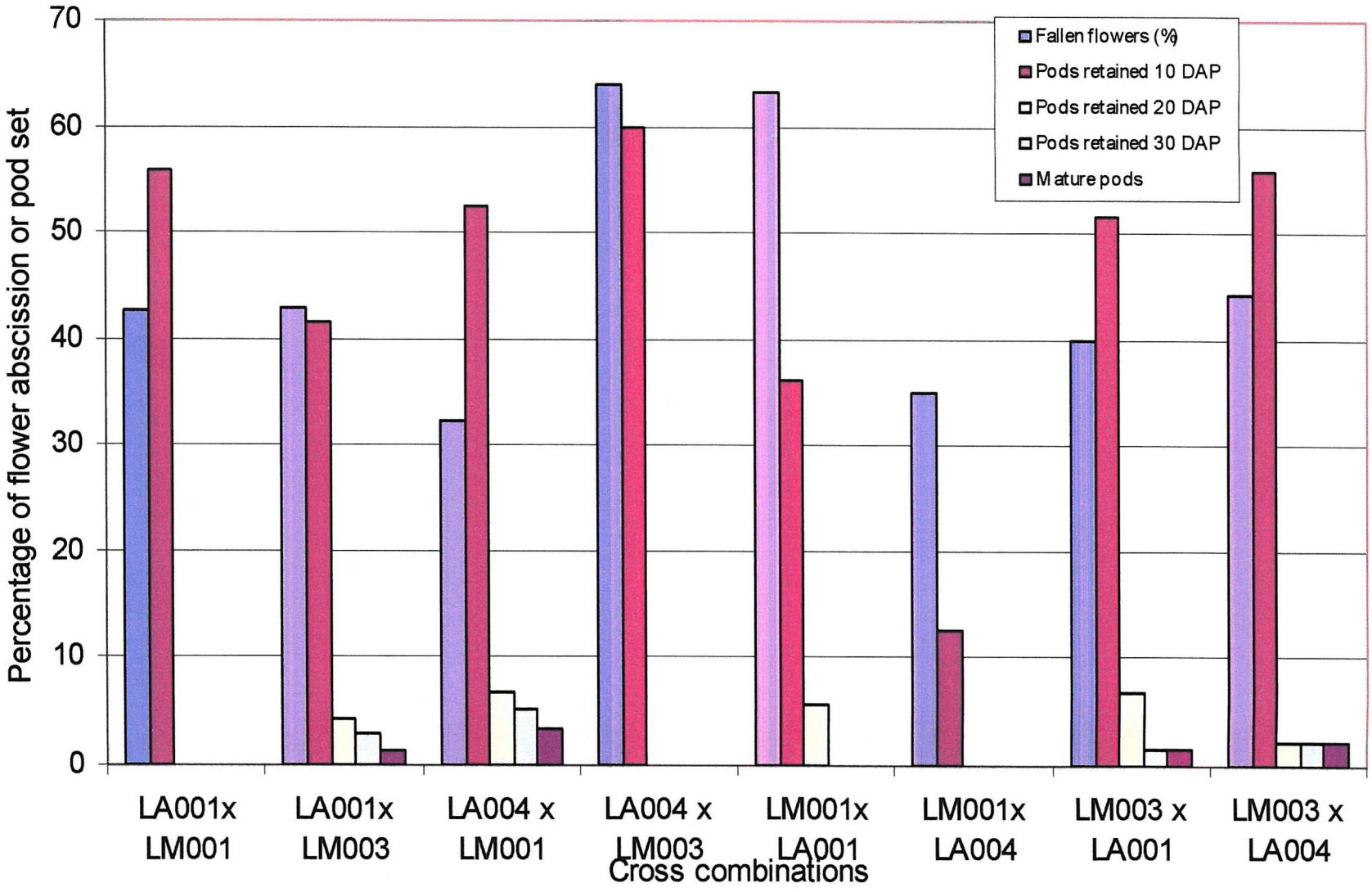


Fig 2.5: Results of interspecific crosses between *L. albus* and *L. mutabilis* with application of growth regulators (S1)

Table 2.12: Results from interspecific crosses with application of growth regulators (S2 = Solution 2 = GA3 100 mg/l, NAA 50 mg/l, Kin 5 mg/l)

		Crosses	Fallen flowers		Pods retained after days of pollination													
Parents					10 days		20 days		30 days		Mature pods		Seeds formed					
Female	Male												Shrivelled		Deformed		Normal	
		No	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
L. albus	***L. mutabilis***																	
LA001	LM001	54.00	22.00	40.74	15.00	27.77	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LA001	LM003	55.00	21.00	38.18	34.00	61.81	6.00	10.90	1.00	1.80	1.00	1.80	2.00	66.6	1.00	33.3	0.00	0.00
LA004	LM001	36.00	19.00	52.77	17.00	47.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LA004	LM003	49.00	20.00	40.81	17.00	34.69	7.00	14.28	4.00	8.16	4.00	8.16	3.00	75	1.00	25	0.00	0.00
Total		**194.00**	**80.00**		**93.00**		**13.00**		**5.00**		**5.00**		**5.00**		**2.00**		**0.00**	
Mean %				**41.23**		**47.93**		**6.70**		**2.57**		**2.57**		**71.4**		**28.6**		**0.00**
L. mutabilis	***L. albus***																	
LM001	LA001	52.00	19.00	36.53	33.00	63.46	2.00	3.84	1.00	1.92	1.00	1.92	2.00		0.00	0.00	0.00	0.00
LM001	LA004	55.00	15.00	27.27	10.00	18.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LM003	LA001	69.00	20.00	28.98	39.00	56.52	11.00	15.94	4.00	5.79	4.00	5.79	5.00	71.4	2.00	28.57	0.00	0.00
LM003	LA004	50.00	31.00	62.00	19.00	38.00	5.00	10.00	2.00	4.00	2.00	4.00	2.00	66.6	1.00	33.3	0.00	0.00
Total		**226.00**	**85.00**		**101.00**		**18.00**		**7.00**		**7.00**		**9.00**		**3.00**		**0.00**	
Mean %				**37.61**		**44.69**		**7.96**		**3.08**		**3.08**		**75**		**25**		**0.00**

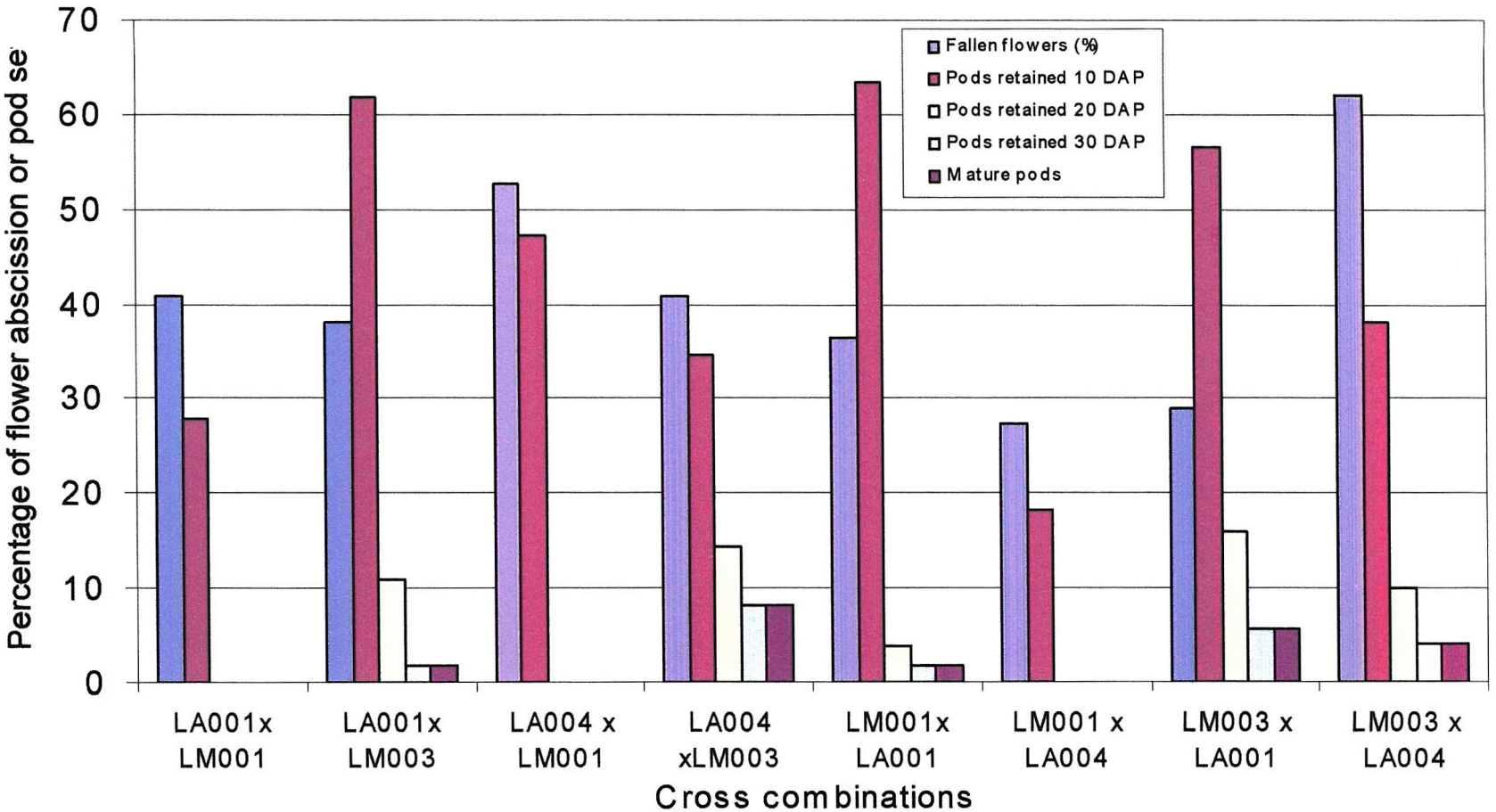


Fig 2.6: Results of interspecific crosses between *L. albus* and *L. mutabilis* species with application of growth regulators (S2)

Table 2.13: Results from Interspecific crosses with application of growth regulators (S3 = Solution 3 = (GA3 120 mg/l, NAA 60 mg/l, Kin 6 mg/l)

		Crosses	Fallen flowers		Pods retained after days of pollination													
Parents					10 days		20 days		30 days		Mature pods		Seeds formed					
Female	Male												Shrivelled		Deformed		Normal	
		No	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
L. albus	***L. mutabilis***																	
LA001	LM001	50.00	38.00	76.00	15.00	30.00	2.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LA001	LM003	48.00	43.00	89.58	12.00	25.00	5.00	10.41	1.00	2.08	1.00	1.80	2.00	100	0.00	0.00	0.00	0.00
LA004	LM001	37.00	25.00	67.56	3.00	8.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LA004	LM003	50.00	29.00	58.00	15.00	30.00	2.00	4.00	1.00	2.00	1.00	8.16	2.00	100	0.00	0.00	0.00	0.00
Total		**185.00**	**135.00**		**45.00**		**9.00**		**3.00**		**2.00**		**4.00**		0.00		0.00	
Mean %				**72.97**		**24.32**		**4.86**		**1.62**		**1.08**		**100**		**0.00**		**0.00**
L. mutabilis	***L. albus***																	
LM001	LA001	50.00	23.00	44.23	9.00	18.00	2.00	3.84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LM001	LA004	53.00	45.00	81.80	11.00	20.75	2.00	3.77	1.00	1.88	1.00	1.88	1.00	100	0.00	0.00	0.00	0.00
LM003	LA001	69.00	28.00	40.57	37.00	53.62	11.00	15.94	4.00	5.79	4.00	5.79	5.00	71.4	2.00	28.6	0.00	0.00
LM003	LA004	45.00	29.00	64.44	19.00	38.00	2.00	4.44	1.00	2.22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total		**217.00**	**125.00**		**76.00**		**17.00**		**6.00**		**5.00**		**6.00**		**2.00**		0.00	
Mean %				**57.60**		**35.02**		**7.83**		**2.76**		**2.30**		**75**		**25**		**0.00**

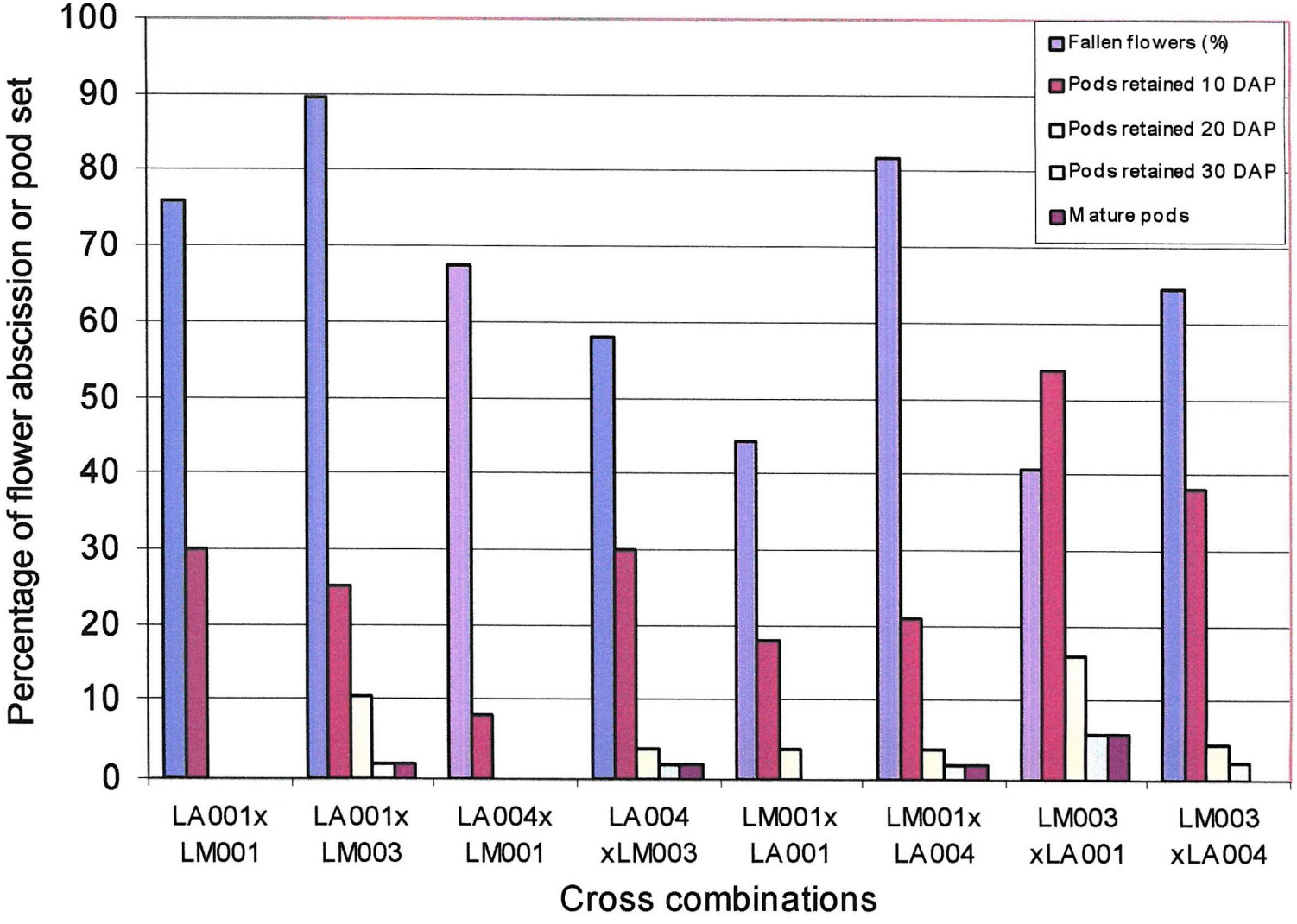


Fig 2.7: Results of interspecific crosses between *L. albus* and *L. mutabilis* with application of growth regulators (S3)

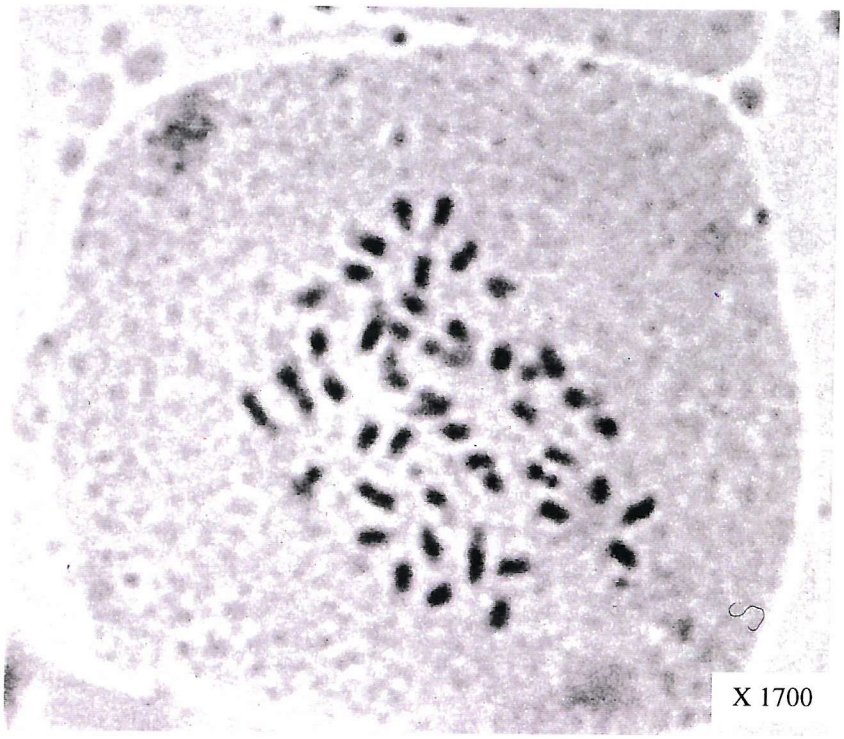


Plate 2 .1: Somatic metaphase plate from a root tip cell of *L mutabilis* (LM003).

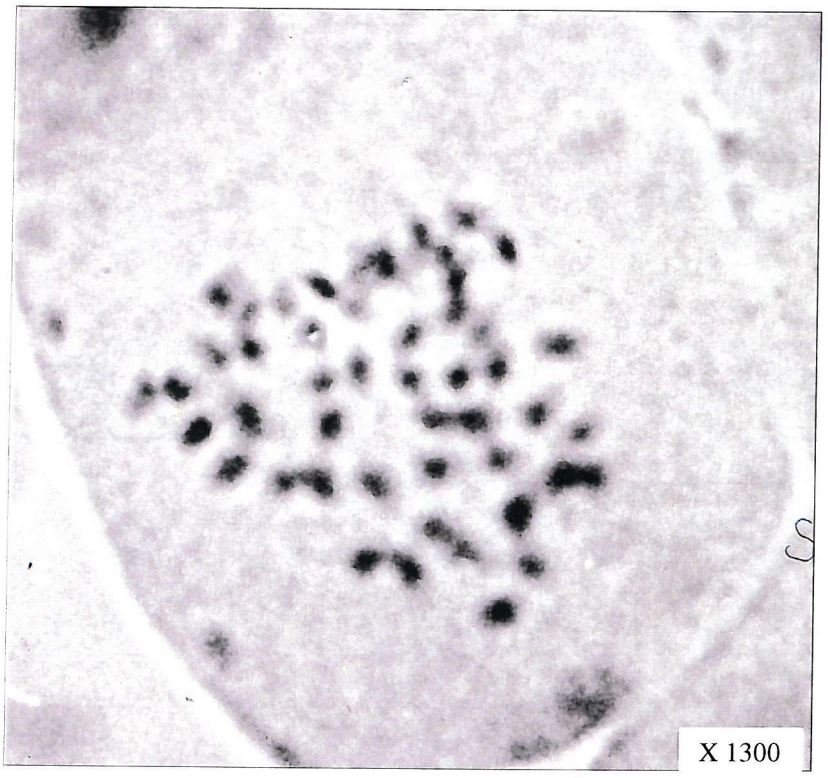


Plate 2.2: Somatic metaphase plate from a root tip cell of *L. albus* (LA001).

Plate 2.3: An aborted pod from the cross of LA003 (♀) and LM003 (♂) 12 DAP.

Plate 2.4: An aborted pod from the cross of LM003 (♀) and LA001 (♂) 16 DAP.

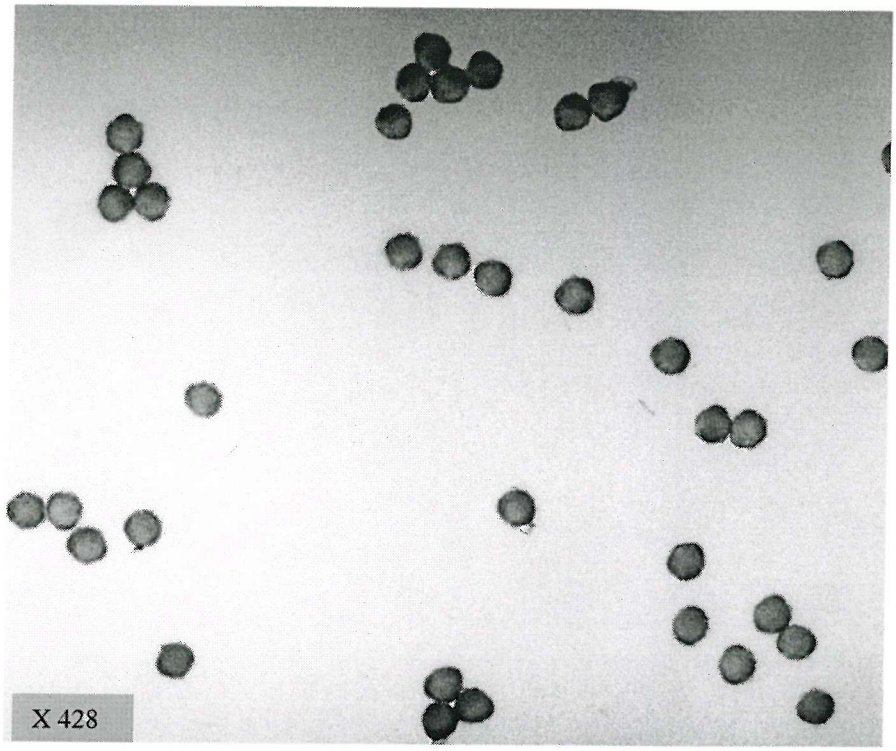


Plate 2.5: Pollen stainability of *Lupinus albus* species (LA001).

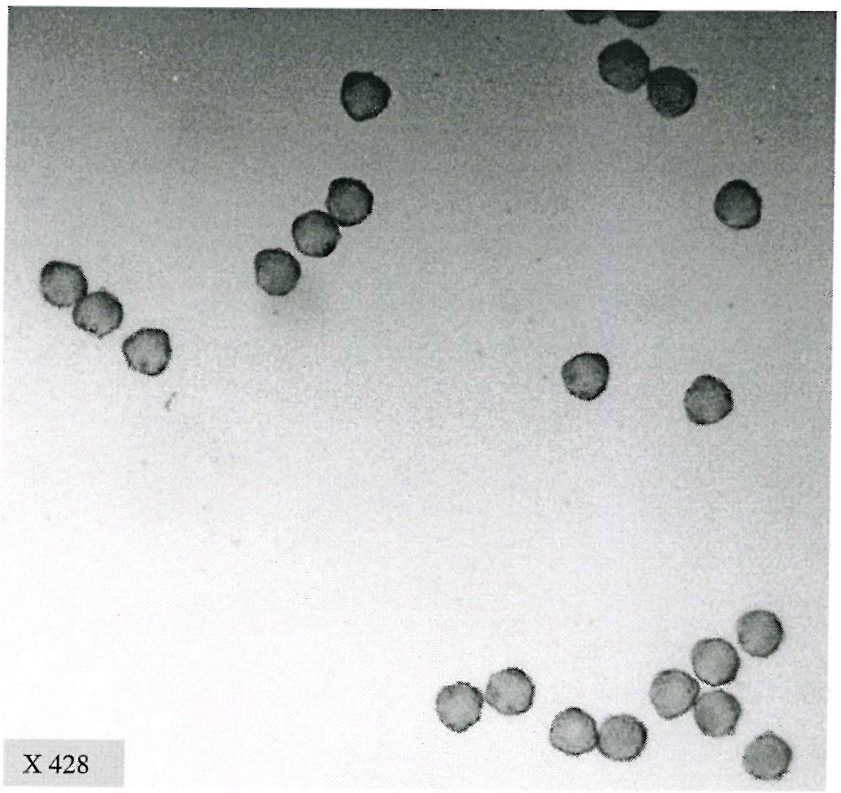


Plate 2.6: Pollen stainability of *L. mutabilis* species (LM003)

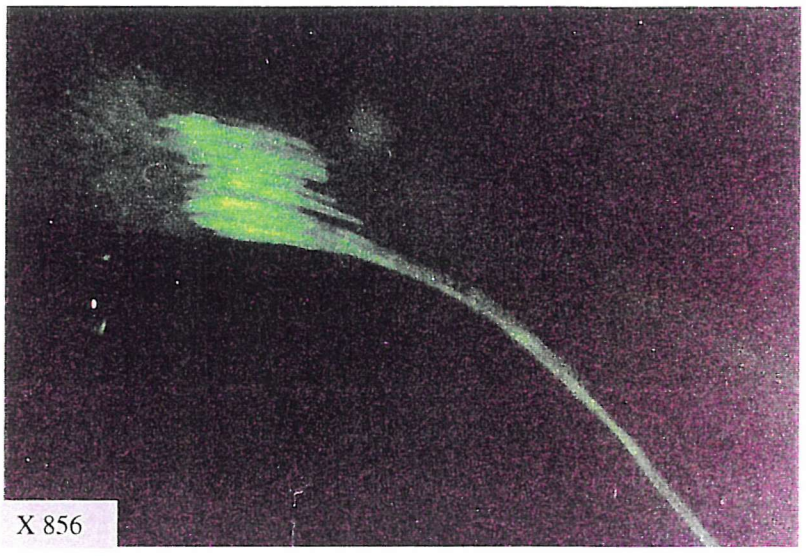


Plate 2.7: Pollen tube growth in selfed cross of *L. albus* LA001 (♀) and LA001 (♂) after 24 hours of self-pollination.

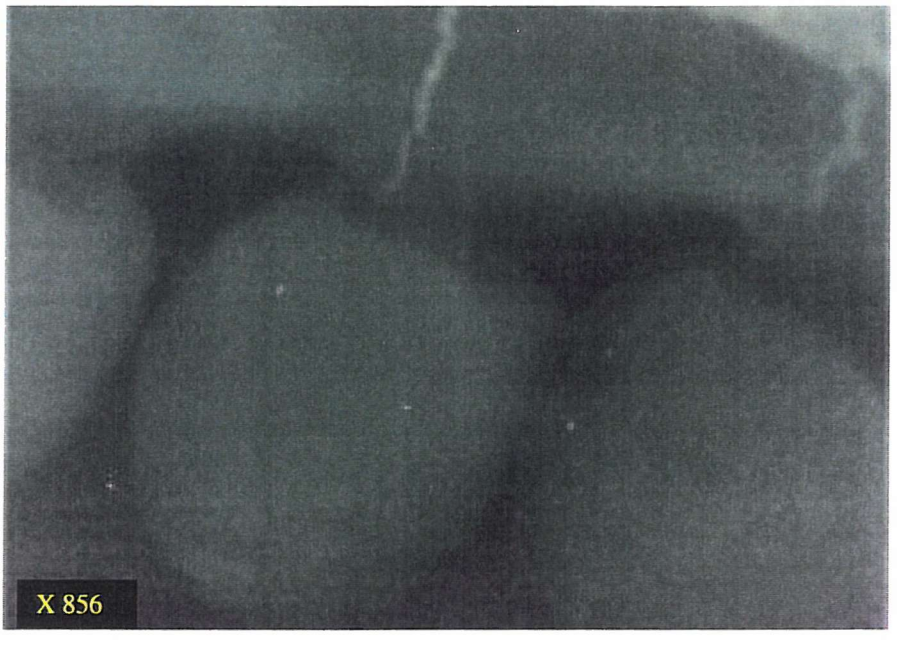


Plate 2.8: Pollen tube approaching ovules in the cross of LM001 (♀) and LA003 (♂) after 36 hours of pollination.

Chapter Three

Pod and seed development

3.1 Introduction

The hybrid pod and seed abortion of interspecific crosses may occur as a result of disharmonious interaction between embryo, endosperm, and maternal tissues. If the hybrid embryo aborts due to starvation by not receiving enough nutrients from its supporting tissues such as suspensor, endosperm, or supporting ovular tissues, it may be possible to rescue it by means of *in vitro* culture. Study of pod and seed development at earlier stages of development can help to recognise the best time and stage when hybrid embryos would be amenable to *in vitro* culture and increase the rate of success for rescue of hybrid embryos through *in vitro* culture.

Interspecific crosses between *L. albus* and *L. mutabilis* studied here showed pod abortion at different stages of embryo development or resulted in production of mature pods containing shrivelled or deformed seeds (chapter 2). To improve chances of rescuing young aborting embryos (section 1.8) it is necessary to determine how fast and for how long they will develop *in vivo*. In addition, comparisons of pod and seed development in self and interspecific crosses may lead to an understanding of the time and reasons for hybrid embryo abortion.

The use of growth hormones to retain pods and enhance embry/seed development *in vivo* may help in obtaining successful interspecific crosses. The role of growth regulators on pod and seed development of different species has been discussed in chapter 1 (section 1.7.1) and their effect on self and interspecific crosses between *L. albus* and *L. mutabilis* species has been presented in chapter two (section 2.3.7).

An investigation on pod and ovule development following self and interspecific crosses of *L. albus* and *L. mutabilis* with and without application of growth regulators was carried out which may lead to an understanding of the process of fertilisation, and shed light on reasons for hybrid pod abortion and the ways may be explored to overcome.

3.2 Materials and methods

Two lupin species, *Lupinus albus* and *Lupinus mutabilis* were used in this study. Plants were grown in a glasshouse as described in chapter 2 (section 2.2.1). Crosses (self and inter-specific) were made following the method of Townsend (1980) explained in chapter 2. All crosses were labelled and measurement was made for each pod of every cross. Immature pods from self and interspecific crosses were harvested and measured for pod length after 1, 2, 3, 4, 5, 10, 15 and 20 days of pollination.

In the second part of this study, length, width and weight of seeds dissected from immature pods following self and interspecific pollination were recorded under a stereo-microscope with the help of a stage and eyepiece micrometer. The pods were harvested from plants at 1, 2, 3, 4, 5, 10, 15 and 20 days after pollination. Stages of embryo development were also recorded where possible (globular, heart stage, torpedo stage and late cotyledonary stage). Seeds from inter-specific crosses were measured till they were dropped from the plants. The ratio of seed length and seed width (L/W) was also calculated.

Investigations on pod development were carried out after application of growth regulators. The growth regulator mixture that was found effective in retaining flower/pods (chapter 3) was used for this experiment. The growth regulator mixture consisted of GA3 100 mg/l, NAA 50 mg/l, and Kin 5 mg/l. Pods were labelled from both cross combinations (*L. albus* ♀ x *L. mutabilis* ♂ and *L. mutabilis* ♀ x *L. albus* ♂) with and without application of growth regulators and pod length was recorded at 1, 2, 3, 4, 5, 10, 15, and 20 days following self and interspecific pollination. Pod development studies carried out without application of growth regulators were treated as control. A number of crosses (inter-specific) did not produce enough data due to early pod abortion.

3.3 Results

3.3.1 Pod development in self and interspecific crosses between *L. albus* and *L. mutabilis* without application of growth regulators

A comparison of pod length of *L. albus* and *L. mutabilis* species following self and interspecific pollination is presented in table 3.1, fig 3.1, and plate 3.1. A gradual increase in pod length of *L. albus* following self-pollination was observed whereas pod length in *L. mutabilis* increased sharply after three days of pollination. Also pod length of *L. mutabilis* was found comparatively higher than *L. albus* in general. Pod length after 20 days of pollination reached up to 54.5 mm in *L. mutabilis* as compared to *L. albus*, which was 41.5 mm after 20 days of self-pollination (fig. 3.1).

In interspecific crosses where *L. albus* was used as seed parent and *L. mutabilis* as pollen parent, pods increased in length for 4 to 5 days after pollination but at a slow rate as compared to selfed pods. Plate 3.1 presents the pod development of selfed and interspecific crosses without application of growth regulators after 11 days of pollination. Pod growth ceased after the fifth day of pollination in interspecific crosses of *L. albus* (♀) and *L. mutabilis* (♂). In the crosses where *L. mutabilis* was used as seed parent, pod development also ceased after 3 days of pollination but occasionally slight growth was observed in limited cases. In interspecific crosses of both species very few pods could be measured due to high rate of flower drop and pod abortion. Therefore, data recorded for pod development in interspecific crosses was not sufficient for statistical analysis.

3.3.2 Pod development in self and interspecific crosses between *L. albus* and *L. mutabilis* with application of growth regulators

Results from pod development in selfed and interspecific crosses of *L. albus* and *L. mutabilis* after application of growth regulators are presented in table 3.1, fig 3.2. In selfed crosses of *L. albus*, a longer pod length (18 mm) were recorded with application of growth regulators as compared to controls (17.7 mm) 10 DAP. In interspecific crosses where *L. albus* was used as seed parent slightly longer pod length (16.9 mm) was recorded as compared to controls (15.5 mm) 10 DAP. Similar results were observed in

selfed and interspecific crosses of *L. mutabilis* (*L. mutabilis* ♀ x *L. albus* ♂). Regardless of the growth regulator treatments and genotypes used most of the hybrid pods aborted within two weeks after pollination. Although growth regulators had some positive effects on hybrid pod retention they eventually abscised and dropped.

3.3.3 Seed development in self and interspecific crosses between *L. albus* and *L.mutabilis* species

The results of seed length, seed width and seed weight in selfed and interspecific crosses of *L. albus* and *L. mutabilis* species are as follows:

3.3.3.1 Seed length in self and interspecific crosses of *L. albus* and *L. mutabilis* species

A comparison between lengths of seed obtained from selfed and interspecific crosses of *L. albus* and *L. mutabilis* species is given in tables 3.2 to 3.5 and fig. 3. 3. After fourth day of self-pollination *L. albus* seeds start to increase in length but seeds of *L. mutabilis* start to increase in length from the third day of pollination. In selfed seeds of *L. mutabilis* a gradual increase in seed length was observed and after 10 days of pollination (self) the maximum seed length was observed to be 2.00 mm. On the other hand seeds from selfed pods of *L. albus* showed a sharp increase in the seed length after the fourth day of pollination. The maximum seed length was 1.15 mm in selfed crosses of *L. albus* after 10 days of pollination.

A slow increase in seed length was observed in interspecific crosses as compared to self-pollinated seeds (fig 3.3). In interspecific crosses involving *L. albus* as seed parent and *L. mutabilis* as pollen parent, seeds increased in length up to three days after pollination. After the third day of pollination seed length increased at a very slow rate and this slight increase in length was observed up to 10 to 12 days after pollination till the pod abscised. Similar seed growth pattern was observed in interspecific crosses of *L. mutabilis* (♀) and *L. albus* (♂). In general a higher seed length was observed in interspecific crosses where *L. mutabilis* was used as seed parent.

3.3.3.2 Seed width in self and interspecific crosses of *L. albus* and *L. mutabilis* species

Comparisons between widths of seeds obtained from selfed and interspecific crosses of *L. albus* and *L. mutabilis* species are given in tables 3.2 to 3.5 and fig. 3. 4. A sharp increase in seed widths after 4th day of pollination was observed in selfed crosses of both *L. albus* and *L. mutabilis* species (fig. 3.4). A higher increase in seed width was observed in ovules of *L. mutabilis* from the 5th day of self-pollination compared to seeds from *L. albus*. The maximum seed width was recorded as 1.00 mm in *L. albus* after 10 DAP.

A slow increase in width was observed in interspecific crosses as compared to self-pollinated seeds (fig 3.4). Seeds increased in length up to three days of pollination and afterwards, gradual increase in width was observed which continued up to 10 to 12 days after pollination till the pods abscised. The maximum seed width was 0.77 mm when *L. mutabilis* was used as seed parent and 0.69 mm width was recorded in the reciprocal cross. In general, seed width was higher when *L. mutabilis* was used as the female parent.

3.3.3.3 Seed weight in self and interspecific crosses of *L. albus* and *L. mutabilis* species

Tables 3.2 to 3.5 and fig. 3.5 summarise the results of weight measurement of the seeds from selfed and interspecific crosses of *L. albus* and *L. mutabilis* species. A slow increase in weight of selfed seeds of both species was observed up to four days after pollination. From the 5th day of self-pollination seeds of *L. albus* as well *as L. mutabilis* increased in weight sharply (fig 3.5). At 10 DAP maximum weight of selfed seeds was observed to be 10.60 mg and 7.60 mg for *L. albus* and *L. mutabilis* species respectively.

In interspecific crosses the seeds showed a relatively small gain in weight as compared to selfed ones (fig. 3.5). In the crosses of *L. albus* (♀) x *L. mutabilis* (♂) and *L. mutabilis* (♀) x *L. albus* (♂) the maximum seed weight recorded after 10 DAP was 2.35 mg and 2.41 mg, respectively. In general, the weight of seeds in interspecific crosses involving *L. mutabilis* as female parent was higher than *L. albus*.

3.4 Discussion

The results from the study on the interspecific crosses of *L. albus* and *L. mutabilis* species (chapter 2) suggested that most of the hybrid pods abscissed within two weeks after pollination. It has been reported that the seed size of the aborted pods shows a good correlation with the size of embryos at a particular developmental stage and can be used as a measure of embryo size (Liu *et al.,* 1993). Hence an investigation of pod and seed development in self and interspecific crosses was carried out to determine the best time for embryo rescue so that a hybrid plant of *L. albus* and *L. mutabilis* species could be achieved. In addition, seed development studies in interspecific crosses could shed light on the establishment of the fertilisation process in the *Lupinus* species.

The investigations on pod development following self-pollination of *L. albus* and *L. mutabilis* species revealed normal pod development. A gradual increase in pod length was observed in selfed crosses of *L. albus* as well as *L. mutabilis* species (fig. 3.1). However, in selfed crosses of both *L. albus* and *L. mutabilis* genotypes a sharp increase in pod length was noticed after the 3rd day of pollination. This may be a natural characteristic of the *Lupinus* species as a similar pattern of pod growth was observed after application of growth regulators (Fig. 3.2). The results of pod development in this study indicate that pod length in selfed crosses of *L. mutabilis* species was longer (24. 5 mm) than *L. albus* (17.7 mm) after 10 DAP.

In the present study, results of reciprocal crosses of *L. albus* and *L. mutabilis* revealed a "non-typical development" of pods. Pod development in interspecific crosses was slow compared to selfed ones. A longer pod length (19 mm) was observed in the cross combinations where *L. mutabilis* was used as the female parent than the combination when *L. albus* was used as the female parent (15.5 mm) after 10 DAP. Unlike selfed crosses, pod growth in interspecific combinations appeared to have ceased after the 3rd day of pollination (Fig. 3.1). The results presented here confirmed the previous reports of slow growth of pods in interspecific crosses of the genus *Lupinus* (Busmann-Loock *et al.,* 1992; Siamasonta and Calligari, 1998). However, in both self and interspecific crosses the period between the 3rd and 4th day after pollination appeared to be crucial for pod development.

In this work "typical seed development" was observed following self-pollination of *L. albus* and *L. mutabilis* species. The seed ratio (L/W) after 5 days of self-pollination was higher in *L. albus* (1.08) than *L. mutabilis* (1.02), which indicates a higher rate of seed development in *L. mutabilis* compared to *L. albus* species. However, for seed development, unlike pod development, the sharp increase in seed length and width was noticed after the 4th day of pollination.

In reciprocal interspecific crosses of *L. albus* and *L. mutabilis* a slow development of seeds was observed (fig 3.3 & 3.4). The selfed seeds continued to grow in length and width whereas seed development in reciprocal crosses of *L. albus* and *L. mutabilis* appeared to have stopped after 5 DAP. This indicates that hybrid seeds of *L. albus* and *L. mutabilis* crosses may grow normally up to 4 or 5 days after pollination. However, Taheri (2000) reported normal development of hybrid pod and seeds for 8 days after pollination in interspecific crosses of the genus *Cicer*.

The investigations on seed weight also indicated similar results to those of pod and seed development. A typical gain in weight of selfed seeds of *L. albus* as well as of *L. mutabilis* was observed (fig. 3.5). The seed weight after 5 days of self-pollination was higher in *L. mutabilis* (3.58 mg) than *L. albus* (3.20 mg). For the first four days of pollination, seeds of both selfed and interspecific crosses showed almost similar pattern of increase in weight. From the 4th day of pollination the weight of seeds from selfed crosses continued to increase whereas in the reciprocal crosses of *L. mutabilis* and *L. albus* the increase appeared to have stopped. The seed weight in the cross combination *L. albus* (♀) x *L. mutabilis* (♂) was higher (1.37 mg) than the reciprocal cross combination (1.30 mg) after 5 days of pollination. The normal development of hybrid seeds for 4-5 days after pollination indicates that fertilisation occurred and at later stages of embryo development "embryo genotype" started to express which caused disharmonious interaction between embryo and its supporting tissues.

The application of growth regulators had some positive effects on pod length. For example pod length 5 DAP in the cross combination of *L. albus* (♀) x *L. mutabilis* (♂) was recorded to be 15.25 mm whereas with application of growth regulators the pod

length was 16.4 mm. From the 5th day of pollination the length of pods from selfed crosses continued to increase whereas in the reciprocal crosses of *L. mutabilis* and *L. albus* the increase appeared to have become slow. Nevertheless, most of the hybrid pods were aborted between 10 – 15 DAP when the application of growth regulators was carried on for 10-15 days after pollination.

A simple correlation between pod and seed development was also observed in this study. When pods stopped growing, the seed development also appeared to have ceased. Williams *et al.*, (1980); and Taheri, (2000) also suggested a positive correlation between pod and seed growth in the genus *Lupinus* and *Cicer* respectively. The results are in agreement with the earlier reports on seed development in selfed and interspecific crosses of the genus *Lupinus* (Williams *et al.*, 1980; Pszyborowski & Packa, 1997; and Siamasonta & Calligari, 1998).

Observations on pod and seed development in the interspecific crosses of *L. albus* and *L. mutabilis* revealed "non-typical" development in comparison to the selfed ones. The period between the 3rd and 5th day after pollination seems to be crucial for interspecific crosses as at this stage pods and seeds start to grow faster in selfed crosses whereas this growth in hybrid pods and seeds of reciprocal cross combinations of *L. albus* and *L. mutabilis* is very slow. It may be concluded that in interspecific crosses between the two species fertilisation occurs but embryo degenerates at an early stage of development (3-5 DAP). This may be due to disharmonious interaction between the embryo, endosperm or maternal tissues. These results are also supported by earlier reports (Schafer-Menhur *et al*, 1988 and Przyborowski & Packa, 1997). The results also suggest that any attempt for hybrid embryo culture has to be made by 5 days after pollination or before the embryo starts to abort. This phenomenon was further confirmed by the results of visual observation of seeds of selfed and interspecific crosses at regular intervals of one day starting from as early as 24 hours of pollination. Seeds from selfed and interspecific crosses were vigorous and green to dark green for the first three days after pollination, but were yellowish green and wrinkled from the 4th day of pollination in cross combinations.

In conclusion, it appeared from the observations that the differences in pod and seed

development at a very early stage may be due to disharmonious interactions between the seed and subsequently pod, and the supporting tissues. These interactions may prohibit supply of nutrients to the growing seeds and pods which indicates that attempts to rescue embryos have to be made at an early stage of seed development, possibly during 4 – 5 days after pollination in the cross between *L. albus* and *L. mutabilis*.

Table 3.1: Mean pod/ovary length (mm) in selfed and interspecific crosses between *L. albus* and *L. mutabilis* with and without application of growth regulators.

DAP	*L. albus* (self)		*L. albus* (♀) X *L. mutabilis* (♂)	*L. albus* (♀) X *L. mutabilis* (♂)	*L. mutabilis* (self)		*L. mutabilis* (♀) X *L. albus* (♂)	*L. mutabilis* (♀) X *L. albus* (♂)
	*(NGR)	*(WGR)	*(NGR)	*(WGR)	*(NGR)	*(WGR)	*(NGR)	*(WGR)
1	14.2	14.3	13	12.95	14.25	14.75	14	13.9
2	14.55	14.60	13.85	13.95	14.5	15.00	14.5	14.5
3	14.6	14.65	14.5	14.7	15	15.00	15.25	15.5
4	15.4	15.6	15.1	15.9	18.75	19.00	17.2	17.9
5	15.75	15.8	15.25	16.4	20	20.50	18.5	20
10	17.7	18.0	15.5	16.9	24.5	27.00	19	22.6
15	21.5	22.5	17	18.25	51	53.00	20.5	23.5
20	41.5	42.5			54.5	55.00		

* Average of five observations
NGR = No growth regulators
WGR = With growth regulators
DAP = Days after pollination

Table 3.2 Seed development in *Lupinus albus* after self-pollination.

DAP	Seed length (mm)			Seed width (mm)			Ratio	Seed weight (mg)		
	*Min	*Max	Mean	*Min	*Max	Mean	(L/W)	*Min	*Max	Mean
1	0.46	0.51	0.48	0.34	0.36	0.35	1.37	0.43	0.48	0.46
2	0.49	0.53	0.51	0.37	0.39	0.38	1.34	0.48	0.52	0.50
3	0.52	0.55	0.53	0.39	0.47	0.43	1.23	0.63	0.69	0.66
4	0.49	0.59	0.54	0.51	0.53	0.52	1.03	0.99	1.16	1.08
5	0.89	1.00	0.94	0.74	1.00	0.87	1.08	2.90	3.50	3.20
10	1.00	1.15	1.07	0.96	1.00	0.98	1.09	20.8	21.6	21.20
15										
20										

L/W = Ovule length/Ovule width
* = Average of five observations

Table 3.3 Seed development in *Lupinus mutabilis* after self-pollination

DAP	Seed length (mm)			Seed width (mm)			Ratio	Seed weight (mg)		
	*Min	*Max	Mean	*Min	*Max	Mean	(L/W)	*Min	*Max	Mean
1	0.47	0.48	0.47	0.19	0.28	0.23	2.04	0.64	0.68	0.66
2	0.49	0.56	0.52	0.30	0.34	0.32	1.62	0.79	0.9	0.84
3	0.58	0.63	0.60	0.38	0.45	0.41	1.46	1.00	1.24	1.12
4	0.78	0.79	0.78	0.51	0.65	0.58	1.34	1.45	1.95	1.7
5	0.95	1.00	0.97	0.91	1.00	0.95	1.02	3.20	3.93	3.58
10	1.85	2.00	1.92	1.70	2.00	1.85	1.03	15.00	15.4	15.2
15										
20										

* Average of five observations

Table 3.4 Seed development in interspecific crosses of *L. albus* (♀) x *L. mutabilis* (♂)

DAP	Seed length (mm)			Seed width (mm)			Ratio	Seed weight (mg)		
	*Min	*Max	Mean	*Min	*Max	Mean	(L/W)	*Min	*Max	Mean
1	0.43	0.50	0.46	0.29	0.34	0.31	1.21	0.35	0.47	0.40
2	0.51	0.54	0.52	0.32	0.35	0.32	1.62	0.41	0.59	0.50
3	0.36	0.54	0.45	0.31	0.33	0.32	1.45	0.56	0.64	0.60
4	0.49	0.55	0.52	0.34	0.35	0.34	1.52	1.13	0.93	1.03
5	0.52	0.55	0.53	0.33	0.35	0.34	1.55	1.3	1.44	1.37
10	0.63	0.90	0.76	0.69	0.70	0.69	1.10	1.97	2.73	2.35
15										
20										

* Average of five observations

Table 3.5 Seed development in interspecific crosses of *L. mutabilis* (♀) x *L. albus* (♂)

DAP	Seed length (mm)			Seed width (mm)			Ratio	Seed weight (mg)		
	*Min	*Max	Mean	*Min	*Max	Mean	(L/W)	*Min	*Max	Mean
1	0.37	0.53	0.45	0.26	0.37	0.31	1.45	0.46	0.66	0.56
2	0.48	0.56	0.52	0.34	0.42	0.38	1.36	0.78	0.86	0.82
3	0.54	0.66	0.60	0.52	0.54	0.53	1.13	1.12	1.19	1.15
4	0.68	0.70	0.69	0.49	0.51	0.50	1.38	1.17	1.23	1.20
5	0.79	0.79	0.79	0.53	0.57	0.55	1.43	1.28	1.32	1.30
10	0.98	1.00	0.99	0.75	0.80	0.77	1.28	2.33	2.49	2.41
15										
20										

* Average of five observations

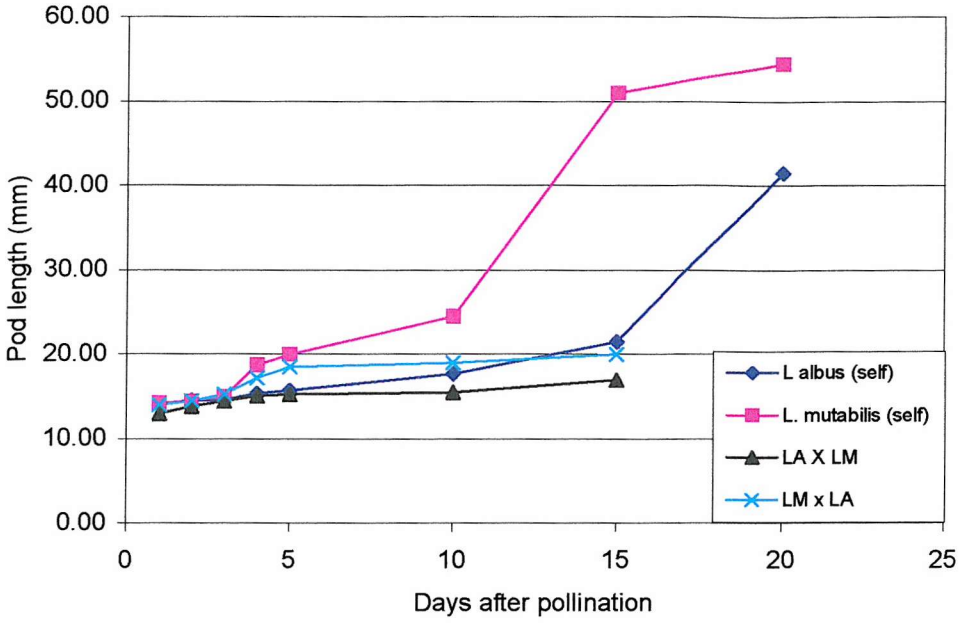


Fig. 3.1: Results of pod length of self and interspecific crosses of *L. albus* and *L. mutabilis* species.

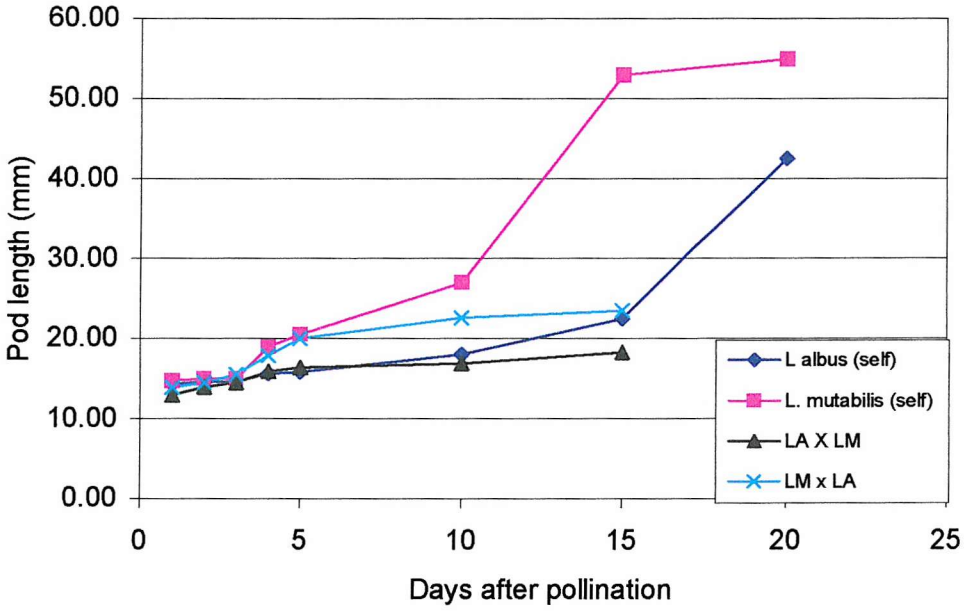


Fig. 3.2: Results of pod length of self and interspecific crosses of *L. albus* and *L. mutabilis* species with application of growth regulators.

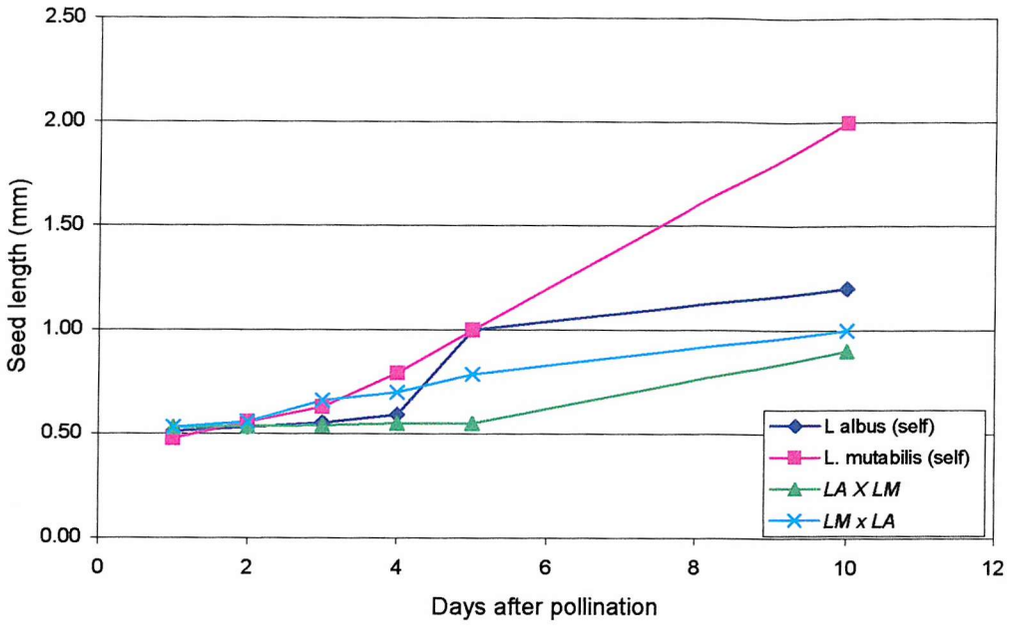


Fig. 3.3: Results of seed length of self and interspecific crosses of *L. albus* and *L. mutabilis* species.

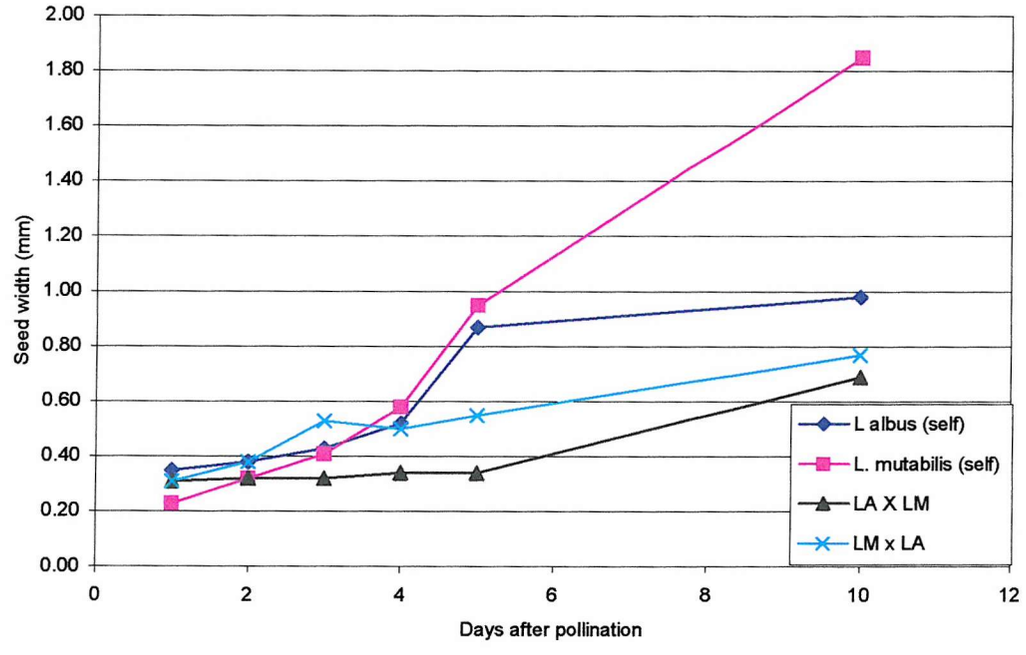


Fig. 3.4: Results of seed width of self and interspecific crosses of *L. albus* and *L. mutabilis* species.

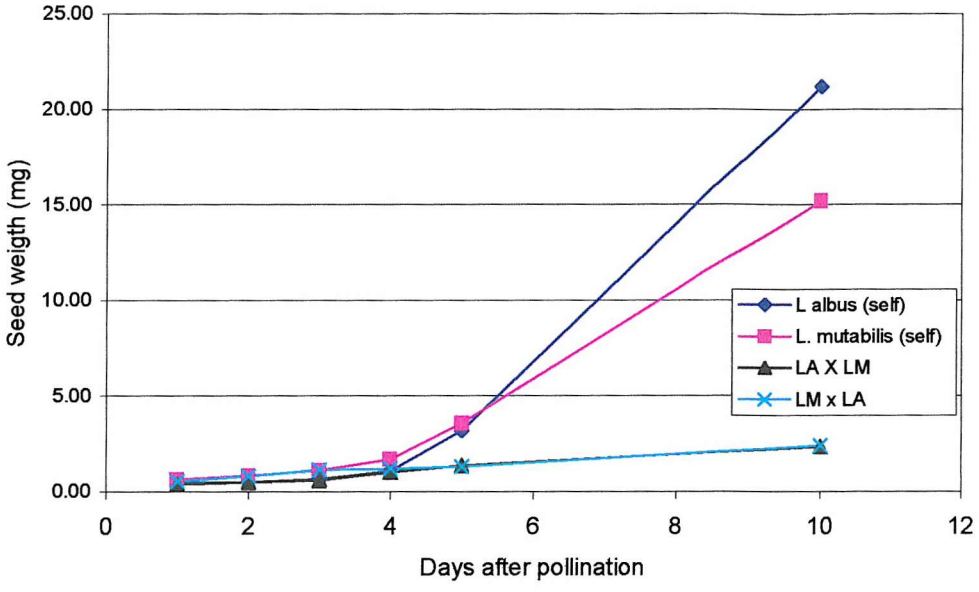


Fig. 3.5: Results of seed weight of self and interspecific crosses of *L. albus* and *L. mutabilis* species

Plate 3.1 Pod development after 11 DAP in *L. albus* following self and interspecific pollination between *L. albus* (♀) x *L. mutabilis* (♂) .

Plate 3.2 Ovule development after 11 DAP in *L. albus* following self and interspecific crosses between *L. albus* (♀) x *L. mutabilis* (♂) .

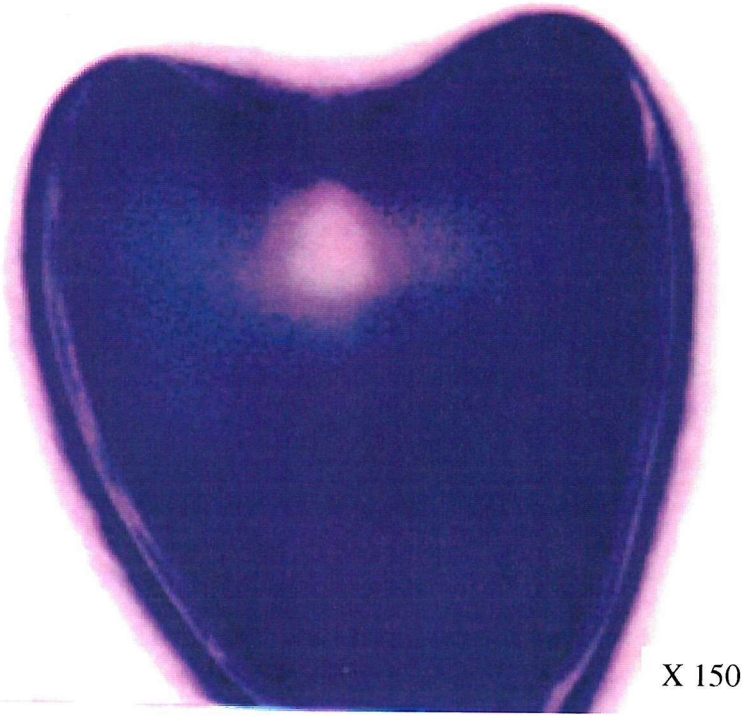


Plate 3.3: Heart stage embryo of *L. albus* species originally excised from selfed pods after 15 DAP.

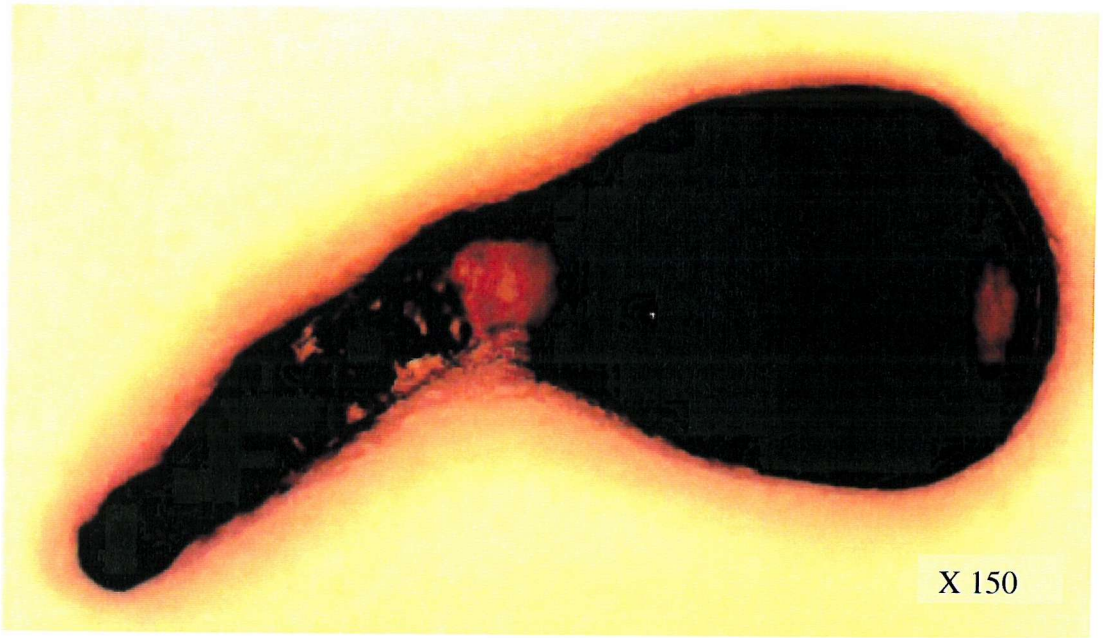


Plate 3.4: Torpedo stage embryo of *L. mutabilis* species originally excised from selfed pods after 15 DAP.

Plate 3.5: Embryos of *L. mutabilis* originally excised from selfed seeds after 22 DAP.

Plate 3.6: Embryos of *L. albus* species originally excised from selfed seeds after 22 DAP

Chapter Four

Embryo/immature seed culture

4.1 Introduction

In interspecific crosses of *Lupinus* species embryo abortion is a common phenomenon. Embryo culture is one of the embryo rescue techniques that involves isolating and growing of an immature zygotic embryo under sterile conditions on an aseptic nutrient medium with the goal of obtaining a viable plant. This technique has successfully been applied in a large number of plant species including legumes such as *Phaseolus* (Kuboyama *et al.,* 1991), *Arachis* (Bajaj *et al*; 1982; Pattel *et al*; 1988) and *Trifolium* (Fergusan *et al*; 1990). In the genus *Lupinus*, interest in the embryo/immature seed culture is mainly focused on its use to facilitate interspecific crosses, which are difficult to achieve through hybridisation.

The successful regeneration of plants through *in vitro* culture of immature embryos of the interspecific crosses between *L. mutabilis* (♀) and *L. hartwegii* (♂) indicates the possibility of achieving hybrid plants of interspecific crosses between the Old World and New World lupin species through embryo/immature seed culture. In addition, investigations carried out on pod and seed development studies of the self and interspecific crosses of *L. albus* and *L. mutabilis* species indicated the appropriate time required (3 – 5 DAP) to rescue hybrid embryos/seeds of the two species. It is apparent from the results of the previous chapter that an attempt to culture embryos/immature seeds of interspecific crosses of *L. albus* and *L. mutabilis* species *in vitro* may enable us to regenerate fertile plant(s).

Following the results of interspecific crosses carried out in the present study (chapter 2) and pod/seed development (chapter 3) which confirmed the previous reports of pod abortion degeneration of embryos in interspecific crosses between *L. albus* and *L. mutabilis* it seems appropriate to rescue aborting embryos through embryo/immature seed culture. Thus, an attempt was made to produce interspecific hybrids between *L. albus* and *L. mutabilis* using *in vitro* culture of embryos/immature seeds.

4.2 Materials and methods

The composition of the culture medium is an important factor for the successful establishment of any tissue culture method. The embryo/immature seed culture was first standardised with selfed embryos/immature seeds of *L. albus* and *L. mutabilis* species, because the experiments showed that the large number of embryos/immature seeds needed for experimentation could not be obtained from interspecific crosses. The basal culture media of Rashid *et al.,* (1988); Kasten *et al.,* (1991); and Malikarjuna (1999) supplemented with various concentrations of auxins and cytokinins ranging from 0.1 to 10 mg per litre (table 4.1 & 4.2) were used. The pH of the media was adjusted to 5.8 using 1 M and 0.1 M sodium hydroxide (NaOH) before adding agar. Agar was added to the medium at required concentrations (7-8 g) and melted in a steamer. The cultured media were then dispensed into glass jars of various sizes (5 ml to 175 ml), which were covered with screw caps. The jars were then sterilised by autoclaving at 121°C for 15 minutes. For liquid cultures, 5 ml jars containing a piece of folded filter paper to support immature seeds, as a bridge was first sterilised in the autoclave. Then, sterilised liquid medium was poured in the jars in a laminar flow-cabinet whenever required. A list of media and all the ingredients used is presented in tables 4.1 and 4.2.

Pods of self and interspecific crosses were collected from the plants grown under glasshouse conditions at different intervals after pollination. The pods were first measured under a stereo-microscope and then they were sterilised. For surface sterilisation, the entire pods were dipped in 70 % ethanol for one minute and then immersed in 7 % domestos (commercial sodium hypochlorite, Unilever, UK) for 25 minutes. The materials were then thoroughly rinsed three times with sterile-distilled water. Immature seeds and embryos were dissected in a sterile solution of 9 % glucose (Liu *et al.,* 1993) or 12 % sucrose (Mergeai *et al.,* 1997) in a petri dish under a stereo-microscope inside a sterile laminar flow-cabinet (Brassair, UK). Care was taken to excise embryos attached to the suspensor from globular to torpedo stages, however, sometimes the suspensor would break and the embryos were cultured without suspensor tissues. Small young embryos (globular-, heart-, and torpedo-stage) were picked up from the liquid with a Pasteur-pipette, large embryos (cotyledonary-stage) and ovules were transferred into the culture vessels with a pair of forceps. The excised embryos and

immature seeds were then cultured on a 2.5 ml nutrient medium in 5 ml glass jars with screw caps. For the successful development of plantlets it was ensured that the embryos were not fully immersed in the medium. Younger ovules and embryos were initially incubated in complete darkness for 5-14 days at 23 ± 2 °C. Then the cultures were maintained in a growth room at 25 ± 2 °C for a 12 hours photo-period with light intensity of 65 $\mu em^{-2} sec^{-1}$ derived from cool fluorescent tubes (F70W/35, UK). Explants were sub-cultured after every two weeks in the same media they were initially cultured. Self-pollinated cultured immature seeds and embryos were treated as controls.

Successful cultured plantlets after formation of shoots and roots were transferred to heat sterilised soil containing 1/3 peat, 1/3 sand, and 1/3 perlite and were hardened in a room with 16 ± 2 hours photo-period provided by cool fluorescent tubes and at 24 °C room temperature. The pots were kept in a propagator or covered with polythene bags to maximise the humidity levels. After three weeks, the plants were introduced to the glasshouse and were gradually acclimatised to lower humidity conditions.

An isozyme analysis of species and hybrid was carried out in order to determine the hybridity of shoots/calli produced. Due to scarcity of hybrid shoots/calli only Estrase (EST) was separated in polyacrylamide gel using a vertical Hoefer SE 600 electrophoresis unit. An extraction buffer, gel and a gel running buffer were derived from the methods of Wendel and Weeden (1990). The extraction buffer consisted of 100 mM Tris-HCL pH 7.5, 5% sucrose (w/v), 5% polyvinylpyrrolidone (PVP-40), 100 mM ascorbic acid, 1% bovine serum albumin and 14 mM (0.1% w/v) mercaptoethanol. Mercaptoethanol was added to the other ingredients just prior to use.

The method was standardised using young leaves of *L. albus* and *L. mutabilis* species. Leaf samples were transferred to liquid nitrogen for quick freezing. Freeze-dried leaf tissues were ground to a fine powder with 30 mg of polyclar insoluble polyvinylpyrrolidone (PVP) using a pre-chilled mortar and a pestle. Five hundred µl of extraction buffer were added to the leaf sample and the sample was further ground. The enzyme extraction was thereafter centrifuged at 12,000 rpm for 15 minutes. Enzyme extracts and plant samples were kept on ice while doing the above-mentioned activities.

Seventy millilitres of 10 % polyacrylamide gel was prepared by mixing 24 ml 30% bis-acrylamide, 45 ml gel buffer, 70 µl 14 M mercaptoethanol, 600 µl of 10% ethyline glycol, 100 µl TEMED and 1 ml of freshly prepared 10% ammonium persulphate. The gel buffer (pH 8.3) was composed of either 0.019 M boric acid, 0.004 M lithium hydroxide, 0.047 M tris-base and 0.007 M citric acid or Trizma base (0.018 g/l) and citric acid (0.0023 g/l). Immediately after mixing the ingredients, 35 ml of gel solution was poured into each gel tray (sandwich assembly) with pre-inserted combs. After gel solidification (about 30 minutes) the combs were removed, creating pre-formed wells. Twenty five µl of enzyme extract supernatant was loaded onto the gel well after adding 15 µl loading dye (20% sucrose, 0.05% w/v bromophenol blue).

After electrophoresis, the gels were removed from the plates. Subsequently, the gel was rinsed twice with distilled water and soaked in enzyme specific staining solution to detect the isozymes. The staining solution consisted of 50 mM sodium acetate (50 ml), α-naphthyle acetate (50 mg), β-naphthyle acetate (25 mg) and Fast Garnet GBC salt 40 g). The staining procedure was carried out in dark conditions until the bands appeared and accordingly gels were fixed in 50% glycerol for 1 hour to preserve the colour reaction product, localizing the position of isozyme bands. Photographs were taken immediately after fixing the gel, placing the gel on a light box.

4.3 Results

4.3.1 *In vitro* embryo culture of *L. albus* and *L. mutabilis* species

A logical step in determining the suitable medium for interspecific hybrid embryo culture is to investigate different compositions of media in which embryos of the parental species grow successfully. Determination of the medium and the conditions in which *Lupinus* embryos could grow was therefore, the first stage of this study. For standardisation of the media, young embryos of globular, heart, torpedo early cotyledonary and late cotyledonary stage were cultured on different media (table 4.1). Although most of the embryos remained green (apparently viable) in liquid media even for 30 days they did not form callus or any shoots. Thereafter, most of the embryos of

selfed crosses of *L. albus* and *L. mutabilis* species were cultured on agar-solidified media supplemented with various concentrations of auxins and cytokinins.

Results from *in vitro* embryo culture of *L. albus* and *L. mutabilis* are presented in the tables 4.3 to 4.6 and plates 4.1A – D and 4.2. Only a few embryos at globular stage were excised from selfed crosses of *L. albus* and *L. mutabilis*. The embryos cultured at globular stage did not grow or form calli in any of the media used. Results from cultures of heart stage embryos are summarised in table 4.3. The heart stage embryos excised from selfed ovules of *L. albus* and *L. mutabilis* species were cultured on the media mentioned in table 4.1 and kept in darkness for 7 – 10 days. Embryos of both species grew on EM1 and EM2 but the growth stopped after the third week of the culture on liquid medium. Embryos of *L. albus* and *L. mutabilis* did not grow on EM3 and EM4 culture media. The percentage of grown embryos of *L. albus* and *L. mutabilis* on EM1 medium was 33.3 % and 44.4 % respectively. The percentage of grown embryos of *L. albus* and *L. mutabilis* on EM2 was recorded as 50 % and 36.36 % respectively. Of the grown embryos of *L. albus* and *L. mutabilis* on EM2 medium, 20 % and 18.2 % stopped growing respectively after three weeks. Only one plantlet each from the grown embryos was obtained but it died soon after its transfer to the green house.

Table 5.4 shows the results from *in vitro* culture of embryos at torpedo stage of their development excised from selfed ovules of *L. albus* and *L. mutabilis* species. The embryos were cultured on five different media. The cultivated embryos were kept in darkness for one to two weeks. On EM1 medium, only 3 (12.5 %) of the cultured embryos of *L. albus* species grew and 21 (87.5 %) did not grow. The embryos stopped growing after three weeks of culture. Embryos of *L. mutabilis* did not grow on EM1 medium. When embryos of *L. albus* and *L. mutabilis* were cultured on EM2 medium, of the total cultured embryos of *L. albus* 75 % (36) did not grow, 25 % (12) grew, 10.41 % (5) stopped growing and 3 (8.33 %) produced plantlets. The cultured embryos of *L. mutabilis* 42 (82.35 %) did not grow, 9 (17.64 %) grew and 4 (7.84 %) produced plantlets. Embryos cultured on the other media either did not grow at all or stopped growing after some time.

Results of cultures from early-cotyledonary stage embryos, which were, excised from selfed ovules of *L. albus* and *L. mutabilis* species are summarised in table 4.5. The embryos were initially cultured on five media and kept in the dark for 5 – 7 days. Of the cultured embryos of *L. albus* 77.7 % did not grow and 22.2 % grew on liquid (EM1) medium. Similar results were observed in the case of *L. mutabilis* embryos. Embryos of both species on EM1 growing turned brown and died after 4 weeks of their culture.

Of the total cultured embryos of *L. albus* and *L. mutabilis* 54.5 % and 24.48 % regenerated on agar-solidified medium (EM2). After three weeks 30.09 % of the grown embryos of *L. albus* and 14.25 % of *L. mutabilis* stopped growing and turned brown. The percentage of plantlets generated from selfed embryos of *L. albus* and *L. mutabilis* was 13.63 % and 10.20 %, respectively. Embryos of both species grew on EM3 for first three weeks but stopped growing afterwards.

Table 4.6 summarises the results of culture of late cotyledonary stage embryos of selfed ovules of *L. albus* and *L. mutabilis* species. Most of the selfed embryos of *L. albus* and *L. mutabilis* species regenerated on EM2 medium without any dark incubation treatment. This may be due to the fact that the embryos were of late developmental stage and cotyledons were green. The embryos also grew on EM1 medium but stopped growing after four weeks. In growing embryos the plumule and radicle started to grow three weeks after culture. The plantlets produced were transferred to soil after 12 – 18 weeks.

4.3.2 *In vitro* immature seed culture

It is apparent from the results of interspecific crosses between *L. albus* and *L. mutabilis* that embryos abort at an early stage of development within 3-5 days after pollination (chapter 3) and too small (early to late globular stage) to be excised and cultured separately. Therefore, the whole immature seeds were excised from hybrid pods and cultured *in vitro*. Immature seeds from pods of interspecific crosses from the 2nd day of pollination until the day when the pods started to show signs of abscission were excised for culture. The media used for this experiment is listed in table 4.2.

4.3.2.1 *In vitro* culture of *L. albus* immature seeds (selfed)

Table 4.7 and plate 4.3A show the results from *in vitro* culture of *L. albus* immature seeds. A total of 253 immature seeds were cultured in ten different media. Of the cultured immature seeds, 160 (63.24 %) did not show any sign of callus formation and only 93 (36.8 %) formed callus. The percentage of immature seeds, which stopped growing after 3-4 weeks of culture, was recorded to be 40 %. A total of 110 immature seeds were cultured on OM2 - OM9 media and the number of grown immature seeds was recorded to be 72 (65.5 %). 21.81 % immature selfed seeds stopped growing after three weeks post culture and only 13.6 % formed callus. The callus produced was transferred to shoot and root-generating medium. The rate of regeneration of immature selfed seeds on liquid media (OM10) was very low compared to agar-solidified media (OM2- OM9). The best results in terms of regeneration of roots and shoots from immature selfed seeds were observed when immature seeds were cultured on a medium (OM2 –OM9) containing agar (8 g/l), sugar (20 mg/l) casein hydrolysate (500 mg/l) supplemented with IAA (0.1 mg/l) and kinetin (0.2 mg/l).

4.3.2.2 *In vitro* culture of selfed immature seeds of *L. mutabilis* species

The results from *in vitro* culture of immature seeds excised from self-pollinated pods of *L. mutabilis* species are presented in table 4.8 and plate 4.3B. Of the total (228) cultured immature seeds, 150 (65.78 %) did not grow at all and only 77 (33.8 %) grew or formed calli. Out of the grown immature seeds 17.54 % stopped growing after four weeks of culture. Of the grown immature seeds 13.2 % formed calli and roots and shoots of 1.75 % of the grown immature seeds regenerated. Immature seeds cultured on liquid media (OM1) remained green even after 30 days of culture but did not show any signs of callus formation. Therefore most of the immature seeds were cultured in medium (OM2 - OM10) with various concentrations of IAA and kinetin. A total of 95 ovules excised from *L. mutabilis* species were cultured on OM2 – OM9 media. The percentage of grown immature seeds was recorded to be 68.4 % (65). After three weeks 36.84 % stopped growing and only 25.3 % formed calli. Roots and shoots were obtained when the calli produced were transferred to media (OM11 and OM12). The highest number of shoots and roots were regenerated from selfed immature seeds of *L. mutabilis* when

cultured on agar-solidified medium (OM9) containing IAA (0.1 mg/l) and kinetin (0.2 mg/l).

4.3.2.3 *In vitro* culture of ovules of interspecific crosses between *L. albus* (♀) and *L. mutabilis* (♂).

The hybrid immature seeds were excised from the pods of interspecific crosses of *L. albus* (♀) and *L. mutabilis* (♂) at an interval of one day starting from as early as 24 hours after pollination till the pods abscised (up to 14 DAP). Due to limitations of time and number of hybrid immature seeds available for culture, hybrid immature seeds were mostly cultured using medium OM9 (Rashid *et al.,* (1989) supplemented with different concentrations of IAA and kinetin as this was found to be effective for culturing selfed embryos/ immature seeds of *L. albus* species in this study.

Table 4.9 and plate 4.4 and plate 4.6 present the results of *in vitro* culture of immature seeds excised from interspecific crosses between *L. albus* (♀) x *L. mutabilis* (♂). A total of 289 ovules were excised from the crosses when *L. albus* was used as seed parent. The percentage of immature seeds, which did not grow varied from 66 to 86 %. The percentage of immature seeds grown varied from 14 to 27 %. 3 to 19 % of the grown immature seeds formed calli and 3–4 % immature seeds produced shoots subsequently. The best results in terms of shoot generation (4.25 %) were obtained from calli produced from the cross between LA003 (♀) and LM003 (♂) when the culture medium contained IAA (0.1 mg/l) and kinetin (0.2 mg/l).

4.3.2.4 *In vitro* culture of ovules of interspecific crosses between *L. mutabilis* (♀) x *L. albus* (♂).

The hybrid immature seeds were excised from the pods of interspecific crosses of *L. mutabilis* (♀) and *L. albus* (♂) with the interval of one day and starting from 24 hours after pollination till the pods abscised (up to 14 DAP). As the number of hybrid immature seeds available for culture was very limited therefore, the excised ovules were mostly cultured in the media previously optimised for culturing of selfed immature seeds of *L. mutabilis* species.

Table 4.9 and plates 4.5 and 4.7 summarise the results of *in vitro* culture of hybrid immature seeds excised from interspecific crosses of *L. mutabilis* (♀) and *L. albus* (♂) species. A total of 119 immature seeds were excised from this cross. The percentage of immature seeds, which did not grow, varied from 56 to 100 %. The percentage of grown immature seeds was observed to be between 16 to 44 %. Of the grown ovules 11 to 12 % formed callus. The best results in terms of callus formation (12 %) were obtained from the cross of LM003 (♀) and LA001 (♂) on a culture medium containing lAA (0.1 mg/l) and Kinetin (0.2 mg/l). Only one shoot was obtained from the calli of the cross LM003 (♀) and LA 001(♂).

An attempt was made to establish the hybridity of the calli formed. An esterase isozyme analysis of the parents and hybrid callus/shoot is presented in the plate 4.8. The plate indicates that the band formation in hybrid callus is different from the parents, which indicates the hybrid nature of the callus/shoot formed.

4.4 Discussion

Results from interspecific hybridisation (chapter 2), and pod/ immature seeds development studies in self and interspecific crosses of *L. albus* and *L. mutabilis* species (Chapter 3) suggested that fertilisation occurred and pods/seeds developed normally for 3 –5 DAP and then embryos started to degenerate. The results also indicated that hybrid embryo abortion was possibly due to endosperm degeneration. Therefore, it seems appropriate to rescue the aborting embryo to obtain a fertile hybrid plant.

In this study the embryo/ immature seeds culture was first standardised with selfed embryos/ovules of *L. albus* and *L. mutabilis* species, because the experiments showed that the large number of embryos/ immature seeds needed for experimentation could not be obtained from interspecific crosses. From the results of embryo (selfed) culture it is apparent that success in plant regeneration through *in vitro* culture of young embryos i.e. globular to torpedo stages was in the range of 0 – 10 % and that of early cotyledonary-stage were 0–13 %. In the present study *L. albus* showed a higher degree of regeneration (13 %) from the embryo culture as compared to *L. mutabilis* (12 %). However,

regeneration of late cotyledonary stage embryos of *L. mutabilis* was higher (22 %) than *L. albus* (19 %). The embryo receives nutrients and solutes for its growth from maternal tissues and endosperm through the suspensor (Nagl, 1990). Studies on embryo development in *Cicer* species (Taheri, 2000) indicated that many hybrid embryos failed to develop due to abnormalities in the suspensor. However, in the present work embryos with broken suspensors were regenerated which indicated that embryos received the essential nutrients. Shoots with well-developed root systems in both species were obtained from cultured embryos of the heart stage or later only and transferred to soil. The plantlets obtained from embryos of *L. albus* and *L. mutabilis* at heart stage did not survive when transferred to a hardening chamber with controlled humidity. Although the shoots regenerated from young embryos were vigorous and had good root systems, unfortunately, they did not survive after their transfer to the soil. Considerable difficulties were experienced in excising the young embryos at earlier stages from selfed immature seeds, therefore; only a few embryos were transferred to nutrient medium. Nonetheless, the embryos at globular stage did not survive in any of the media used.

The results of embryo culture indicate that media of Rashid *et al.,* (1989) used here were suitable for generating plants from cultured embryos of *L. albus* and *L. mutabilis* species. However, some modifications in the concentrations of IAA and kinetin were made to suit the materials used for this study. An optimal balance of growth hormones is an essential requirement for differentiation of immature embryos into both shoot and root and eventual regeneration into plantlets. Growth regulators have been extensively used in embryo culture studies. Since the effect of these substances is not nutritional, it is likely that their action is related to osmotic concentration and they are linked in some way with cell permeability and uptake of ions (Sharma *et al.,* 1996). The agar-solidified medium (EM2) containing IAA and kinetin appeared to have essential nutrients to promote shoot and root proliferation. When agar was omitted from the culture medium (EM1 & EM5) a slow growth of embryos was observed.

Early embryo abortion seems to be the case in interspecific crosses of *L. albus* and *L. mutabilis* species. The hybrid immature seeds excised from pods of interspecific crosses were very small or shrivelled and concomitantly resulted in small embryos. Therefore, it was not possible to excise the hybrid embryos from them. Embryo culture techniques

may be more useful to overcome the post-fertilisation barriers in the genus *Lupinus* if it were possible to promote the size of hybrid embryos *in vivo* to the stage that they were big enough to be excised and cultured *in vitro*. Badami *et al.*, (1997) managed to obtain hybrid ovules of 7 – 8 mm in length from pods 18 DAP through application of growth regulators to the cross pollinated flowers of crosses between *Cicer* species. Similarly, Mallikarjuna (1999) was able to excise and culture hybrid immature seeds of *C. arietinum* x *C. pinatifedum* from 18 – 25 DAP. After 30 days the embryos were dissected and transferred to a fresh medium. Mallikarajuna (1999) reported that the degree of success in regenerating the hybrid ovules was also dependent on the type of parental genotypes used. In the present work, although growth regulators were applied to the pods, most of the hybrid pods aborted within 5 - 10 DAP. Hybrid immature seeds excised at this stage were 2 - 4 mm in length and did not grow much in the media. Attempts were made to excise embryos from developing seeds *in vitro* but the seeds had become brittle and the embryos were readily damaged. Results from a study of hybrid embryo development with and without application of growth regulators (Chapter 2) showed that hybrid embryos did not develop further than the globular stage. This may suggest that genetic incompatibility between the species is, at least partially the reason for embryo abortion so that the use of exogenous hormones could not help in rescuing the hybrid embryos. Therefore, whole hybrid immature seeds were cultured *in vitro*.

OM9 medium used for culturing embryos of *L. albus* and *L. mutabilis* species was also found suitable for culturing immature seeds of these species. In the present work shoots and roots were regenerated from the cultured immature seeds of the species on agar-solidified medium (OM9). The success in generating plants from selfed immature seeds of *L. mutabilis* (4 %) was higher than *L. albus* (3 %). The liquid media used in this study for culturing selfed immature seeds of *L. albus* and *L. mutabilis* species did not give satisfactory results. Most of the selfed immature seeds cultured on liquid media turned brown and died.

As mentioned, the modified medium of Rashid *et al.*, 1989 (OM9) was used for culturing most of the hybrid ovules of *L. albus* and *L. mutabilis* because embryos from the species were successfully grown on this medium. When immature seeds were cultured on a filter paper bridge in liquid media (OM1 and OM10) only three seeds of the cross between *L.*

albus (♀) x *L. mutabilis* (♂) formed calli and produced shoots. It may be worthwhile to try this technique with different media composition for culture of *Lupinus* immature seeds in future work. Mallikarjuna (1999) reported plant regeneration from interspecific hybrids of *Cicer* species through ovule-embryo culture on a filter paper bridge using the liquid medium. Taheri (2000) also obtained similar results while working with *Cicer* species.

In the medium OM9 hybrid immature seeds excised from the cross combination *L. albus* (♀) x *L. mutabilis* (♂) showed a higher degree of callus formation (8.3 %) compared to *L. mutabilis* (♀) x *L. albus* (♂) (7.5 %). However, due to limited time and scarcity of hybrid immature seeds the number of immature seeds cultured was restricted. In general, 7 – 8 % of the cultured immature seeds of the two cross combinations (*L. albus* (♀) x *L. mutabilis* (♂) and *L. mutabilis* (♀) x *L. albus* (♂) formed calli. Shoot initiation was observed only in the immature seeds of the cross *L. albus* (♀) x *L. mutabilis* (♂) following callus formation but did not regenerate into shoots and/or roots. This needs further work.

Since there was no root formation in the calli produced from hybrid ovules of the cross between *L. albus* (♀) x *L. mutabilis* (♂) an attempt was made to establish the hybridity of the callus/shoot using isozyme method. However, due to scarcity of hybrid material, a comprehensive isozyme analysis of the hybrid callus/shoots could not be carried out. The characterisation of the hybrid material produced through *in vitro* culture of embryos/ immature seeds is always difficult due to the limited number of plantlets, as previously reported by Atkins *et al.*, (1998). In the present study, limited number of hybrid immature seeds produced shoots, which restricted the isozyme studies to one enzyme (Estrase) only. In addition, the problem of fungal infestation was experienced throughout the course of *in vitro* embryo/ immature seeds culture investigations that further reduced the number of hybrid shoots formed. However, isolation of Estrase of the hybrid calli and shoots through PAGE revealed the different pattern of band formation, which indicates the different genetic composition of the hybrid material than those of the parents. Further work is needed to improve the method to make any conclusive identification of hybrids using isozyme analysis.

It appears from the study that the culture of very young embryos is difficult but if embryo growth of interspecific crosses of *L. albus* and *L. mutabilis* can be sustained until the heart shaped stage then the hybrid embryo/ immature seeds could be cultured and shoots and roots can be obtained in an appropriate medium. Further work is needed to standardise the method for a regular culture, and root and shoot regeneration to establish hybrid plant(s).

Table 4.1: Media used for *in vitro* embryo culture of *L. albus* and *L. mutabilis*.

Medium 1(EM1)	Medium 2 (EM2)	Medium 3 (EM3)	Medium (EM4)	Medium 5 (EM5)	Medium 6 (EM6)
Gamborg's B5 = 3.08 gr. Agar = 7 gr. Sugar = 20 gr. + Liquid layer Gamborg's B5 = 1.5 conc. Glutamine = 15 mg. Casmino acids = 725 mg GA3 = 0.525 mg IBA = 1.5 mg pH = 5.5	M & S = 4.7 g Agar = 8 gr. Sugar = 20 gr. Casein acid hydrolysate = 500 mg IAA = 0.1 - 10 mg Kinetin = 0.1 – 10 mg pH = 5.8	Gamborg's B5 basal salts = 3.1 gr. Iron EDTA: [Na2EDTA,2H2O = 37 mg FeSO4, 7H2O] = 28 mg NH4NO3 = 400 mg L-glutamine = 1000 mg Nicotinic acid = 5 mg Myo-inositol = 100 mg Thiamine HCl = 1.0 mg Pyridoxine = 0.5 mg Casein hydrolystae = 1000 mg N6-benyladenine (BA) = 0.028 mg Sucrose = 30 g Difco, Bacto-agar = 8 g pH = 5.8	M&S = 4.7 gr. Agar = 8 gr. Sucrose = 30 gr. BAP = 2 mg Adenine sulphate = 40 mg Kin = 0.5 mg pH = 5.8	M& S = 3.5 g Glutamine = 7.5 mg Casmino acids = 362.5 mg IAA = 1 mg Zeatin = 0.5 mg pH = 5.6	M&S basal salt = 4.7 g Sucrose = 30 g

All media are made per litre
M1= Modified media of Kasten and Kunert (1991).
M2= Modified media of Rashid (1988).
M3= Modified media of Mergeai et al. (1997)
M4= Modified media of Gupta et al., (1998)
M5= Modified media of Malikarjuna (1999)
M6= Hormone and vitamin free ½ strength B5 basal liquid medium

Table 4.2: Media used for *in vitro* immature seed (selfed and hybrid) culture of *L. albus* and *L. mutabilis*.

Medium No	Auxin (mg/l)	Cytokinin (mg/l)	Basal salts (MS or B5) g/l	Agar (g/l)	Casein hydrolysate (mg/l)	Sucrose (g/l)	pH
OM1	GA3 =1.5 ABA= 15	Zeatin = 0.5	B5 = 3.1	-	500	20	5.5
OM2	GA3 =1	TDZ = 0.1	B5 = 3.1	8	500	20	5.8
OM3	IAA = 0.5	KIN = 0.2	MS = 4.7	8	500	20	5.8
OM4	NAA = 0.1	KIN = 0.2	MS = 4.7	8	500	20	5.8
OM5	IAA =0.1	BA = 2	MS = 4.7	8	500	20	5.8
OM6	2, 4-D =2	BA = 2	MS = 4.7	8	500	20	5.8
OM7	2,4-D =2	KIN = 0.2	MS = 4.7	8	500	20	5.8
OM8	GA3 = 1.5	KIN = 0.2	MS = 4.7	8	500	20	5.8
OM9	EM2- Modified media (Rashid, 1988) used for embryo culture						
OM10	EM5 – Modified media (Nalini et al., 1999.) previously used for embryo culture						
OM11	BAP	-----	MS = 4.7	8	----------	20	5.8
OM12	-------	-----	MS = 4.7	8	----------	20	5.8

All media are made per litre
OM1= Modified media of Kasten and Kunert (1991).
OM2 - M9 = Modified media of Rashid (1988).
OM10 = Modified media of Malikarjuna (1999).
OM11 & OM12 = Media for shoot and root generation respectively.

Table 4.3: Results of heart-stage embryos of *Lupinus* species cultured on different media

Medium / Species	Age of excised Embryos (DAP)	No. of excised embryos	Cultured embryos				Grown embryos							
			Did not grow		germinated		stopped growing		contaminated		produced plantlets		transferred to soil	
			no	%	no	%	no	%	no	%	no	%	no	%
EM1														
L. albus	10 - 14	12	8	66.6	4	33.3	4	33.3						
L. mutabilis	13 - 15	9	5	55.5	4	44.4	4	44.4						
EM2														
L. albus	10 - 14	10	5	50	5	50	2	20	0	0	1	10	1	10
L. mutabilis	13 - 15	11	7	63.6	4	36.36	2	18.2	0	0	1	9.09	1	9.09
EM3														
L. albus	10 - 14	11	10	90.9	1	9.09	1	9.09						
L. mutabilis	13 - 15	10	10	100										
EM4														
L. albus	10 - 14	9	9	100										
L. mutabilis	13 - 15	14	14	100										
EM5														
L. albus	10 - 14	13	13	100										
L. mutabilis	13 - 15	11	11	100										

Table 4.4: Results of torpedo-stage embryos of *Lupinus* species cultured on different media

Medium / Species	Age of excised Embryos (DAP)	No. of excised embryos	Cultured embryos				Grown embryos							
			Did not grow		germinated		stopped growing		contaminated		produced plantlets		transferred to soil	
			no	%	no	%	no	%	no	%	no	%	no	%
EM1														
L. albus	13 -19	24	21	87.5	3	12.5	3	12.5						
L. mutabilis	15 - 17	19	19	100										
EM2														
L. albus	13 -19	48	36	75	12	25	5	10.41	4	8.33	3	8.33	1	
L. mutabilis	15 - 17	51	42	82.35	9	17.64	4	7.84	1	1.96	4	7.84		
EM3														
L. albus	13 -19	10	10	100										
L. mutabilis	15 - 17	17	16	94.1	1	5.88								
EM4														
L. albus	13 -19	14	14	100										
L. mutabilis	15 - 17	15	15	100										
EM5														
L. albus	13 -19	27	24	88.8	3	11.11	3	11.11						
L. mutabilis	15 - 17	13	11	84.61	2	15.38	2	15.38						

Table 4.5: Results of early cotyledonary-stage embryos of *Lupinus* species cultured on different media

Medium / Species	Age of excised Embryos (DAP)	No. of excised embryos	Cultured embryos				Grown embryos							
			Did not grow		germinated		stopped growing		Contaminated		produced plantlets		transferred to soil	
			no	%	no	%	no	%	No	%	no	%	no	%
EM1														
L. albus	15 – 20	36	28	77.7	8	22.2	8	22.2						
L. mutabilis	15 – 20	21	18	85.71	3	14.21	3	14.2						
EM2														
L. albus	15 – 20	44	20	45.5	24	54.5	15	30.09	9	20.45	6	13.63	1	2.27
L. mutabilis	15 – 20	49	37	75.5	12	24.48	6	12.2	1	2.04	5	10.20	2	4.08
EM3														
L. albus	15 – 20	28	10	35.71	18	64.28	12	64.28						
L. mutabilis	15 – 20	19	16	84.21	3	15.78	3	15.78						
EM4														
L. albus	15 – 20	8	8	100										
L. mutabilis	15 – 20	15	15	100										
EM5														
L. albus	15 – 20	15	15	100										
L. mutabilis	15 – 20	10	10	100										

Table 4.6: Results of late cotyledonary-stage embryos of *Lupinus* species cultured on different media

Medium / Species	Age of excised Embryos (DAP)	No. of excised embryos	Cultured embryos				Grown embryos							
			Did not grow		germinated		stopped growing		Contaminated		produced plantlets		transferred to soil	
			no	%	no	%	no	%	No	%	no	%	no	%
EM1														
L. albus	18 – 22	42	14	33.3	28	66.6	28	66.6						
L. mutabilis	18 – 25	38	21	55.26	17	44.73	17	44.73						
EM2														
L. albus	18 – 22	36	13	36.11	23	63.88	11	30.55	4	11.11	7	19.44	3	8.33
L. mutabilis	18 – 25	36	7	19.44	29	80.55	16	44.4	5	13.88	8	22.20	2	5.55
EM3														
L. albus	18 – 22	24	24	100										
L. mutabilis	18 – 25	36	36	100										
EM4														
L. albus	18 – 22	26	26	100										
L. mutabilis	18 – 25	21	21	100										
EM5														
L. albus	18 – 22	19	19	100										
L. mutabilis	18 – 25	14	14	100										

Table 4.7: Results of *in vitro* immature seed culture of *L. albus*

Media used	No. of cultured immature seeds	DAP	Cultured immature seeds				Grown immature seeds							
			Did not grow		grown		Stopped growing		Formed callus		Produced plantlets		Transferred to soil	
			no	%	no	%	no	%	no	%	no	%	no	%
OM1	78	2 – 14	75	96.15	3	3.84	3	3.84						
OM2-OM9	110	2 – 14	38	34.5	72	65.5	24	21.81	15	13.6	5	4.54	2	1.81
OM10	65	2 - 14	47	72.30	18	27.7	13	20	4	6.15	1	1.53		
Total	**253**		**160**	**63.24**	**93**	**36.8**	**40**	**15.81**	**19**	**7.50**	**6**	**2.53**	**2**	**0.7**

Table 4.8: Results of *in vitro* immature seed culture of *L. mutabilis*

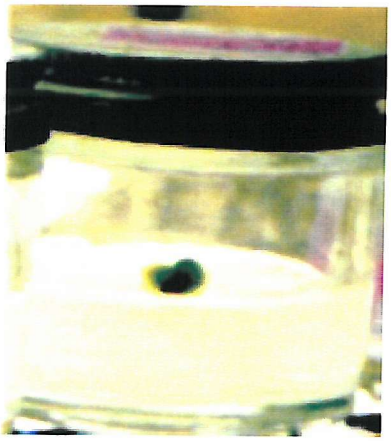
Media used	No. of cultured immature seeds	DAP	Cultured immature seeds				Grown immature seeds							
			Did not grow		Grown		Stopped growing		Formed callus		Produced plantlets		Transferred to soil	
			no	%	no	%	no	%	no	%	no	%	No	%
OM1	80	2 – 14	79	98.75										
OM2-OM9	95	2 – 14	30	31.75	65	68.4	35	36.84	24	25.3	7	7.36	4	4.21
OM10	53	2 - 14	41	77.53	12	22.6	5	9.43	6	11.3	1	1.88		
Total	**228**		**150**	**65.78**	**77**	**33.8**	**40**	**17.54**	**30**	**13.2**	**8**	**3.50**	**4**	**1.75**

OM1 = Modified media of Kasten and Kunert (1991)
OM2 – OM9 = Modified media of Rashid *et al*, (1989).
OM5 = Modified media of Malikarjuna (1999).

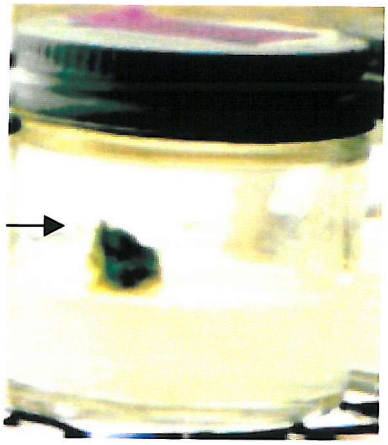
Table 4.9: Results from *in vitro* culture of hybrid immature seeds of *L. albus* (♀) x *L. mutabilis* (♂) *and L. mutabilis* (♀) x *L albus* (♂).

Cross combinations	Media Used	Cultured immature seeds	Age of cultured Immature seeds	Cultured immature seeds				Grown immature seeds					
				Did not grow		Grown		Stopped growing		Formed callus		Produced shoots	
		(No)		No	%	No	%	no	%	no	%	no	%
LA (acc003) x LM (acc003)	OM9	47	8 - 10	32	68.08	15	31.91	6	12.76	9	19.14	2	4.25
		26	2– 10	19	73.07	7	26.92	3	11.53	4	15.38	00	00
	OM10	36	8 – 10	27	75	9	25	6	16.66	3	8.3	1	2.77
		15	6 – 10	10	66.6	5	33.3	4	26.66	1	6.7	00	00
LA (acc004) x LM (accoo1)	OM9	53	8 – 10	45	84.90	8	15.04	4	7.54	3	5.66	00	00
		49	2 – 10	39	79.59	10	20.40	7	14.28	3	6.12	00	00
	OM10	61	8 - 10	52	85.24	9	14.75	9	00	00	00	00	00
		36	2 - 10	31	86.11	5	13.88	4	11.1	1	2.77	00	00
Total		**289**		**255**	**88.23**	**68**	**23.52**	**43**	**14.87**	**24**	**8.30**	**3**	**1.03**
LM (acc003) x LA (acc001)	OM9	13	2 – 6	13	100								
		58	6 – 10	49	84.48	9	15.5	2	3.44	7	12.06	00	00
	OM10	21	2 – 6	20	95.23	1	4.76	1	4.76				
		18	6 – 10	10	55.55	8	44.4	5	27.7	2	11.11	00	00
	OM9	9	13	9	100								
Total		**119**		**101**	**84.87**	**18**	**15.1**	**8**	**6.72**	**9**	**7.56**	**00**	**00**

OM2 = Modified media of Rashid *et al*, (1988)
OM10 = Modified media of Malikarjuna (1999).



A: Callus formation (4 weeks post culture



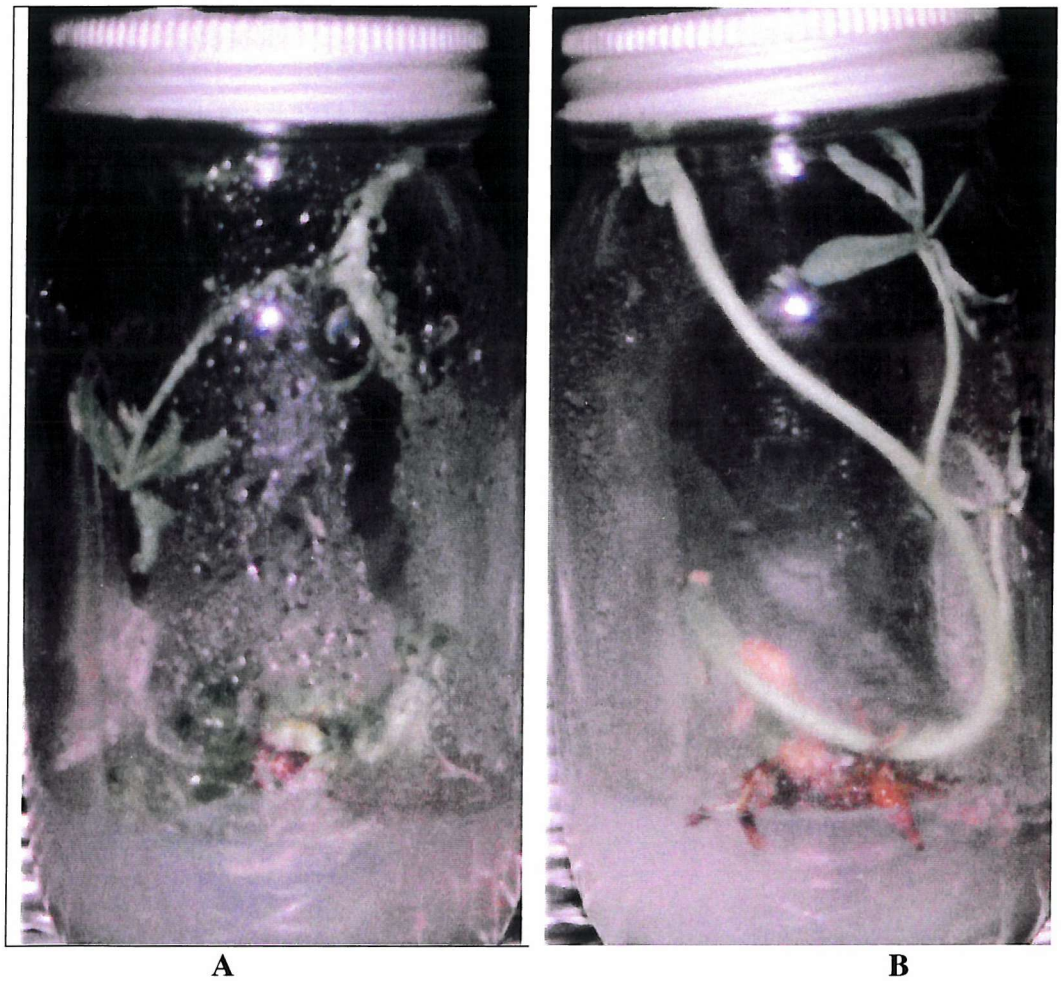
B: Shoot initiation (7 weeks post culture)

C: Shoot generation (11 weeks post culture)

D: Root & shoot formation (18 weeks post culture)

Plate 4.1(A to D): Root and shoot formation in *L. mutabilis* embryo (10 DAP)

Plate 4.2: Root and shoot generation from a selfed embryo (14 DAP) of *L albus* at 10 weeks post culture



A **B**

Plate 4.3: A plantlet produced from ovules of
A: *L. albus* selfed immature seed (14 DAP)
B: *L. mutabilis* selfed immature seed (13 DAP)

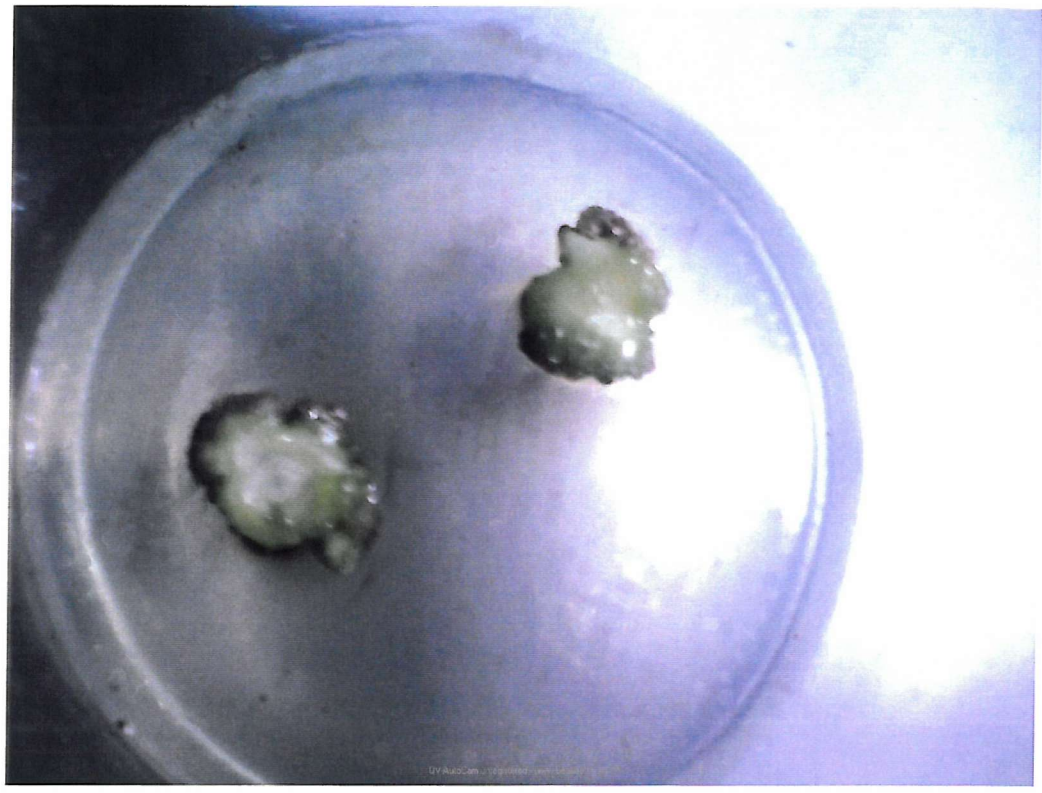


Plate 4.4: Callus formation in an immature seed of interspecific cross between *L. albus* (♀) and *L. mutabilis* (♂) originally excised from a pod 9 DAP

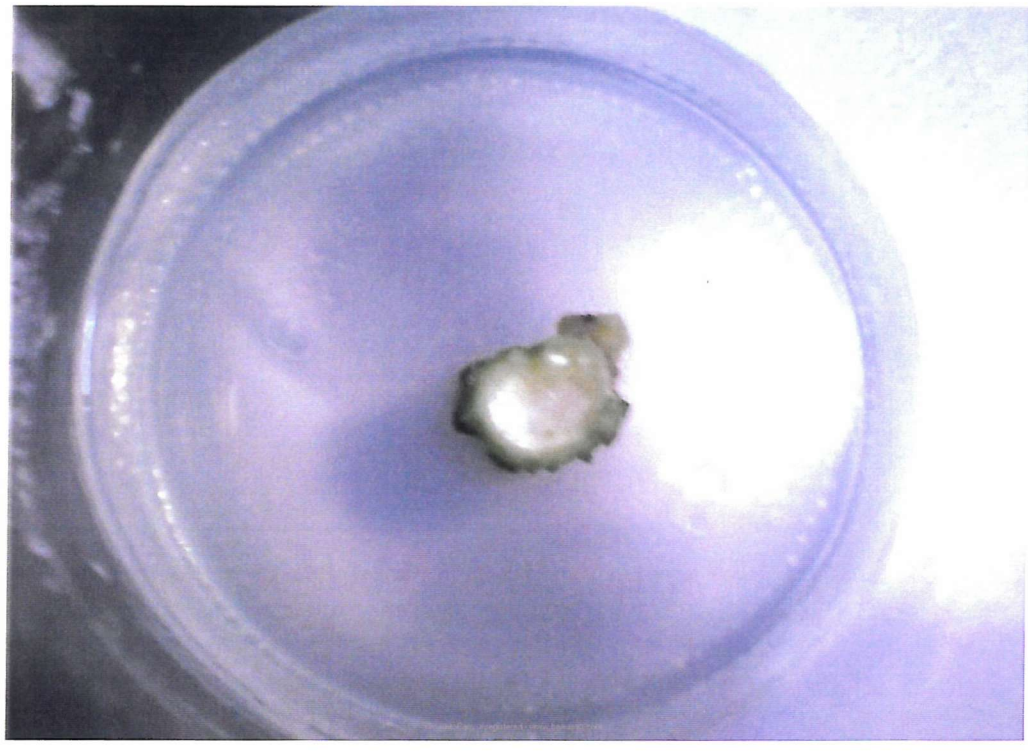


Plate 4.5: Callus formation in an immature seed of interspecific cross between *L. mutabilis* (♀) and *L. albus* (♂) originally excised from a pod 8 DAP.

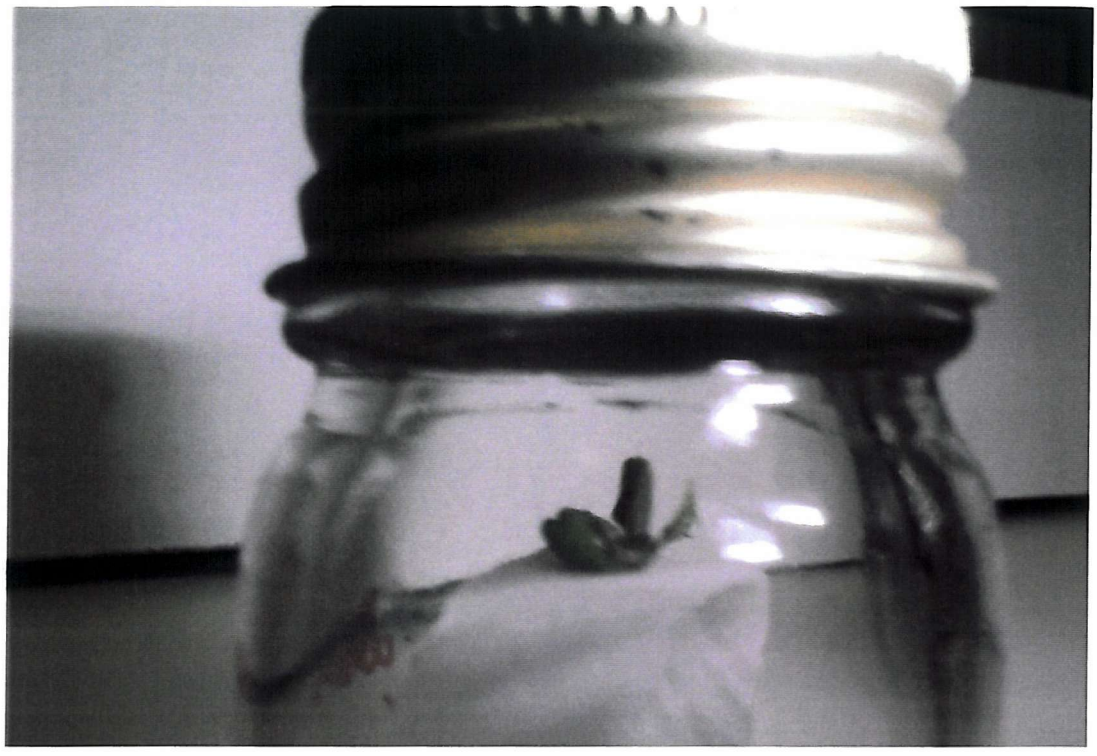


Plate 4.6: Shoot generation from an immature seed of the cross *L. albus* (♀) x *L. mutabilis* (♂) at 6 weeks post culture on a liquid medium and originally excised from a pod 10 DAP.

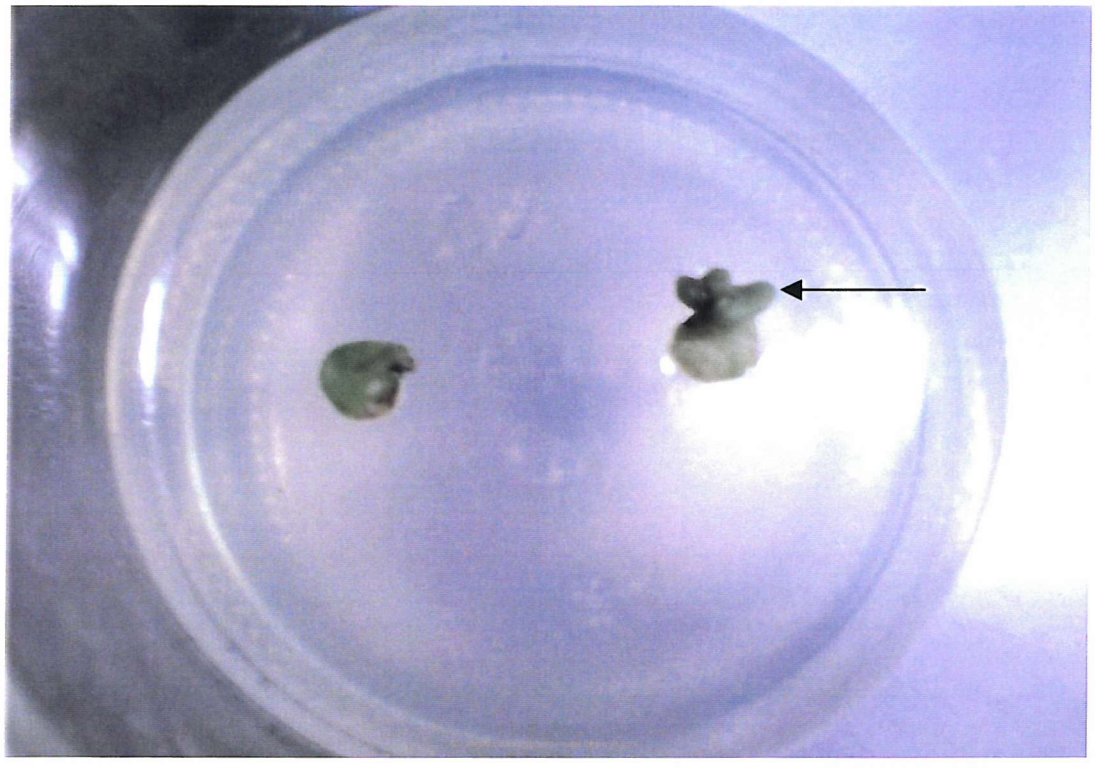


Plate 4.7: Shoot initiation from an immature seed of *L. mutabilis* (♀) x *L. albus* (♂) at 4 weeks post culture and originally excised from a pod 9 DAP.

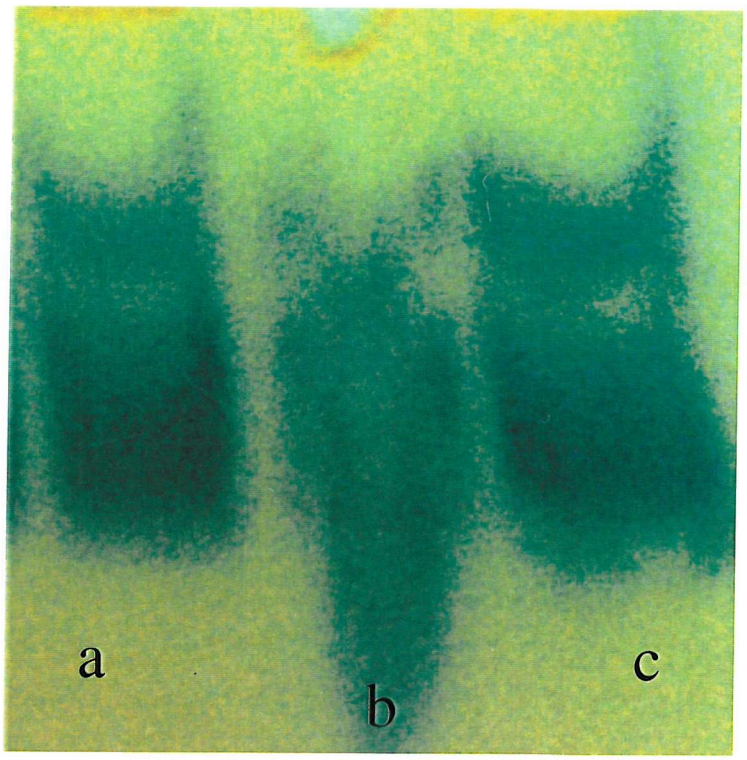


Plate 4.8: Esterase isozyme pattern of the parents and the hybrids. a. *L. albus* (LA001), b. Hybrid callus LA001 (♀) x LM003 (♂), c. *L. mutabilis* (LM003).

Chapter Five

Isolation and fusion of gametes of *Lupinus albus* and *Lupinus mutabilis*

5.1 Introduction

During the past century, significant improvements in crops have resulted from the successful wide crosses (interspecific and intergeneric) between wild and cultivated species (Haq, 1996). Thus, wide hybridisation is a significant plant-breeding tool for modification of crop plants to improve their suitability for cultivation. However, in many plants, cytogenetical and physiological barriers limit the free genetic recombination of species through wide hybridisation. These barriers include: pollen germination, poor/no fertilisation, premature embryo abortion, hybrid sterility, and hybrid break down in F1 generations. Many techniques such as embryo rescue, and other novel techniques such as protoplast fusion, direct gene transfer, *in vitro* fertilisation, and plant transformation have been used to overcome these constraints. *In vitro* fertilisation and regeneration of plants from fused gametes however, have given a new impetus in overcoming the above barriers (Dumas & Russell, 1992; Kranz *et al* 1992; and Dumas *et al.*, 1998). *In vitro* fertilisation methods have already been developed for other plant species such as wheat (Kovcas *et al.* 1995), *Plumbago* (Huang and Russell, 1989) *Zea* (Kranz *et al.*, 1991) and *Nicotiana* (Tian & Russell, 1997). In many species, maize for example, regeneration of plants from isolated, fused gametes is now routine (Kranz *et al.*, 1998).

In the genus *Lupinus* although interspecific hybridisation has been attempted over the past century (Atkins *et al.*, 1998) the crossing behaviour between American species and European species is not fully resolved. The results from previous chapters show that premature embryo abortion in interspecific crosses of *L. albus* and *L. mutabilis* species like other legumes is due to the failure of the endosperm and supporting tissues (Haq *et al.*, 1973; Siamasonta & Calligari, 1998). Attempts were made to rescue the aborting hybrid embryos of various interspecific crosses of the genus *Lupinus* through embryo culture, but apart from limited success reported by Schafer-Menhur *et al.,* (1988); Kasten *et al.*, (1991); and Kasten & Kunert, (1991) most of the attempts failed. This failure, as

indicated earlier, may be attributable to the fact that grain legumes in general are difficult to regenerate *in vitro* (Smartt, 1979; Schafer-Menhur *et al.*, 1991). Furthermore, in interspecific crosses of *L. albus* and *L. mutabilis* species hybrid embryo abortion occurs at an early stage of development and embryos do not reach a size sufficient enough to be excised and cultured (Siamasonta & Calligari, 1998). The results of the interspecific crosses attempted during the course of the present study (chapter 2 and 3) further confirmed the previous reports of embryo abortion. Although success was achieved in culturing the hybrid ovules of interspecific crosses of *L. albus* and *L. mutabilis* species (chapter 4) but the success rate was very low.

Since the embryo abortion at an early stage has been identified as one of the reasons for failure of hybrid embryo development in lupins (chapter 2 and 3) and the success in vitro culture of embryos at early stages has been difficult, an attempt was made to obtain hybrid plant (s) using the *in vitro* fertilisation method. The technique includes the isolation of male and female gametes, in vitro fusion of the gametes and the regeneration of plantlets from fused gametes. Gametes (male and female) of the two species were isolated. *In vitro* gametic fusion of *L. albus* and *L. mutabilis* was attempted using different calcium concentrations and various levels of pH in the culture medium with the aim of achieving genetic recombination of the two species.

5.2 Materials and methods

5.2.1 Isolation of sperm cells

The isolation of sperm cells of *L. albus* and *L. mutabilis* was carried out following the protocol of Tian and Russell (1997). However, some modifications (table 5.1) were made in the concentrations of the ingredients in the culture medium and the agar-solidified medium was replaced with liquid medium. Pollen grains from fully opened flowers were collected in the morning before anthesis (in *Lupinus* species anthesis takes place during 0800 – 0900 hours). The quality of pollen grains was tested using the method described in chapter 2 (section 2.2.7). Pollen grains, with viability of 90 % or above, were collected and cultured in petri dishes containing 2 ml of isolation medium at room temperature (20 ± 2 °C) for 48 hours. The composition of the media used for pollen grain culture is given

in table 5.1. Each treatment was repeated three times to minimise experimental errors. Data on pollen germination, pollen tube growth (length) and number of pollen tubes burst (discharge) after 6,12, 24, 36 and 48 hours of culture were recorded.

Sperm cells were collected from the filtrate after filtering the suspension through 20 - μm gauze. The viability of sperm cells was determined by staining with 0.1 % FDA (diluted in acetone at 1:300 in culture medium) and observed under a phase contrast microscope (Zeiss Axioskop, Germany). The presence of cell wall was detected by analine blue staining (Tanaka, 1988).

5.2.2 Isolation of egg cells

The isolation procedure of egg cells was similar to that used by Kranz *et al.,* (1991) and Tian and Russell (1997). This was carried out in two steps. In the first step, embryo sacs were isolated from unfertilised ovules, which were collected from flowers a day before and after the anthesis. The surface of the ovaries was sterilized with 95 % alcohol for 30 seconds and with 15 % bleach for 5 minutes. About 50 ovules were dissected out from the placenta of young ovaries and put into a 2 ml enzymatic maceration mixture containing 5 mM $CaCl_2$, 5 mM KH_2PO_4, 0.7 mM $MgSO_4$, 3mM 2- (N-morpholino) ethanesulfonic acid and 9 % (w/v) mannitol at pH 5.5. The concentrations of different enzymes used are given in table 5.2. The ovules were incubated at 30 °C for 2, 4, 6, 8 and 10 hours with gentle shaking (30-rpm). After an appropriate incubation period, the embryo sacs were collected from the maceration mixture using a Pasteur pipette.

In the second step, embryo sacs were collected from the first maceration mixture under a stereo-microscope (Nikon, Germany) with the help of a micropipette and transferred into an isolation medium containing enzymes cellulase (1%) and pectinase (0.5%). Then the material was incubated again at 30 °C for half an hour with gentle shaking (30 rpm). The suspension of loose cells and protoplasts was analysed to determine the number of egg cells using light and fluorescence (FDA) microscopy (Heslop-Harrison *et al.,* 1984). The egg cells were then isolated from the rest of the cells using a very thin glass rod and the comparative yield of the isolated egg cells was estimated by the following formula:

$$Yield\ of\ isolated\ egg\ cells\ (\%) = \frac{Number\ of\ egg\ cells\ isolated}{Number\ of\ ovules\ treated} \times 100$$

5.2.3 *In vitro* gametic fusion

The fusion of isolated gametes of *L. albus* and *L. mutabilis* species was carried out following the protocol of Kranz & Lorz, (1994) for maize. The fusion of an egg and a sperm protoplast was performed in two separate sets of experiments. In the first set of experiments, gametic fusion was attempted by varying the calcium concentration (5 - 15 mM $CaCl_2$) in the fusion medium while keeping the pH constant (pH = 5.5). In the second set of experiments calcium concentration was kept constant while pH of the medium was varied from 5 to 11. The fusion medium contained 5 mM potassium hydro phosphate (KH_2PO_4), 3 mM 2 – (N-morpholine) ethane sulfonic acid (MES), 5 % mannitol, 15 % polyethylene glycol mannitol solution, 3 % sucrose, and 6.5 % glucose. A droplet (about 1 µl) of fusion solution was placed on a microscopic slide with two cavities on it. The isolated sperm and egg protoplasts were transferred into the fusion medium with the help of a micropipette. The *in vitro* gametic fusion process was carried out under a light microscope (Leitz- Diplan, Germany) interfaced to a computer with Image Pro Plus software. A micromanipulator (Etabls, Beaudouin, Paris, France) was used to align two protoplasts together. After fusion the fused gametes were transferred to a culture medium containing M&S basal salt (Murashige and Skoog, 1962) supplemented with 3 % sucrose, 3 % glucose, polyethylene glycol (PEG 5 %), and mannitol 7 % (Kao and Micayluk, 1975).

5.2.4 Regeneration of fused gametes

Regeneration of fused gametes was attempted following the method described by Kranz and Lorz (1993). The culture medium contained MS (Murashigue and Skoog, 1962) basal salt (4.7 g/l), NAA (1.0 mg/l), 2, 4-D (0.5 mg/l), sucrose (3 % w/v), glucose (3 % w/v) and agarose (0.5 % w/v). The pH of the medium was adjusted to 5.8. The culture medium was sterilised by autoclaving the jars (15 ml) containing 10 ml of media at 120 °C. The fused gametes were picked up from the fusion medium with the help of a pasture

pipette and transferred into jars under sterile conditions. The material was then incubated in complete dark at 25± 2 °C.

5.3 Results

5.3.1 Sperm cell isolation

The results from experiments on *in vitro* isolation of sperm cells of lupins are presented in tables 5.3 to 5.6, figures 5.1 to 5.12, and plates 5.1 to 5.4. In general, the diameter of pollen tubes averaged about 30 µm. After 2- 3 hours of *in vitro* pollen culture the sperm cells were visible near the tip of the pollen tube (plate 5.1). Sperm cells were released from pollen tubes through osmotic shock induced by the isolation medium (plate 5.2). After isolation, the sperm cells were found to be FDA positive and showed dense cytoplasm (plate 5.4). No positive reaction was observed when isolated sperm cells were stained with analine blue.

In vitro isolation of large quantities of mature, viable, male gametoplasts depends on a number of factors such as pollen germination, pollen tube growth, and pollen tube discharge. The composition of the medium was found to be crucial for *in vitro* pollen germination, pollen tube growth and pollen tube discharge. Maximum pollen germination, pollen tube growth and pollen tube discharge (burst) was observed when pollen grains were cultured in a medium containing 0.03 % (w/v) H_3BO_3, 0.04 % $CaCl_2$, 0.01 % (w/v) KH_2PO_4, and 10 % (w/v) sucrose. In lupins, fertilisation (*in vivo*) occurs between 24 to 36 hours after pollination, therefore, to achieve the *in vitro* conditions comparable to those *in vivo*, the culture medium was optimised for best results after 24 hours of culture. The effects of various concentrations of boric acid, calcium chloride, potassium phosphate and sugar on pollen germination, pollen tube growth, and pollen tube discharge after 24 hours of incubation are discussed as follows:

5.3.1.1 Effect of boric acid on pollen germination and pollen tube discharge

The effect of boric acid concentrations on pollen germination, pollen tube length and pollen tube discharge are presented in the table 5.3 and figures 5.1 to 5.3. Maximum pollen grain germination (98.7 %) and pollen tube length (255μm) was observed when boric acid was used at a minimum concentration (0.03 %). When the concentration of boric acid was increased the pollen germination decreased, which indicates that higher concentration of boric acid has a negative effect on pollen germination. Interestingly the highest percentage of pollen tube burst (release of sperm cells) was observed when no boric acid was used. The results indicate that a high concentration of boric acid was not suitable for pollen germination.

5.3.1.2 Effect of calcium chloride on pollen germination and pollen tube discharge

Results from this experiment are presented in table 5.4 and figures 5.4 to 5.6. After 24 hours of *in vitro* culture of pollen grains maximum pollen germination (100%), pollen tube growth (190 μm) and pollen tube burst (100 %) were observed when calcium was used at 0.04 % (w/v) concentration. The results confirmed the previous reports on the appropriate calcium concentration for isolation of sperm cells (Tian and Russell, 1997). Abnormal pollen tube growth and pollen tube burst was observed in the same medium where no calcium was used. Abnormal pollen tube elongation, swelling and pollen tube breakage other than the tips was observed when calcium was used at lower concentrations (Plate 5.3).

5.3.1.3 Effect of potassium phosphate on pollen germination and pollen tube discharge

Results from the experiment are given in the table 5.5 and figs. 5.7 to 5.9. After 24 hours, maximum pollen germination (92.87 %), pollen tube burst (83.94 %) and pollen tube length (116 μm) was observed when potassium phosphate in the culture medium was used at 0.01 % (w/v) concentration level. It appeared that potassium plays a vital role in pollen germination as no pollen germination was observed when potassium was omitted from the isolation media. It was also observed that higher concentrations of potassium in

the culture medium had negative effect on pollen germination. Pollen germination decreased when potassium concentration was increased in the isolation medium.

5.3.1.4 Effect of sucrose on pollen germination and pollen tube discharge

Table 5.6 and figures 5.10 to 5.12 summarise the effects of different concentrations of sucrose in the isolation media, on *in vitro* pollen germination, pollen tube length and pollen tube discharge (burst). The optimal concentration of sucrose in isolation media was found to be 10 % (w/v) for pollen germination. At this concentration of sucrose the pollen grains germinated even after 6 hours of culture. When sucrose was used at 10 % concentration level maximum pollen germination (100 %), pollen tube length (130 μm) and pollen tube discharge (63.35 %) was observed after 24 hours of culture.

5.3.2 Egg cell isolation

The results from the egg cell isolation experiments are presented in the table 5.2 and plates 5.5 to 5.9. When the ovules were subjected to enzyme incubation, the ovules disintegrated into loose cells and embryo sacs were visible (Plate 5.5 and 5.6). Embryo sacs were released after five hours of incubation with all concentrations of enzymes except when cellulase was used at 0.5 % (w/v) or below. The egg cells in embryo sacs could easily be distinguished from the bulk of the saprophytic cells by their size, shape and appearance. Following the mechanical manipulation of embryo sacs the egg cells were isolated and detected as single spherical cells (Plate 5.7 - 5.9). Egg cells were always detected as a single spherical cell, which may be confused with synergids, as they also retain spherical shape after isolation (Plate 5.8). However, synergids were smaller in size (2.5 - 3 μm) as compared to egg cells (6 – 7μm). All the contents of the embryo sac were isolated successfully through enzymatic maceration followed by mechanical manipulation. The highest number of egg cells (20 %) were obtained when ovules were incubated for 5 hours with 1 % cellulase, 0.5 % pectinase, 7 mM $CaCl_2$, 5 mM KH_2PO_4, 0.7 mM $MgSO_4$, 3 mM MES {2 – (N-morpholine) ethane sulphonic acid} and 10 % mannitol mixtures (table 5.2). In general 10 – 15 (20 – 30 %) egg cells were obtained from 50 ovules. A positive correlation was found between embryo sac/egg cell yield and enzyme concentrations. When the concentration of enzymes, especially cellulase in

combination with pectinase was increased then the released number of embryo sacs also increased for less incubation time. The effects of other components of maceration solution were not significant compared to enzymes concentrations. After staining with FDA a large faintly fluorescing nucleus was observed and thus egg cells were identified. The egg cells, which showed brighter fluorescence after one hour of isolation, were considered to be viable.

5.3.3 Fusion of gametes

Results from *in vitro* gametic fusion experiments are presented in tables 5.7 and 5.8 and plates 5.9 & 5.10. The fusion process itself was very rapid and fusion occurred within two seconds. The best results in terms of fully fused gametes (46.15 %) were obtained by using a fusion medium containing 10 mM calcium chloride. It was observed that when the calcium concentration was higher than 10 mM in the fusion medium, gametoplast movement became difficult and also gametoplasts tended to shrink. The highest percentage of fused gametes (53.84 %) was achieved when a fusion medium containing 10 mM concentration of calcium chloride and the pH was 7. No fusion was observed when the pH of the medium was less than 7 and the number of fully fused gametes reduced as the pH was increased.

5.3.4 Regeneration of fused gametes

Due to time constraints a limited number of attempts were made to culture the fused products *in vitro*. It was observed that most of the cultured products were either contaminated or did not show any sign of callus or shoot formation. A step towards the establishment of an *in vitro* system for culturing the fused gametes of *Lupinus* species comparable to those in animal biology (Yanagimachi, 1994) will be extremely useful to achieve the genetic recombination of *L. albus* and *L. mutabilis* species.

5.4 Discussion

The methods such as *in vitro* fertilisation and regeneration of plants from fused gametes have opened up new ways for direct gene transfer between different crop plants (Dumas *et al.,* 1998). The objective of this work was to develop optimal techniques for isolation of gametes and *in vitro* fertilisation to facilitate gene transfer between *L. albus* and *L. mutabilis* species. A method was developed to isolate viable sperm and egg cells of *Lupinus* species and *in vitro* gametic fusion was attempted using various levels of calcium and pH in the isolation medium.

In the present study *in vitro* pollen grain culture method was chosen for the isolation of sperm cells due to the fact that in the family *Fabaceae*, sperm cells are formed in growing pollen tubes (Russell, 1998). From the pollen tubes sperm cells were isolated through osmotic shock induced by the culture medium. Isolation of large numbers of sperm cells from tricellualr pollen species through osmotic shock have been reported for *Plumbago* (Russell, 1986) and maize (Dupuis *et al.*, 1987).

A number of parameters were tested for pollen germination and pollen tube discharge (release of sperm cells from pollen tubes) *in vitro*. In this study the most important factor recognised in the pollen germination of *Lupinus* species was that of potassium phosphate and calcium chloride, and their concentrations in the isolation medium. When potassium was omitted from the isolation media no pollen germination was observed. Abnormal swelling, pollen tubes bursts from the points other than the tips were observed when calcium concentration in the same medium was kept lower than 0.04 % (w/v). The role of calcium in fertilisation of gametes is well documented (Dumas *et al*, 1998). Mo and Young (1992) obtained large numbers of purified sperm cells by incorporating calcium in the growth medium. Tian and Russell (1997) reported similar results. However, the significance of potassium for germination of pollen grains of *Lupinus* species *in vitro* was realised first time in this study.

The diameter of the pollen tubes averaged about 30 µm. and the two sperm cells were located near the tip of the pollen tubes (plate 5.6). At initial stages the sperm cells in pollen tubes were visible as spherical shaped and covered by a cytoplasmic layer. But

when the sperm cells were released from the pollen tubes after ~ 2 seconds they became circular shaped (Plate 5.4). In most plant species, the *in situ* sperm cells are spindle shaped and loose their shape during or just after isolation (Russell *et al.*, 1990). Loss of connection between two sperm cells and change in the shape may be due to breakdown of microtubular cytoskeleton (Tanaka, 1988). Theunis *et al.*, (1991) reported similar observations in *Spinacia oleracea.*

The cytological characterisation of sperm cells was necessary for identification and viability of sperm cells. When the sperm cells are in the natural conditions *(in situ)*, their lifetime can be long. However, when isolated *in vitro* the male gametes may loose viability. Therefore, viability of the isolated sperm cells of *Lupinus* species was tested by staining with FDA (Fluroscein diacetate). Further characterisation of sperm cells of *Lupinus* species was carried out using analine blue stain. The negative results of the analine blue stain indicate the absence of callose and it may be concluded that male gametes of lupins have no cell walls and are prepared to fuse with female target cells. This has also been reported for other species such as *Zea* and *Lolium* (Dumas *et al.*, 1998).

The female gametes (egg cell) of *Lupinus* species, like most of the angiosperms, are surrounded by thousands of cells, which form ovules. Therefore, the direct access to the female gametes of lupins is difficult and the egg cells can only be released either by micro-dissection or by enzymatic maceration of ovules. Embryo sac isolation is a prerequisite for egg cell isolation therefore, prior to obtaining the egg cell; it is necessary to isolate the embryo sac from the ovule. The yield of embryo sac depends on many factors like time of ovaries/ovule collection (anthesis), concentration of enzymes, and incubation period. In this study the results of experiments on embryo/egg cell isolation of lupins indicate that the efficiency of embryo/egg cell isolation was higher when the incubation period was higher. However, the longer incubation period may be harmful for the viability of egg cells. On the other hand, yield of egg cells was low when incubation period was less than one hour. Therefore an appropriate incubation period (5 hours) was necessary to isolate embryo sacs in viable and in tact original shape (Plates 5.1 and 5.2).

For isolation of the egg cells from isolated embryo sacs two techniques were used: (1) isolated embryo sacs were transferred into a new enzyme solution containing cellulase and pectinase to remove the embryo sac cell wall. (2) mechanical manipulation of the isolated embryo sacs. However, in this study incubation period of embryo sacs with enzymes had to be kept to a minium (30 minutes) as longer incubation periods caused other sporophytic cells to lose their cell walls and become protoplasts, which made egg cell protplast identification difficult.

The isolated egg cells of *L. albus* and *L. mutabilis* species were recognised as spherically shaped. The egg cells were identified among the saprophytic cells originating from the macerated ovules because of the specific cellular organisation. The granular structure of the cytoplasm is probably due to starch grains (Plate 5.5), as has been reported previously for egg cells of maize (Kranz *et al.,* 1993), ryegrass (van der Mass, *et al*, 1993), and tobacco (Tian & Russell, 1997). The faint fluorescent of the nucleus after DNA staining indicates the presence of a large nucleus, which is a characteristic feature of the nucleus of egg cells in angiosperms (Willemse and van Went, 1984).

A number of methods such as fusion of isolated gametes mediated by calcium and pH level (Faure *et al.*, 1994; Kranz and Lorz, 1994), and electro-fusion (Kranz *et al.,* 1991a, b) have been developed for *in vitro* fertilisation in angiosperms (Rougier, *et al.,* 1996; Dumas *et al.*, 1998). In this work however, *in vitro* fusion of isolated gametes was attempted using various levels of calcium and pH in the fusion medium. This was due to the fact that unlike electro-fusion, fusing gametes *in vitro* conditions using high calcium and pH in the fusion medium gamete specificity can be retained (Faur *et al.*, 1994). Using 5 mM calcium in the fusion medium Faur *et al.*, (1994) obtained 80 % fused pairs of maize gametes.

In the present study various concentrations of calcium were used in the fusion experiments of *Lupinus* species and the frequent fusion was observed when calcium concentration was fairly high (10 mM) in the fusion medium. A higher concentration of calcium (10 – 50 mM) in the fusion medium reportedly promotes *in vitro* gametic fusion (Kranz and Lorz, 1994; Dumas *et al.*, 1998). However, the results of *in vitro* gametic fusion experiments carried out on isolated gametes of *L. albus* and *L. mutabilis* species

indicate that fusion occurred neither on higher nor on lower but at neutral pH (7). This may be due to the fact that biological functions of the gametes themselves help in the fusion process. The second reason for this fusion at neutral pH may be due to the electrostatic charges on the gametes. Furthermore, in the present work high calcium concentrations lead sperm and egg cells of lupins to shrink and made the movement of gametoplasts difficult. Faure *et al.*, (1994) obtained frequent fusion of maize gametes in fusion media containing 5 mM calcium. The involvement of calcium ions in membrane fusion is well known and has been reported in earlier reports on somatic protoplast fusion (Keller and Melchers, 1973). Therefore, it may be suggested that the technique needs to be improved considerably specially the gametoplasts alignment.

In conclusion, methods for the isolation of male and female gametes and in vitro fertilisation of *Lupinus* species have been developed. The methods could be extremely useful to exchange the genetic materials between different *Lupinus* species and could also be extended to other leguminous species. The fused zygotes have potential to organize and develop into new plants with *in vitro* methods. In addition, the techniques open up new way for chromosome transfer between two gametes and create new opportunities to study the interaction between nucleus and the cytoplasmic organelles (Dumas *et al.*, 1998). Although *in vitro* gametic fusion was achieved using different calcium concentrations and various levels of pH in the fusion medium however, characterization of calcium-fused zygotes and their regeneration from embryos to fertile plants need further work. The recent progress in *in vitro* fertilisation of single isolated sperm cell and central cells of maize (Kranz *et al*, 1998) has shown possible mechanisms of adhesion, recognition, fusion and early embryo development from fused gametes. This would provide adequate opportunities to manipulate isolated and fused gametes for introgression of desirable traits from one species to the other in *Lupinus* species.

Table 5.1: Media composition for *in vitro* pollen grain culture for sperm cell isolation

Medium	Agar	H_3BO_3	$CaCl_2$	KH_2PO_4	Sucrose
	(%)	(%)	(%)	(%)	(%)
M1(C*)	4	0.01	0.04	0.01	15
M2	-	0.01	0.04	0.01	15
M3 - M8	-	0.01	0.04	0.01	0 - 25
M9 - M14	-	0.01	0.04	0 - 0.1	10
M15 - M20	-	0.01	0 - 0.04	0.01	10
M21 - M25	-	0 - 0.9	0.04	0.01	10
M26	-	0.03	0.04	0.01	10

C* = Control

Table 5.2: Concentrations of enzymes in the maceration medium and their relationship to embryo sac/egg cell isolation in *Lupinus* species

Treatment	Cellulase	Pectinase	Pectolyase	No of ovules	Embryo sacs released		Egg cells isolated	
	(%)	(%)	(%)	macerated	No	%	No	%
1	0.65	0.55	0.25	50	2	4	1	2
2	0.5	0.55	0.25	35	2	5.71	0	0
3	0.6	0.55	0.25	45	5	11.11	3	6.66
4	0.7	0.55	0.25	45	12	26.66	5	11.11
5	0.8	0.55	0.25	50	25	50	14	28
6	0.9	0.55	0.25	40	14	35	5	12.5
7	1	0.55	0.25	35	11	31.42	7	20
8	2	0.55	0.25	20	7	35	2	10

Table 5.3: Effect of different concentrations of boric acid on *in vitro* pollen germination, pollen tube growth and pollen tube discharge (burst)

Boric acid (%)	Time after culture											
	6 hours			12 hours			24 hours			48 hours		
	Ger. (%)	Burst (%)	Ptl (μm)	Ger. (%)	Burst (%)	Ptl (μm)	Ger. (%)	Burst (%)	Ptl (μ)	Ger. (%)	Burst (%)	Ptl (μ)
0.00	60.01	72.20	62.20	86.55	24.98	167.00	94.37	91.54	178.00	95.65	86.14	211.00
0.03	93.50	74.15	115.50	74.57	49.19	137.00	98.71	70.14	255.00	96.95	81.53	262.00
0.05	86.55	54.74	88.40	87.15	41.00	56.60	67.55	64.88	145.00	93.51	80.63	160.00
0.07	93.30	46.65	96.20	91.42	37.13	168.00	65.96	57.47	180.00	96.53	93.11	184.00
0.09	0.00	0.00	0.00	69.53	46.96	122.00	87.47	72.46	127.00	50.94	80.35	131.00

Ger = Germination
Ptl = Pollen tube length
μm = micrometer

Table 5.4: Effect of different concentrations of calcium chloride on *in vitro* pollen germination, pollen tube growth and pollen tube discharge (burst).

Calcium chloride (%)	Time after culture											
	6 hours			12 hours			24 hours			48 hours		
	Ger. (%)	Burst (%)	Ptl (μm)	Ger. (%)	Burst (%)	Ptl (μm)	Ger. (%)	Burst (%)	Ptl (μ)	Ger. (%)	Burst (%)	Ptl (μ)
0.00	57.11	54.28	63.60	86.23	55.52	83.30	92.01	58.47	120.00	94.16	60.83	55.00
0.03	66.62	20.75	50.00	78.32	52.21	56.60	94.78	89.41	53.30	95.16	93.9	110.00
0.05	72.76	27.28	76.60	87.41	47.39	73.30	94.17	53.00	113.00	95.27	53	70.20
0.07	57.12	71.45	50.00	60.11	54.78	80.00	86.68	57.23	217.00	87.99	100	63.00
0.09	100	60.06	46.60	100	77.71	143.00	100	100	190	93.20	100	85.00

Ger = Germination
Ptl = Pollen tube length
μm = micrometer

Table 5.5: Effect of different concentrations of potassium phosphate on *in vitro* pollen germination, pollen tube growth and pollen tube discharge (burst).

Potassium phosphate (%)	Time after culture											
	12 hours			24 hours			36 hours			48 hours		
	Ger (%)	Burst (%)	Ptl (µm)	Ger (%)	Burst (%)	Ptl (µm)	Ger (%)	Burst (%)	Ptl (µm)	Ger (%)	Burst (%)	Ptl (µm)
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	-
0.01	90.01	81.99	100.00	92.87	83.94	116.00	93.24	83.98	135.00	-	-	-
0.05	55.82	41.87	70.00	70.71	48.75	925.00	97.39	92.10	95.00	-	-	-
0.10	76.92	56.38	104.00	79.43	55.86	130.00	84.03	75.99	126.00	-	-	-
0.50	6.66	2.22	50.00	2.56	0.00	50.00	90.70	9.28	126.60	-	-	-

Ger = Germination
Ptl = Pollen tube length
µm = micrometer

Table 5.6: Effect of different concentrations of sucrose on *in vitro* pollen germination, pollen tube growth and pollen tube discharge (burst).

Sucrose (%)	Time after culture											
	3 hours			6 hours			12 hours			24 hours		
	Ger (%)	Burst (%)	Ptl (µm)	Ger (%)	Burst (%)	Ptl (µm)	Ger (%)	Burst (%)	Ptl (µm)	Ger (%)	Burst (%)	Ptl (µm)
0.00	6.14	0.00	80.00	9.28	6.97	73.30	16.25	0.00	80.00	3.63	4.60	95.00
5.00	16.62	0.00	40.00	18.18	8.33	66.00	27.75	0.00	100.00	40.42	4.71	60.00
10.00	92.80	26.90	264.00	96.18	57.12	108.00	96.46	64.30	96.00	100.00	63.35	130.00
15.00	77.49	24.98	125.00	84.81	44.82	165.00	89.64	51.45	166.00	94.33	54.28	140.00
20.00	25.61	7.69	123.00	78.98	44.44	207.00	91.17	73.52	218.00	92.50	39.49	161.00
25.00	79.43	8.82	146.00	90.26	12.15	115.00	94.56	47.74	200.00	97.74	21.75	123.00

Ger = Germination
Ptl = Pollen tube length
µm = micrometer

Table 5.7: Results of *in vitro* fusion of lupin gametes in various concentrations of calcium.

Treatment	Calcium concentration	Egg cells brought into contact with sperm cells	Not fused		Partially fused		Fully fused	
	mM	No	No	%	No	%	No	%
1	5	11	6	54.54	2	18.2	2	18.18
2	10	13	3	23.07	4	30.76	6	46.15
3	15	17	12	70.58	4	23.52	1	5.88
Total		41	21	51.21	10	24.39	9	21.95

Table 5.8: Results from *in vitro* fusion of lupin gametes with various levels of pH in fusion medium.

Treatment	pH	Egg cells brought into contact with sperm cells	Not fused		Partially fused		Fully fused	
		No	No	%	No	%	No	%
1	5	11	7	63.63	4	36.36	00	00
2	7	13	3	23.07	3	23.07	7	53.84
3	9	16	6	37.5	4	25	6	37.5
	11	19	12	63.15	4	21.05	3	15.78
Total		59	28	47.45	15	25.4	16	27.11

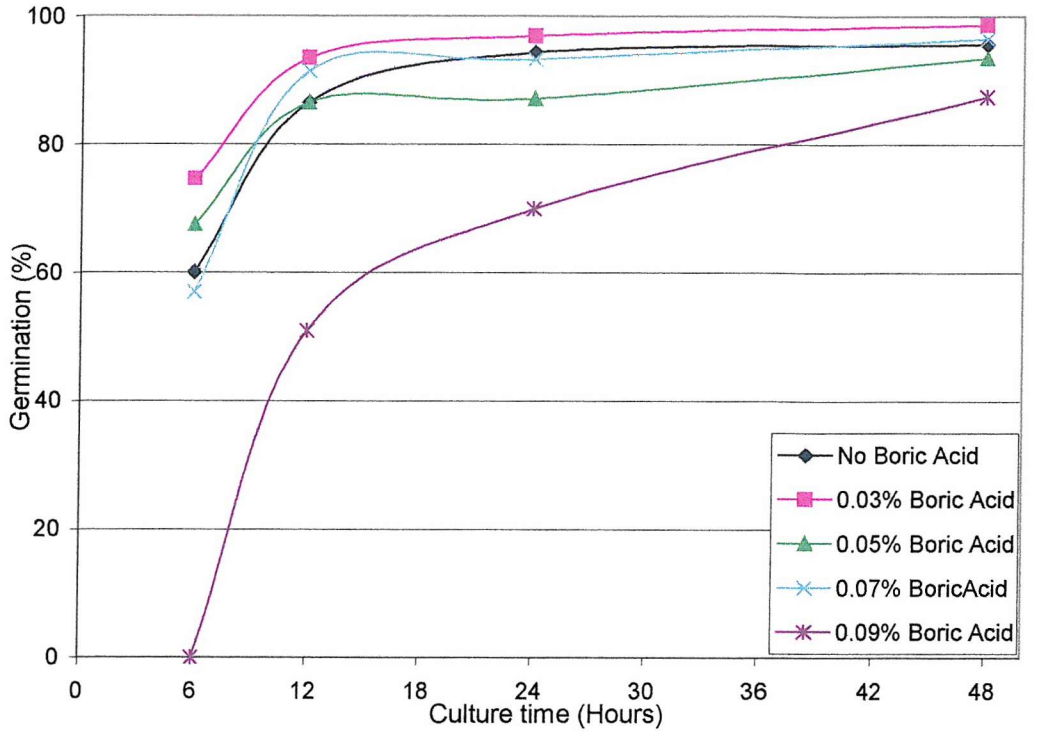


Fig 5.1: Effect of boric acid on in *vitro* pollen germination of *Lupinus* species.

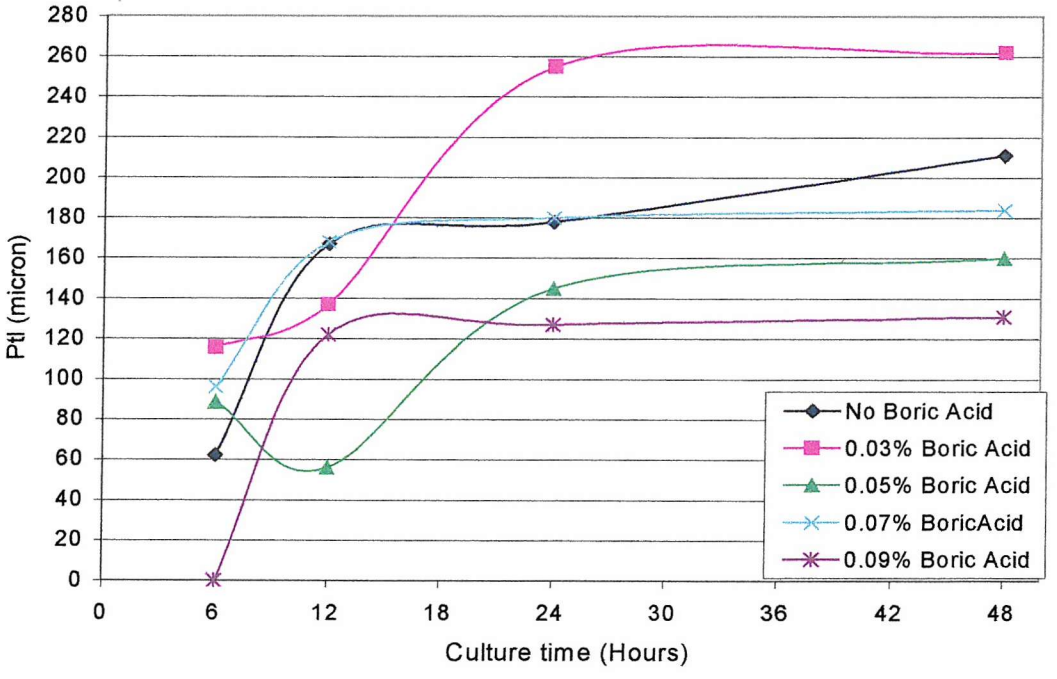


Fig 5.2: Effect of boric acid concentrations on *in vitro* pollen tube growth (length) of *Lupinus* species.

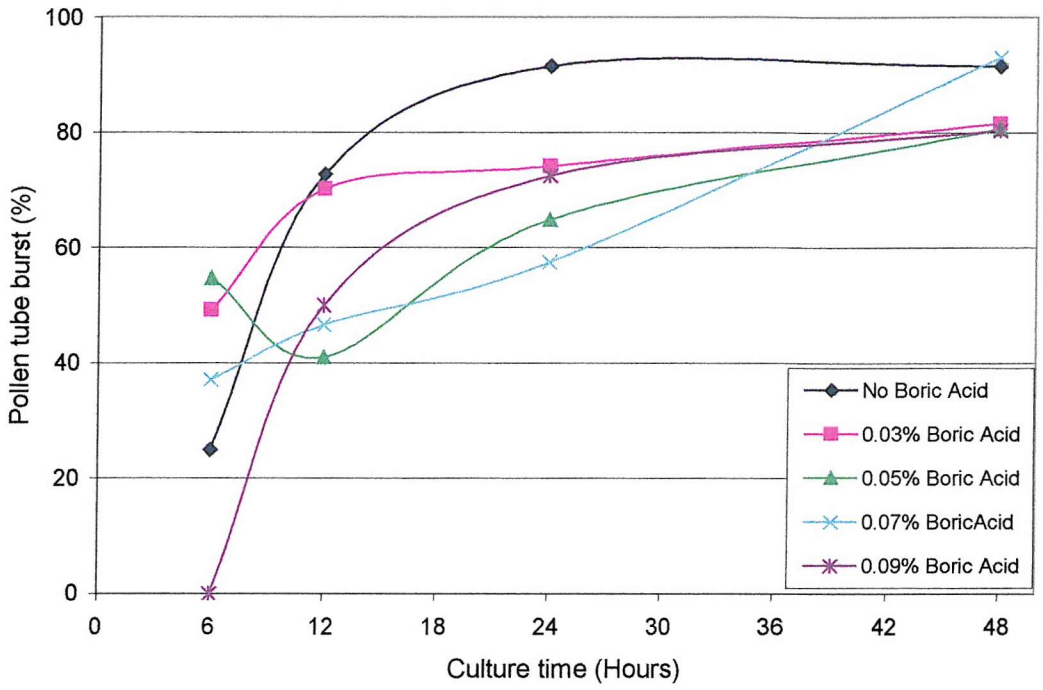


Fig 5.3: Effect of boric acid on *in vitro* pollen tube discharge (burst) of *Lupinus* species.

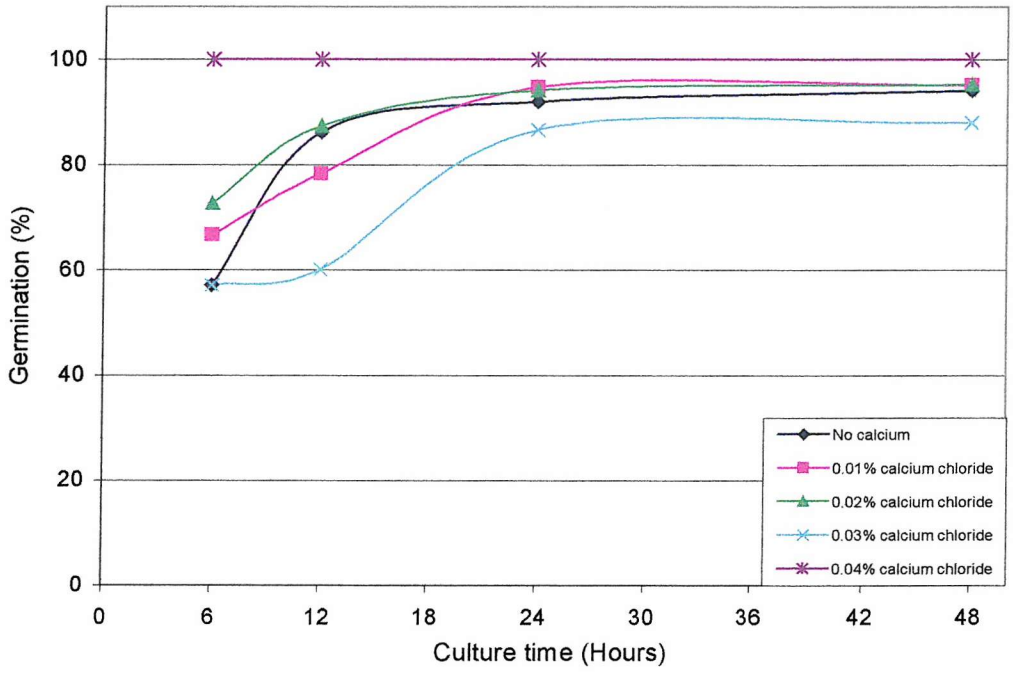


Fig 5.4: Effect of calcium chloride on *in vitro* pollen germination of *Lupinus* species.

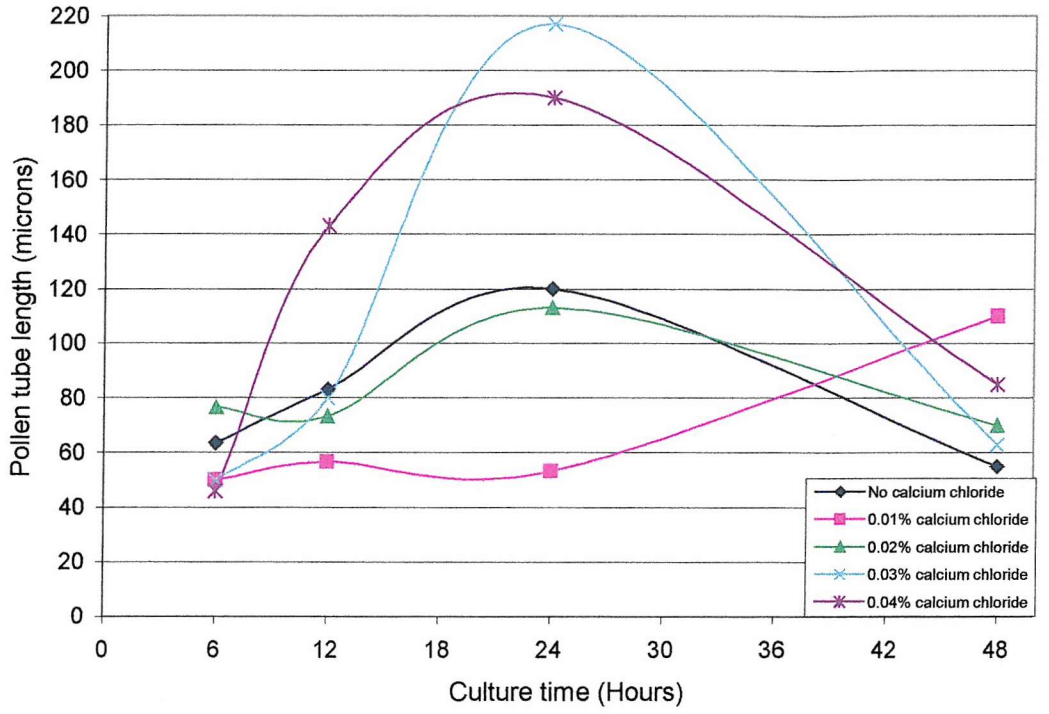


Fig 5.5: Effect of calcium chloride on *in vitro* pollen tube growth (length) of *Lupinus* species.

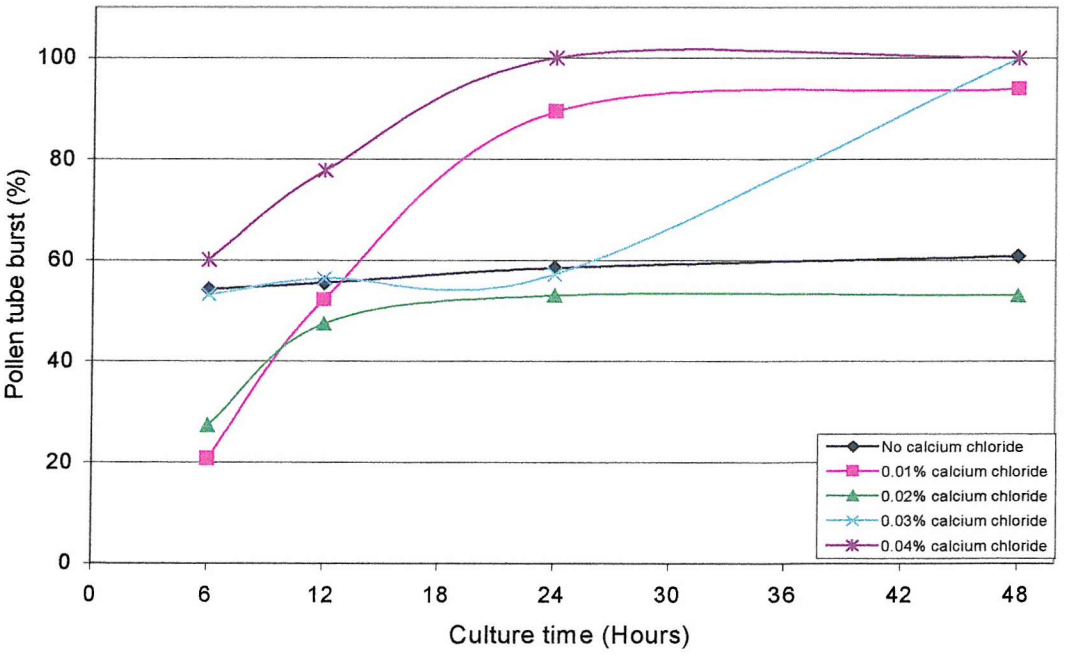


Fig 5.6: Effect of calcium chloride on *in vitro* discharge of pollen tube contents (burst) of *Lupinus* species.

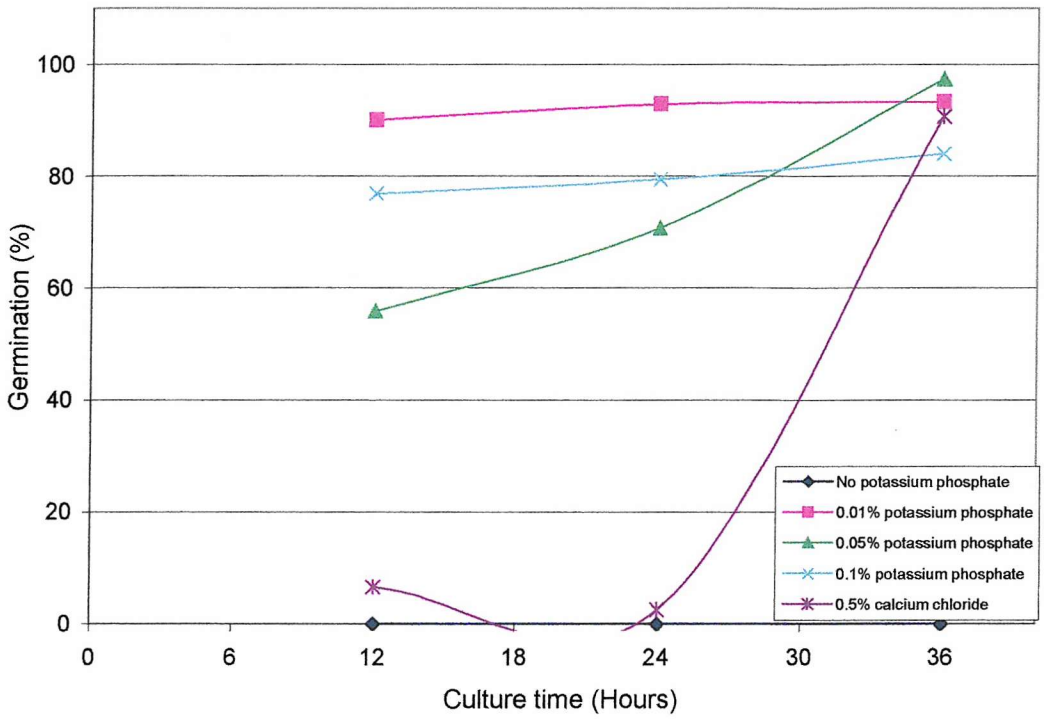


Fig 5.7: Effect of potassium phosphate on *in vitro* pollen germination of *Lupinus* species.

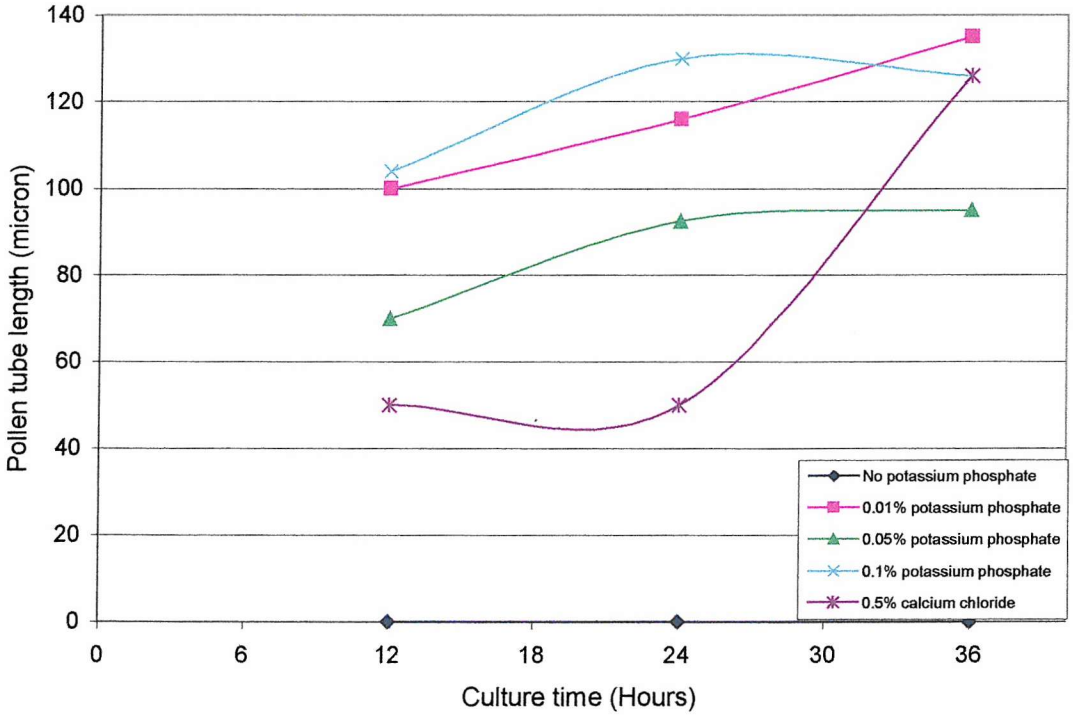


Fig 5.8: Effect of potassium phosphate on *in vitro* pollen tube growth of *Lupinus* species.

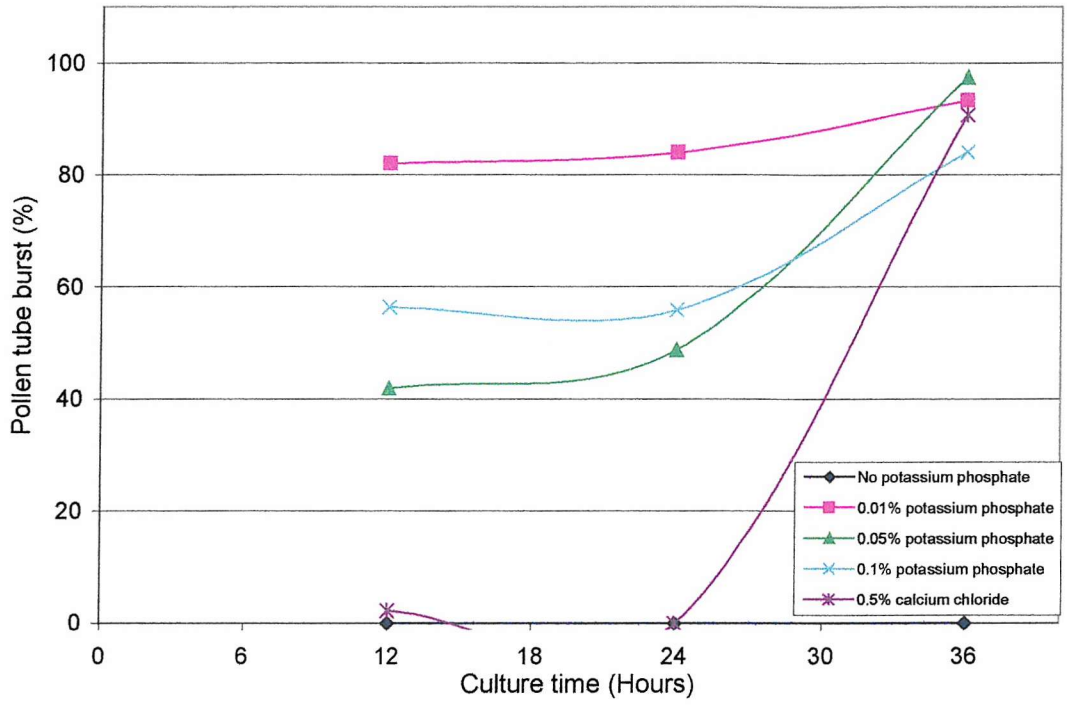


Fig 5.9: Effect of potassium phosphate on *in vitro* discharge of pollen tube contents (burst) of *Lupinus* species

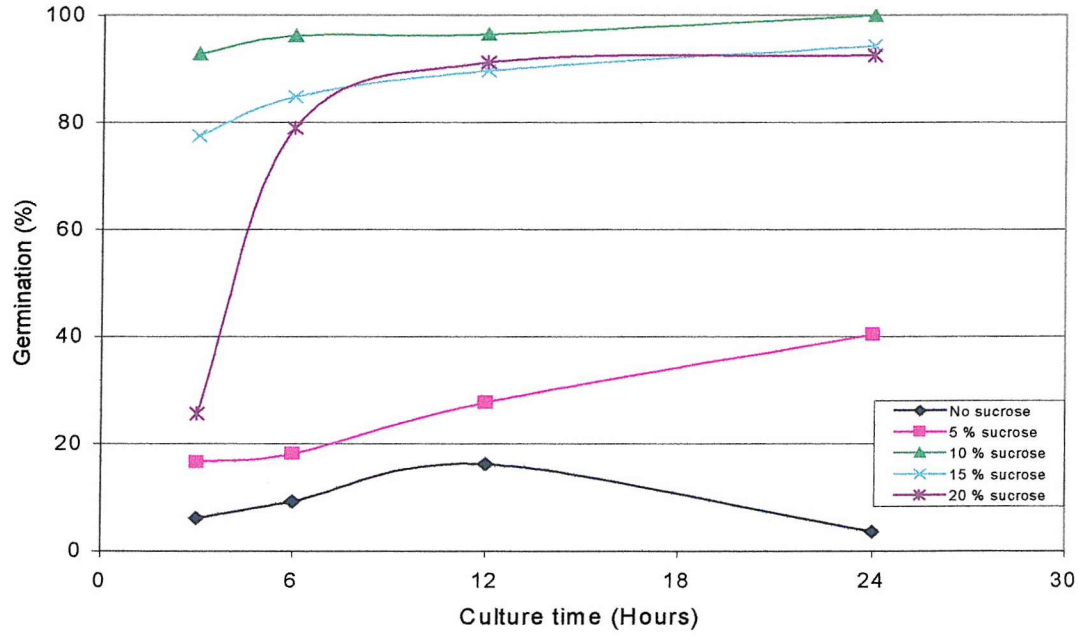


Fig 5.10: Effect of sucrose on *in vitro* pollen germination of *Lupinus* species.

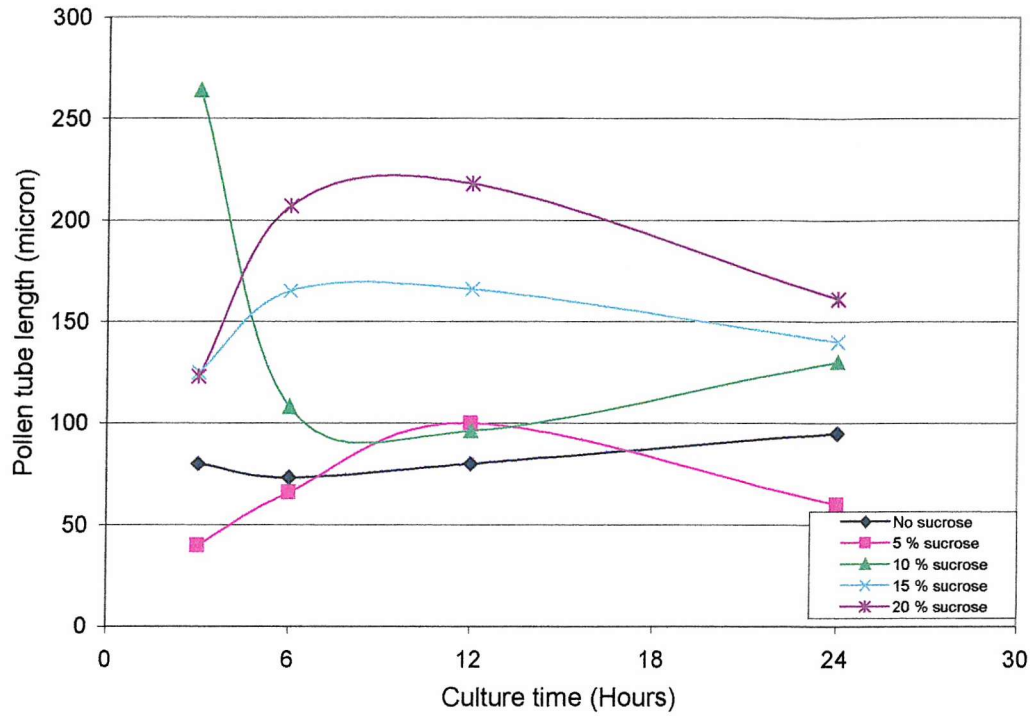


Fig 5.11: Effect of sucrose on *in vitro* pollen tube growth of *Lupinus* species.

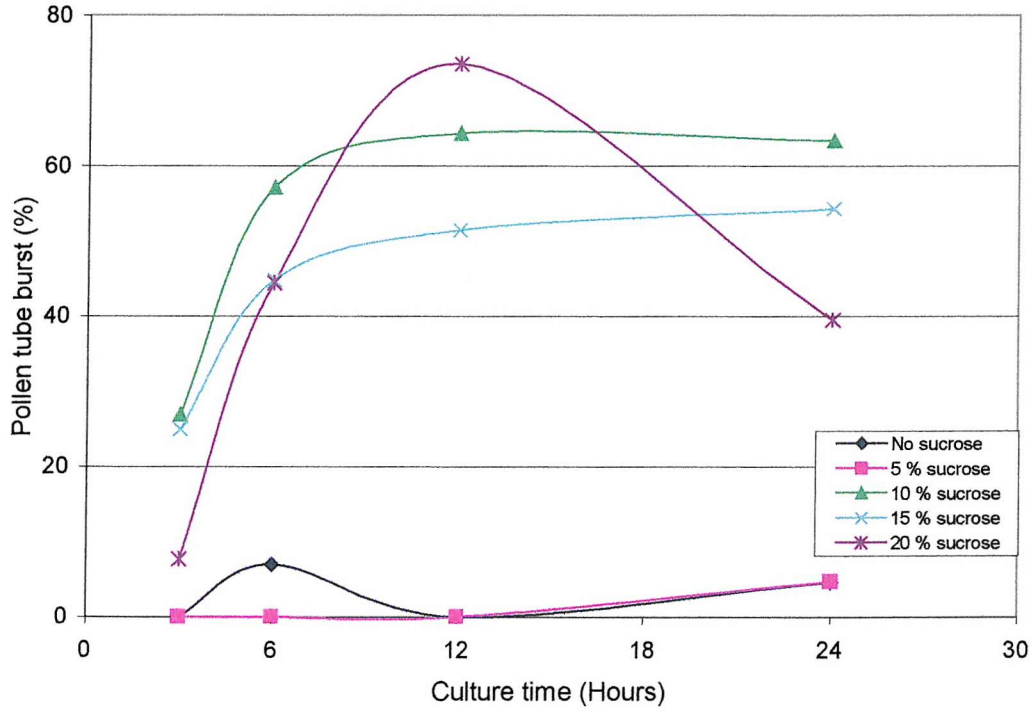


Fig 5.12: Effect of sucrose on *in vitro* discharge of pollen tube contents (burst) of *Lupinus* species

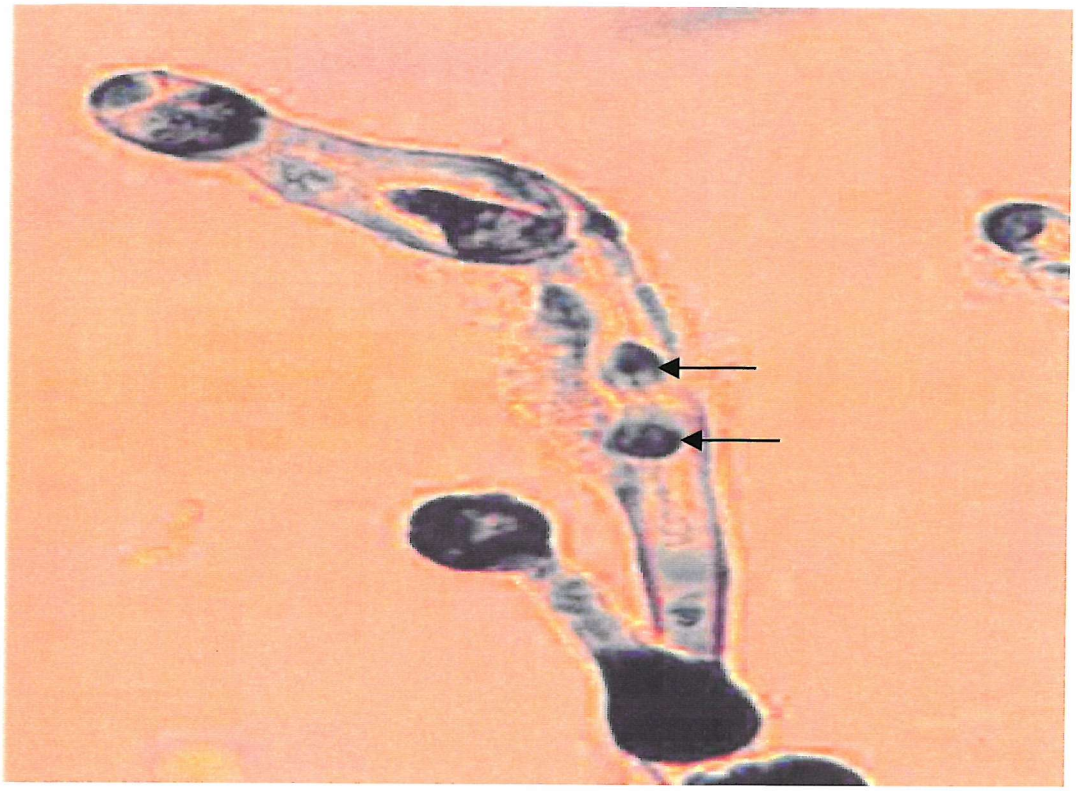


Plate 5.1: Pollen tubes of *Lupinus mutabilis* showing two sperm cells (X 428).

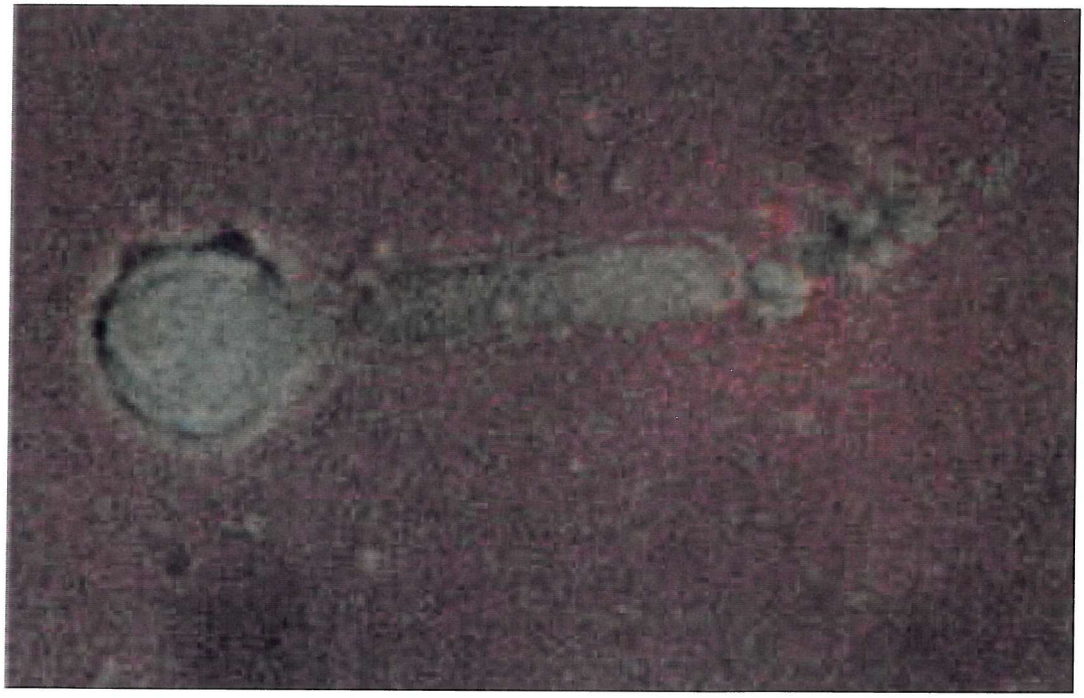


Plate 5.2: Contents of pollen tube being released from *L. mutabilis* pollen tubes (X 856).

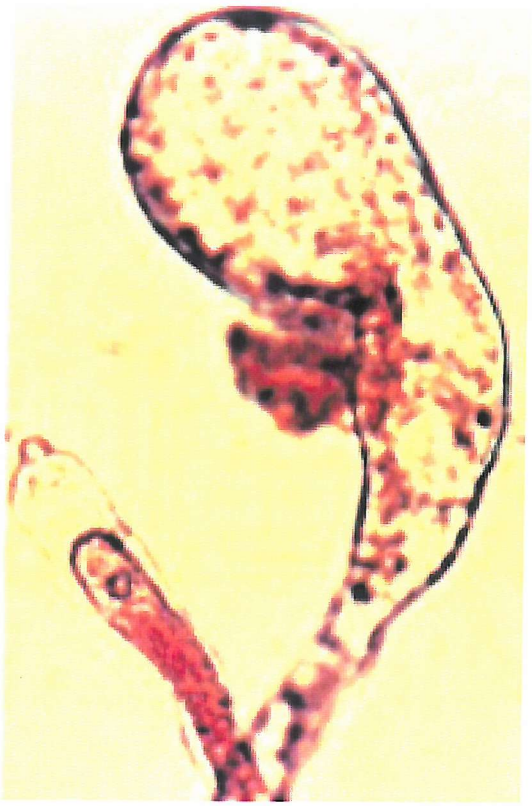


Plate 5.3: Abnormal pollen tube growth, swelling, and bust in culture medium containing 0.02 % (w/v) calcium chloride (X 856).

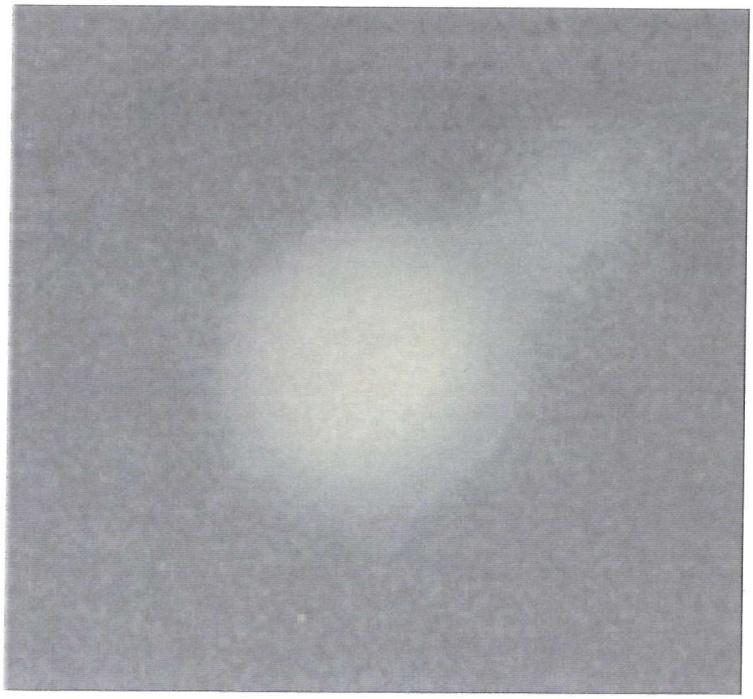


Plate 5.4 An isolated sperm cell of *L. mutabilis* stained with FDA (X 856).

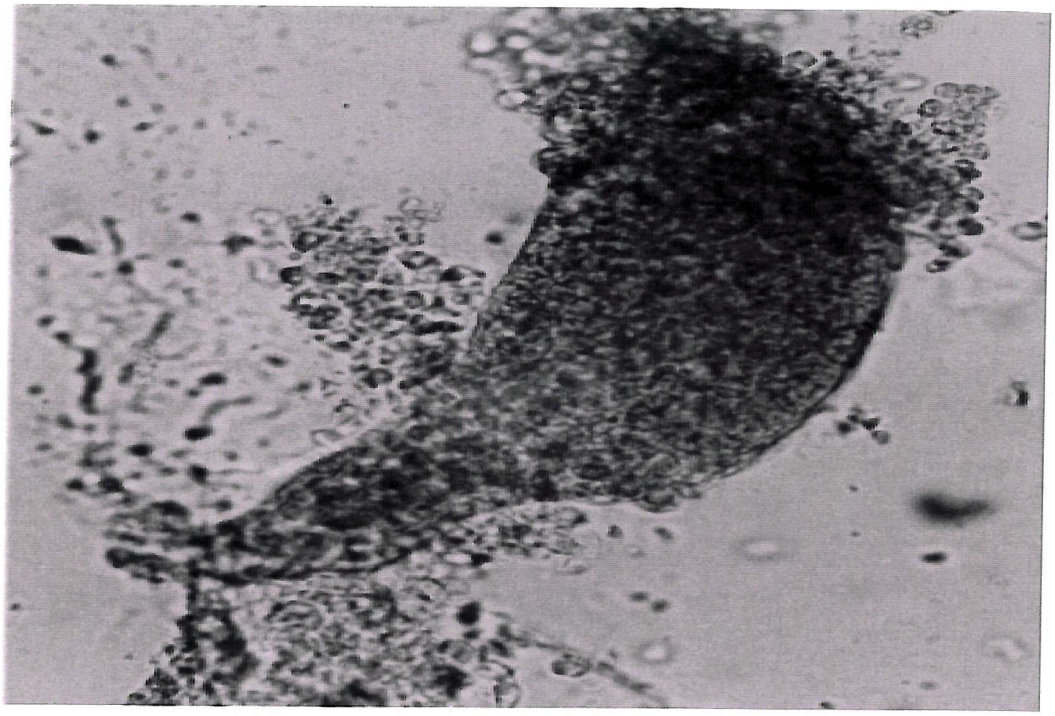


Plate 5.5 An isolated embryo sac of *L. albus* showing egg cell of *L. albus* (X 428)

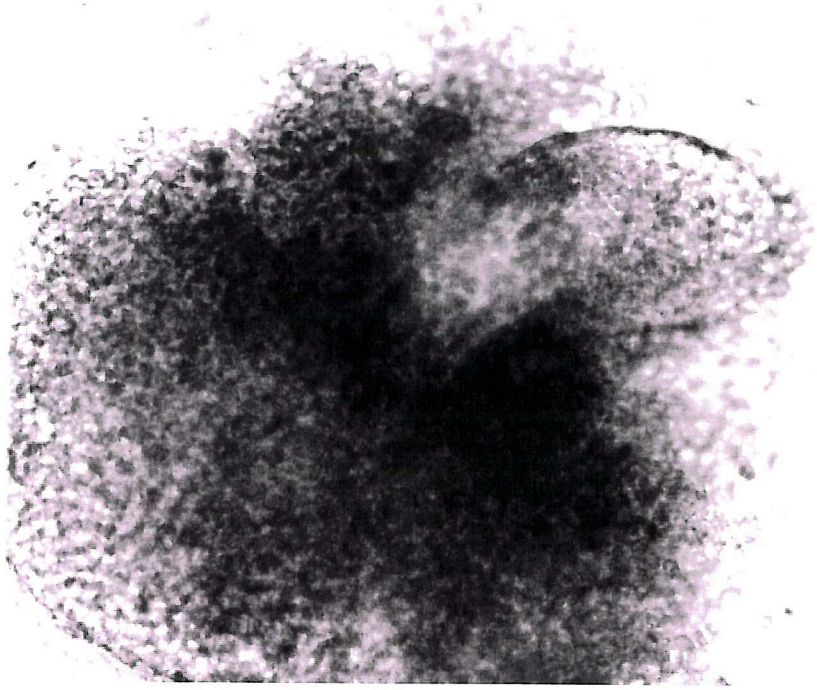


Plate 5.6 An embryo sac of *L. mutabilis* during isolation (X 428)

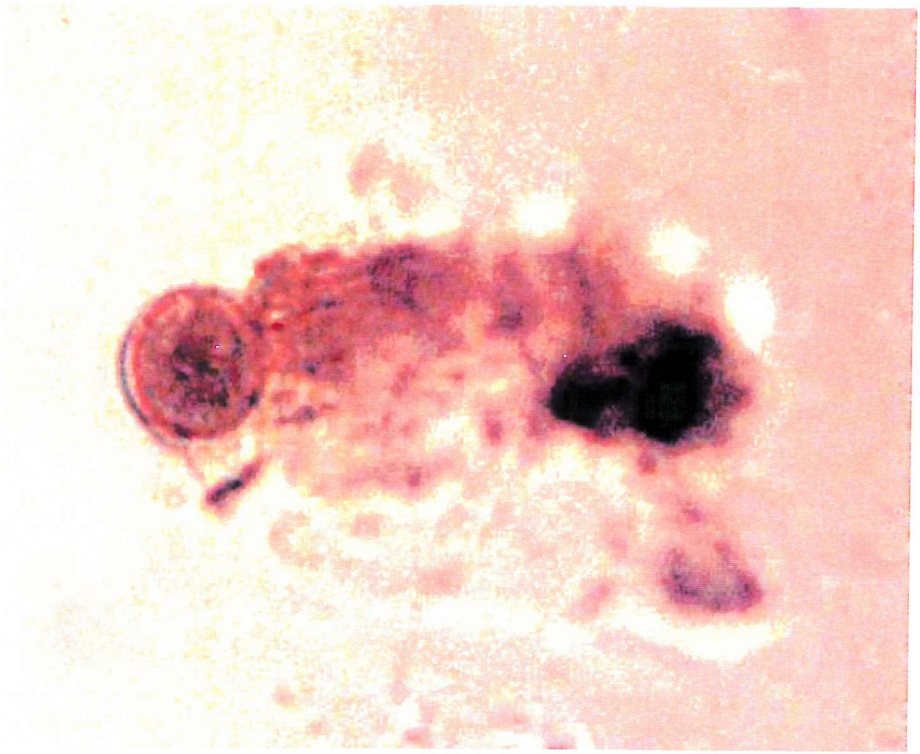


Plate 5.7: Embryo sac showing egg cell of *L. mutabilis* during isolation (X 456).

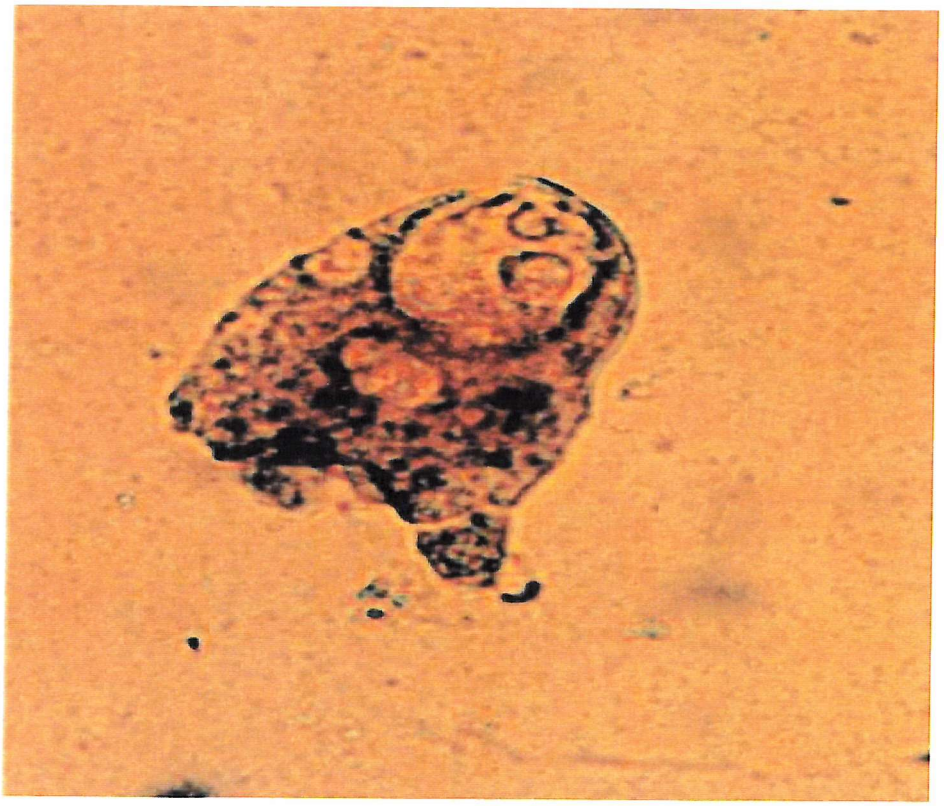


Plate 5.8: Egg cell of *Lupinus mutabilis* with two synergids (X 856)

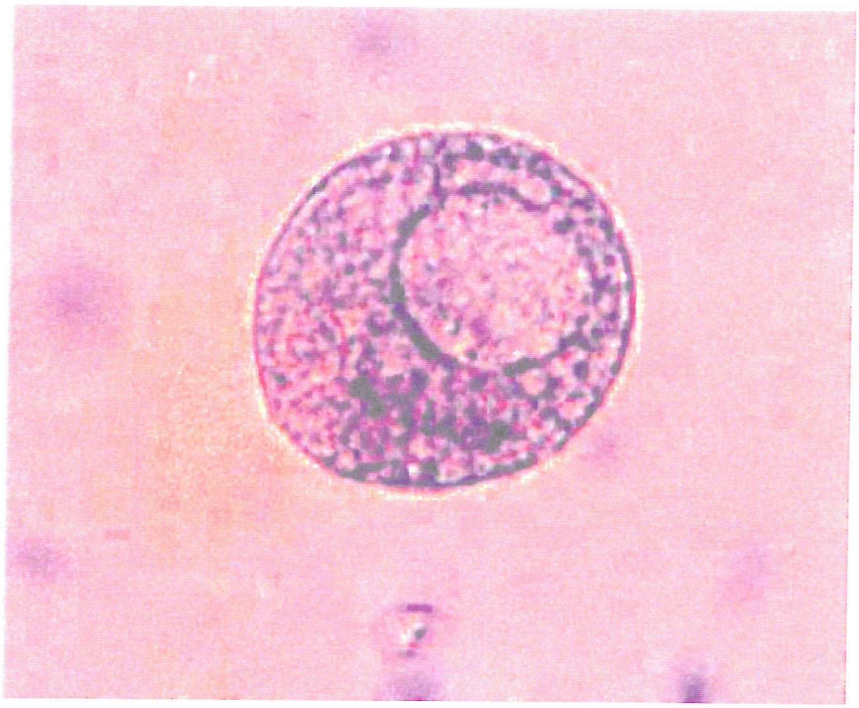


Plate 5.9: Egg cell of *Lupinus albus* with nucleus (X 856)

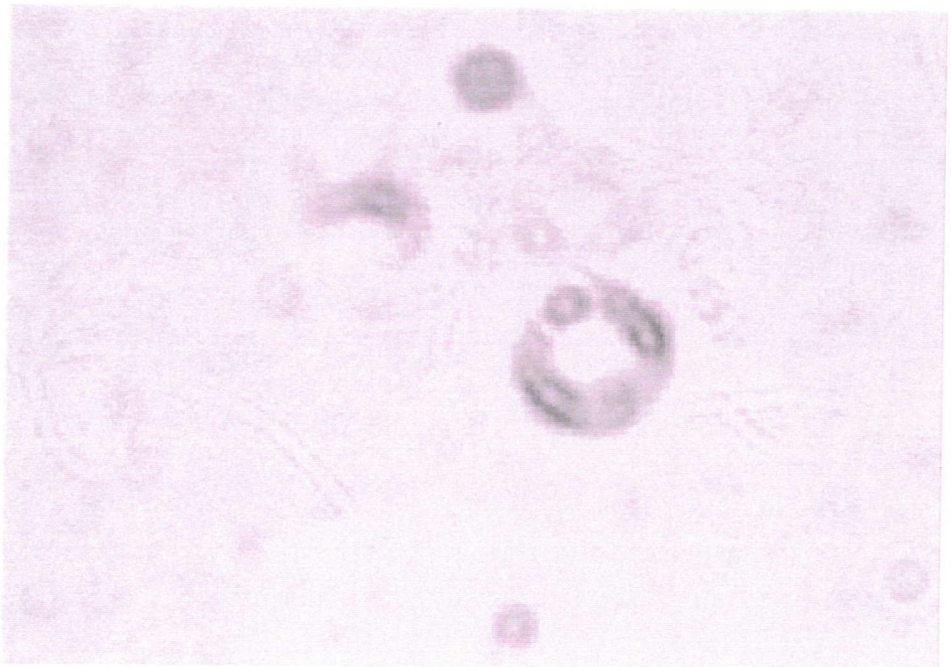


Plate 5.10: Fusion of sperm cell and egg cell in *Lupinus* species (X 600)

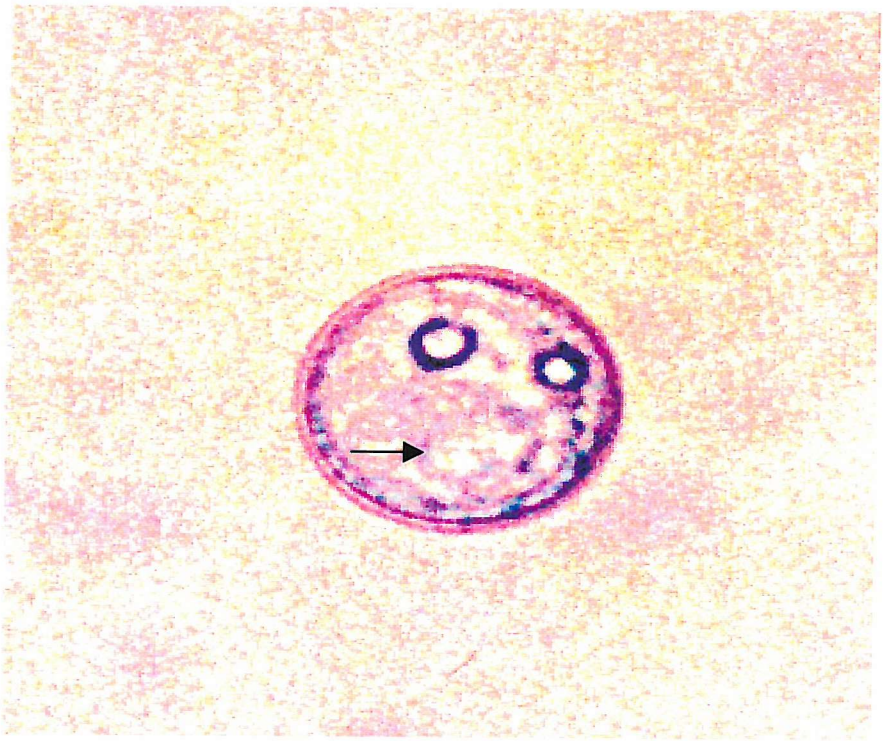


Plate 5.11: A fused product of sperm cell and egg cell of *Lupinus* species (X856).

Chapter Six

General discussion and conclusion

Modification of crop plants to improve their suitability for cultivation have been made for thousands of years. During the past century plant breeders have become more rigorous in their approach to develop new varieties of crops. Significant improvements were made in the crop plants through conventional as well as novel plant-breeding techniques.

Lupins are a potential source of protein for both animal and human consumption. However, *Lupinus* species remain underutilised due to certain undesirable characteristics such as alkaloids, uneven maturity and susceptibility to pests and diseases. It is believed that lupins (*Lupinus* species) can also be improved through introgression of desirable genes for wider adaptation, resistance to diseases and pests, and increased yield. The Andean lupin (*L. mutabilis*) has been identified as a potential breeding material for a number of desirable traits, which would enhance agronomic improvement of cultivated *L. albus* if they could be transferred through interspecific hybridisation. These traits include resistance to viral and other diseases, high oil and protein content and higher quality of harvested grain, tolerance of environmental stress, including water-logging, and adaptation to a wider range of climate and soil types. The literature reviewed showed that attempts to hybridise different lupin species started as early as the first half of the last century and some of them succeeded in obtaining hybrid plants (Gollmick 1937; Roy & Gladstones, 1985; Schafer-Menhur *et al.* 1988; Kasten *et al.,* 1991). This success has clear merits (Gladstones, 1998) but many of these attempts have unfortunately failed and thus transfer of desirable traits between *Lupinus albus* (Old World) and *Lupinus mutabilis* (New World) was not achieved (Williams *et al.*, 1980; Siamasonta & Calligari 1998). These studies have concluded that barriers to genetic recombination of these two species exist at post-fertilisation stage.

This study has established barriers in interspecific crosses between the two *Lupinus* species i.e. *L. albus* and *L. mutabilis.* Morphological characteristics of both species were

studied for selection of important traits and for comparison of characters between species and hybrids. However, because of the unavailability of hybrid materials, heritability studies of these characters could not be made. Genotypes with equal number of chromosomes were selected for the success of interspecific crosses in this study. Pollen tube growth, pod and seed development were investigated in self and interspecific crosses to determine the fertilisation process. Techniques such as application of growth regulators and *in vitro* embryo/immature seed culture were used to facilitate the production of hybrids between the two species. Apart from traditional hybridisation techniques, *in vitro* fertilisation of gametes was also used to achieve the genetic recombination of Old World (*L. albus*) and New World (*L. mutabilis*) lupin species.

The crossing behaviour of different genotypes was determined through hand self-pollination. In self-pollinated crosses a higher level of flower abscission was observed in both *L. albus* as well as *L. mutabilis* species. Genotypic differences were observed within the species in all accessions of *L. albus* and *L. mutabilis* used in terms of flower abscission, pod set and number of mature pods.

Interspecific crosses of *L. albus* and *L. mutabilis* species were initially attempted using a wide range of genotypes. However, in most of the interspecific crosses attempted in the present study the flowers/pods abscised at an early stage (during the first ten days after pollination). It has been reported that interspecific crosses attempted between species, containing the same number of chromosomes had higher success than those containing different chromosome numbers (Singh *et al.,* 1990). Therefore, the number of somatic chromosomes (2n) of a wide range of genotypes was investigated and eight accessions of *L. albus* and *L. mutabilis* with equal numbers of somatic chromosomes (2n = 48) were selected for interspecific crosses. Further selection of accessions was made on the basis of their morphological characters. Phenotypic correlation between important yield characters such as plant height, pods per plant, number of seeds per pod was calculated to determine the possible association between them.

In interspecific crosses most of the flowers/pods abscised within two weeks after pollination. Shrivelled seeds were produced only when *L. mutabilis* was used as female parent. From the reciprocal cross only deformed seeds were produced. From the results

of interspecific crosses between *L. albus* and *L. mutabilis* in this study it appears that there is a post-fertilisation incompatibility between these two species as previously reported by Williams *et al.*, (1980); Kasten & Kunert, (1991); Gupta, (1996); Simasonta & Calligari, (1998).

Initially, cross-incompatibility between these two species was investigated to establish whether inhibition of pollen tube growth or its cessation at the pistil base had occurred. Therefore, pollen germination and pollen tube growth in self and interspecific crosses of the two species was studied. The results obtained from this study suggested the normal pollen germination, pollen tube growth and fertilisation in the reciprocal interspecific crosses of *L. albus* and *L. mutabilis*, as was previously reported (Atkins *et al.*, 1998). This suggests that incompatibility is probably operating at the post-fertilisation level and disharmonious interaction between embryo, endosperm, and maternal tissues or disharmonies within the hybrid zygote are the primary causes of the hybrid failure. A few shrivelled hybrid seeds were produced from the cross combination of *L. mutabilis* (♀) and *L. albus* (♂) whereas the reciprocal cross resulted in deformed seeds.

A high level of flower abscission and pod abortion was observed in self and inter-specific combinations at an early stage of development. Therefore, further interspecific crosses were carried out with the application of a mixture of growth regulators (Chapter 2) to retain the pods. Although pod retention was enhanced by the application of growth regulators the hybrid embryos were not large enough to excise for *in vitro* culture. Although growth regulators had some positive effect on decreasing the rate of flower drop and enhancing pod retention only a few pods were retained on the plant after two weeks of pollination. The longest period of hybrid pod retention was found to be 22 DAP when *L. mutabilis* was used as female parent. Many mature pods were produced, containing shrivelled and deformed seeds and a few mature pods had no fertilised ovules and were probably formed by parthenocarpy because of the use of growth regulators. Ahmed *et al.*, (1995) and Taheri (2000) reported similar observations in the genus *Cicer*. Another significant factor in the failure of crosses worth mentioning is that even in the selfed pods (flowers from same plant pollinated with pollen of the same plant) the number of seed set was very low (1-3 seeds/pod) which further reduces the probability of achieving a hybrid seed.

The interspecific crosses only resulted in the production of shrivelled or deformed seeds. It is worth noting that shrivelled seeds were only obtained when *L. mutabilis* was used as seed parent. In the cross combination where *L. albus* was used as female parent only deformed seeds were obtained. The production of shrivelled or deformed seeds suggested that hybrid failure occurred due to embryo abortion. Maternal tissues and the endosperm are the main sources of nutrients for the embryo at its early development stages. If transportation of nutrients from these tissues to the young embryo is arrested due to disharmonious interaction between them it will result in embryo starvation. The genetic differences between accessions may cause disharmonious interaction between parental genomes or between the genome and cytoplasm and subsequently degeneration of endosperm. These differences may affect the normal metabolism of nucleic acids and enzymatic proteins or it may cause abnormalities in hormonal metabolism (Shii *et al.,* 1982; Lester & Kang, 1998). Abnormal interaction between the endosperm and maternal tissues may also prevent the flow of nutrients from maternal tissues to the endosperm (Sangduen *et al.,* 1983) and cause endosperm degeneration.

An investigation of pod and seed development in selfed and interspecific crosses was carried out to determine the process of fertilisation and the suitable time to rescue hybrid embryos through *in vitro* culture. In selfed crosses of *L. albus* and *L. mutabilis* species normal pod and seed development was observed. The rate of pod development was somewhat faster in *L. mutabilis* as compared to *L. albus*. Pod development in interspecific crosses was slow compared to selfed ones as was previously reported by Kasten & Kunert (1991); and Przyborowski & Packa (1997). However, in the present study unlike selfed crosses pod growth in interspecific combinations appeared to have stopped after third day of pollination. In the present study investigations carried out on seed development in self and interspecific crosses revealed a similar pattern of development as of pods. However, unlike hybrid pods, hybrid seeds developed normally for 4 -5 days after pollination and then stopped growing. In the present study the normal development of pods and ovules for 3-5 DAP indicates that fertilisation occurred in interspecific crosses of the two species and that barrier to interspecific crosses existed at post-fertilisation level. Furthermore, the results of this study suggest that any attempt to rescue hybrid embryos of *Lupinus* species has to be made within the first week after pollination. The study of hybrid pods also revealed that application of growth regulators,

as mentioned earlier, did not stop pod abscission although there was some positive effect on pod retention.

From the results of pod and seed development studies of selfed and reciprocal crosses of *L. albus* and *L. mutabilis* (chapter 3) it became apparent that the process of degeneration of hybrid embryos starts at an early stage (3 – 5 DAP). Immature seed/embryo culture was attempted to develop a suitable *in vitro* method for production of hybrids. Young embryos of selfed crosses of *L. albus* and *L. mutabilis* species were cultured *in vitro* to establish a suitable culture system for rescuing *Lupinus* hybrid embryos. Plantlets were successfully produced from selfed embryos at heart to cotyledonary stages of development *in vitro*. The rate of success in culturing selfed embryos of *L. albus* and *L. mutabilis* species was 13 % and 10 % respectively. However, hybrid embryos were too small to be excised from hybrid immature seeds. Hence the whole immature seeds from interspecific crosses were dissected from pods and cultured. Immature seeds from selfed pods were excised and cultured as control. Hybrid immature seeds did not regenerate and only in few cases callus was formed. The isozyme analysis of the calli produced through *in vitro* culture revealed different patterns of band formation, which indicated the hybrid nature of callus. However, a comprehensive analysis could not be carried out due to limitations in time and availability of hybrid material.

The early abortion of embryos in the genus *Lupinus*, difficulties in excising young hybrid embryos and low regeneration rate due to recalcitrant nature of *Lupinus* species, makes it even more difficult to produce *Lupinus* hybrids through *in vitro* embryo/ immature seed culture. Therefore, the use of *in vitro* fertilisation and regeneration of fused gametes to plants seemed appropriate to facilitate the gene transfer between *L. albus* and *L. mutabilis* species.

As a prerequisite for *in vitro* fertilisation, methods for the *in vitro* isolation of viable male and female gametes of *Lupinus* species were developed. The sperm cells of *L. albus* and *L. mutabilis* were isolated from growing pollen tubes in a culture medium containing 0.01 % (w/v) potassium phosphate, 0.03 % (w/v) boric acid, 0.04 % (w/v) calcium chloride, 10 % sucrose (w/v) and pH 5.4. In the present study the vital role of calcium and potassium concentrations for *in vitro* pollen germination and pollen tube growth was

realised. Although the role of calcium concentrations in gametes and fertilisation is well documented for other species (Dumas *et al.*, 1998) the importance of potassium for *in vitro* pollen germination of *Lupinus* species was seen for the first time in this study. Pollen grains of *Lupinus* species did not germinate when potassium was omitted from the culture medium. For the isolation of egg cells of *Lupinus* species, unfertilised ovules were macerated with different concentrations of cell wall degrading enzymes followed by mechanical manipulation. The maximum yield of egg cells (28 %) was achieved when cellulase and pectinase were used at 1 % (w/v) and 0.5 % (w/v) respectively.

In vitro gametic fusion of sperm and egg cells of *L. albus* and *L. mutabilis* was attempted using different calcium concentrations and different levels of pH in the fusion medium. Frequent fusion of gametes was observed when calcium was used at a concentration of 10 mM in the fusion medium. When concentration of calcium was increased more than 10 mM in the fusion the movement of gametoplasts became difficult and hence fusion frequency decreased. In fusion experiments pH level of the fusion media was also found to be crucial. In this study frequent fusion of gametes occurred neither on higher nor on lower but at neutral pH. However, this aspect of the research needs further investigation.

In summary the results of this study confirm the previous reports of incompatibility in interspecific crosses of *L. albus* and *L. mutabilis*. The number of chromosomes did not seem to be an important factor in the success or failure of interspecific crosses in this study. From the observations of pollen germination and pollen tube growth, and pod and ovule development of interspecific crosses it appears that interspecific incompatibility between Old World and New World *Lupinus* species is due to post-fertilisation barriers. The investigations on pod and ovule development were also useful in detecting the precise time of embryo degeneration and suitable time for embryo rescue. The results from application of growth regulators to sustain pod/seed growth *in vivo* indicated that duration or composition of the growth regulators applied may need to be altered to allow further growth of embryo *in vivo* (if it is not the genetic incompatibility). Although embryos and immature seeds of selfed crosses were cultured successfully using *in vitro* technique further work is needed to rescue hybrid embryos particularly for the regeneration of hybrid embryos/ immature seeds of the interspecific crosses. The establishment of suitable isolation and gametic fusion methods for *Lupinus* species have

opened up new ways to overcome barriers in interspecific hybridisation of lupins in particular and legumes in general provided the regeneration methods of plants are established. This technique is not only efficient but also provides an opportunity to look into the process of fertilisation itself, which is difficult, *in vivo*. Further work on the improvement of methods for isolation, fusion and regeneration of fused gametes to a fully developed plant is needed.

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