Incidence and Inheritance of

Hard-seededness and

Early Maturity in

Ornithopus sativus

Bradley John Nutt

B. Sc. (Agric.) Hons, University of Western Australia

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Division of Science and Engineering, School of Biological Sciences and Biotechnology, Murdoch University
Abstract

*Ornithopus sativus* Brot. (French serradella, pink seradella) is an annual legume in the Leguminosae sub-family Papilionoideae that has value in dryland agriculture as a forage or green manure. It is cultivated in Mediterranean and temperate climates where it is favored for the production of high quality fodder, robust nitrogen fixing symbiosis, and adaptation to acidic and relatively infertile soils. In common with grain legumes, cultivated forms of *O. sativus* are soft-seeded, while wild relatives of the genus are generally hard-seeded. The dynamics of these opposite seed characteristics are explored in *O. sativus* along with the environmental control over the initiation of flowering.

In two cultivated populations of *O. sativus*, rare hard-seeds which did not imbibe moisture were isolated. Plants (*S*₁) grown in an open field from these residual hard-seeds produced either all soft-seeds (0% hard-seed) or a proportion of hard-seeds (4 – 100%). The offspring (*S*₂) from a subset of these plants showed a similar distribution and correlation between parent and offspring production of hard-seeds. However, the proportion of hard-seed produced and the parent-offspring relationship was different for the two source populations.

The hard-seeds selected from accession 97ZAF5sat produced *S*₁ plants expressing the extremes in hard-seed production and *S*₂ plants segregating to the opposite expression of their maternal parent. All of these selections
commenced flowering relatively early with little variation between individuals and no apparent association of timing of flowering with hard-seed production.

Plants grown from the hard-seeds selected from cv. Emena also produced either all soft-seeds or some proportion of hard-seeds. However, among the hard-seed producing individuals there was a broad range in the proportion of hard-seeds produced and there was less segregation away from the maternal expression in a subsequent generation of offspring (compared to the 97ZAF5sat selections). There was also a broad range in the timing of flowering in the selections from cv. Emena and a positive correlation between the timing of flowering with the proportion of hard-seed produced by both the $S_1$ and $S_2$ generations.

The variation observed in the *O. sativus* populations studied for hard-seed production and timing of flowering (both between individual plants and the segregation away from maternal plant expression) was the result of cross pollination. Between-plant cross pollination in *O. sativus*, in an open field with honey-bee activity, was measured at 25.3% (± 0.8). This was assessed by examining the pollen flow from pink-flowered plants to white-flowered maternal plants. This result demonstrated that although *O. sativus* is a self-compatible species, it should be considered to have a mixed breeding system with allogamic cross pollination possible in the presence of pollinating insects.
The inheritance of seed coat permeability was examined by hybridization between plants that were homozygous for their respective seed character. A hard-seed parent plant was selected from each of the 97ZAF5sat (S₁ plant A1.1) and cv. Emena (S₁ plant B1.2) populations based on consistent hard-seed production in S₂ offspring. Hybrids were generated between these plants and also with two soft-seeded parents. One of the soft-seeded parents was selected from cv. Cadiz (C) and other was a very early flowering selection from this cultivar (D1). Sequential planting of these parent plants in a controlled temperature glasshouse showed variation in the timing of flowering to be control by a basic accumulation of days (at constant temperature) that is modified by photoperiod. Differences between the four genotypes in the timing of flowering were generated by inhibition of flowering under short photoperiods. In the early flowering selection, D1, the influence of photoperiod was reduced.

All F₁ seed from hand pollination was either soft-seeded or hard-seeded depending on the phenotype of the maternal parent. This confirmed that the permeability of a seed coat (a maternal tissue), is determined by the genotype of the maternal plant, regardless of the genotype of the seed embryo. F₁ hybrid plants with A1.1 maternal or paternal parentage produced very high proportions of hard-seeds (greater than 80%) regardless of the expression of the other parent (hard or soft-seeded). However, the hybrid between a soft-seeded D1 maternal plant and a paternal hard-seeded B1.2 produced soft-seeds.
Selfed F₂ hybrids between hard-seeded A1.1 and soft-seeded parents segregated to 26.6% (± 1.3%) of plants producing all soft-seeds and the remainder producing high levels of hard-seed (>80% hard-seed). All selfed F₂ hybrids between hard-seeded A1.1 and hard-seeded B1.2 produced hard-seed, however the proportion of hard-seed produced by these hybrids ranged between the two parent expressions, with the majority producing the high percentages observed in A1.1. The F2 hybrids between B1.2 and D1 segregated to 77% of plants producing soft-seed and 23% of individuals producing some proportion of hard-seed (12% to 100% hard-seed).

The segregation evident in F2 hybrids for hard-seed production between A1.1 and the soft-seeded genotypes predicts its type of hard-seededness is conferred by a single dominant allele. Hybrids between the other hard-seeded parent, B1.2, and the soft-seeded D1 suggest the inheritance of a single recessive locus being associated with hard-seed production. Further, the F₂ hybrids between the two hard-seeded genotypes showed a dominance of A1.1 type hard-seededness. However, there was no obvious truncation point across the variation among these individual F2 hybrids to facilitate estimation of a segregation ratio.

The simplest model of inheritance of the hard or soft-seed expression is a single, qualitative locus with three possible alleles. In order of dominance; SCP₁ > SCP₂ > SCP₃. SCP₁ is associated with the hard-seed character selected from accession 97ZAF5sat which is expressed as high proportions of hard-seeds (impermeable seed coats) with little variation among
individuals; SCP\textsubscript{2} is associated with soft-seed production and all seed is permeable to moisture; and SCP\textsubscript{3} is associated with the hard-seed character selected from cv. Emena, expressed as variable proportions of hard-seed between individuals.

Seeds produced by the soft-seeded genotype D1 freely and rapidly exchanged moisture with the environment, and this exchange was not influenced by seed moisture content. Staining revealed areas permeable to moisture at sites randomly distributed over the whole seed coat, but not at the lens or hilum regions.

Hardseededness of the A1.1 genotype was stable at a moisture content of 6.6\% (seed DM) and the number of hard-seeds did not change with further drying. Re-adsorption of moisture at 76\% relative humidity was minimal in these seeds at this, or drier seed moisture contents. Staining revealed that when hard-seed breakdown is induced, these seeds first absorb moisture through the lens, and then through gradual hydration and expansion of the seed coat radiating away from the lens.

The number of hard-seeds in seed lots produced by Genotype B1.2 progressively increased as they were dried to approximately 4\% moisture content. Above 4\%, the seeds displayed a protracted imbibition and germination pattern. Staining showed that moisture was absorbed by these seeds at sites randomly distributed over the whole seed coat, but not at the lens or hilum regions. However, below 4\% moisture content, when hard-seed
breakdown was induced, these seeds developed a breach at the lens region and moisture uptake and germination was rapid.

Seasonal changes in seed germination were measured by exposing seed (in lomenta) in mesh pockets either on the soil surface or covered with 1 cm of soil. Three cultivars of *O. sativus*, Cadiz (soft-seeded), Erica (hard-seeded) and Margurita (hard-seeded) were compared along with a hard-seeded cultivar of *O. compressus* (Santorini) (yellow serradella). The seed was produced at two sites with different soil types. The seed germination behaviour of cv.s Erica and Margurita are best described by the dominant SCP₁ type hard-seed character, and therefore the recessive SCP₃ type hard-seed was not represented in this experiment. Seed produced by the soft-seeded cultivar deteriorated on the soil surface, but this was reduced when seed was slightly covered with soil. Breakdown of hard-seeded cultivars determined that germination was distributed across at least three growing seasons after the year of production.

The single locus, three allele concept of inheritance was applied in a stepwise model to predict generational/seasonal population changes in hard and soft-seed production. Biological and environmental factors included in the model include initial (fresh) germination, cross-pollination, changes in seed germination *in situ* (seasonal hard-seed breakdown, seedling viability), and frequency of seasonal seed production. An important consideration in the model is the generational lag that occurs due to the control of the
maternal phenotype on seed germination, particularly when allogamic cross pollination occurs.

In the absence of conditions or treatments to break hard-seed dormancy, the model predicts *O. sativus* populations, cultivated in the traditional way of repeated harvesting for re-sowing, will rapidly become dominated by soft-seeded genotypes. The rate of loss of a dominant form of hard-seededness will be greater than for a recessive form. In both cases, the rate of change is reduced by cross-pollination.

Selective pressure favors hard-seed production when *O. sativus* is left to self-regenerate *in situ*. In a permanent pasture, the rate of change in a mixed hard and soft-seed producing population will, in the short term, be determined by the balance between seed deterioration of soft-seeded phenotypes and the rate of hard-seed breakdown (until soil seed reserves reach an equilibrium). As the hard-seed reserve in the soil approaches equilibrium, the selective pressure is driven wholly by rate of deterioration in soft-seeds.

The greatest rate of change in a population structure occurs with reproductive failure in a single or series of reproductive cycles. In these scenarios, the rate of change is more rapid with the recessive $S_3$ type hard-seed than $S_1$, because the dominant effect protects the allele for soft-seed. Cross-pollination reduces the rate of change in both cases of hard-seed. However, because of its absolute expression, all soft-seed alleles will be
removed from a population with a single season of failed reproduction in the absence of heterozygosis and cross-pollination.

This research showed that there are two genetically-controlled forms of hard-seed in *O. sativus*, each with a different expression of inheritance, and interaction with seed moisture. This has implications for the management of hard-seedness in *O. sativus* and in other legume species where moderate or variable proportions of hard-seeds occur. To maintain a proportion of hard-seeded genotypes in a cultivated population, that is sown and harvested each year, the harvested seed must be treated to remove the hard-seed dormancy before re-sowing.

The opportunities to expand the use of *O. sativus* in agricultural systems are discussed along with appropriate protocols that could be used in *O. sativus* breeding programs to achieve the full potential of the species.
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Declaration

I declare that all sources are acknowledged and this thesis is my own composition and the result of my own work.
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Chapter 1. General Introduction

“The land must rest every second year, or be sown with lighter kinds of seed, which prove less exhausting to the soil. A field is not sown entirely for the crop which is to be obtained the same year, but partly for the effect to be produced in the following; because there are many plants which, when cut down and left on the land, improve the soil”

Marcus Terentius Varro

(116 BC – 27 BC)

author of

Rerum Rusticarum de Agri Cultura
Serradella is the common name applied to species of genus *Ornithopus* L. They are low growing, annual legumes, producing small seeds tightly bound in indehiscent woody pods. Although they could be considered weeds in some situations, they also provide benefits that encourage cultivation. Their principle benefit to agriculture is the ability to form a symbiotic association with soil bacteria that fix atmospheric nitrogen. The nitrogen requirement of the serradella is provided by this relationship and the plant residues thereby improve soil fertility. Serradella therefore fits the description of plants beneficial to (rotational) plant cultivation documented by Varro more than two thousand years ago. They also provide high quality forage which is not compromised by anti-nutritional properties, and can be utilised *in situ* or conserved as hay or silage.

Species of *Ornithopus*, and their nitrogen fixing symbionts, are adapted to coarse textured, neutral to highly acidic soils of low fertility. This attracts the attention of agriculturalists because they are more productive on these soil types than most other pasture or crop legumes available to agriculture. Several species, *O. sativus* Brot., *O. compressus* L., *O. pinnatus* Druce, *O. perpusillus* L., and *O. microanthus* Arechav. have been evaluated for value to agriculture. (Gladstones and Barrett-Lenard, 1964; Gladstones and McKeown, 1977b; Ovalle et al., 2006). These evaluations report promise for serradella as a legume component of pastures on poor soils in regions with Mediterranean or temperate climates.
With the exception of *O. sativus*, serradellas are “wild plants” that produce high proportions of hard-seed. The production of “hard-seeds” (seeds impermeable to moisture) is a common mechanism of seed dormancy in the Papilionoideae sub-family of legumes to which *Ornithopus* belongs. It provides persistence of the species by distributing germination across several seasons. This is also a desirable feature in agricultural systems that utilise the self-regenerating capacity of hard-seeded legume species. In these systems, the legumes may be required to persist through one or more cycles of failed reproduction. This may arise following poor seasonal conditions, negative biotic influences or through rotational crop production, where the legumes and other weeds are removed by tillage or herbicides.

The production of hard-seed can present problems when attempting to cultivate or establish a species as a forage or seed crop in new areas (Aitken, 1939). Harvested seed with high proportions of hard-seed will have low levels of germination, and this is unlikely to change under normal storage conditions. In hard-seed producing species this is usually overcome in commercial quantities by the mechanical scratching of the seed coat. As this process requires access to the seed coat to be effective, any coverings over the seed must first be removed. In species where the covering is a tightly bound pod, such as in *Ornithopus*, this can be difficult to achieve without damage to the seed. It requires specialist equipment, and additional processing (Weeldenburg and Smith, 1969; Sanders, 1996a, 1996b). All of these factors can attract considerable cost in the provision of seed in commercial quantities with high germination.
In contrast to the wild species of *Ornithopus*, *O. sativus* could be considered a domesticated species that does not possess many of the ecological fitness characters that compromise commercial utilization. The most obvious of these is the production of seed which readily germinates (Gladstones and McKeown, 1977a; Bolland and Gladstones, 1987; Fu *et al.*, 1994a). But there are also several other more subtle characteristics that relate to seed capture and handling in bulk quantities that suit *O. sativus* to commerce. The wild species generally shed their seed pods as they senesce which makes harvesting inefficient. Further, the shape and structure of the pod, which is curved and has a hooked beak, does not move well in bulk handling equipment such as augers and elevators. *O. sativus* in contrast has good retention of the pods on the stems after complete plant senescence. These pods readily segment into small, evenly sized units and the pod-beak is small and generally straight. This allows *O. sativus* to be harvested by conventional crop machinery, without modification, and the harvested material is easily handled and cleaned in bulk (Nutt and Loi, 1999). It is because of this combination of characteristics that *O. sativus* is attributed with the greatest use and value to agriculture of all *Ornithopus* spp. (Frame *et al.*, 1998).

Cultivation of *O. sativus* was common in central and northern Europe from at least the 1800’s (Schofield, 1950). More recently, it has been cultivated in South Africa as a landrace known as Emena, on course textured soils in the Western Cape region. The cultivar Koha was developed in New Zealand for use on the more infertile soils where subterranean clover fails (De Lautour
and Rumball, 1986). In Australia, early commercial introductions of *O. sativus* for use as a fodder legume failed. In the traditional pasture-cereal rotation, agronomists concluded that late maturity and the lack of seed dormancy were the key weaknesses of the species and the most likely cause of failure (Gladstones and Barrett-Lennard, 1964). For these reasons, incorporation of serradella into Australian farming systems focused on the hard-seed producing species, *O. compressus*, *O. pinnatus* and *O. perpusillus*. Cultivars of each of these species were released to commerce in Australia between 1950 and 1990, however, the problems of extracting and scarifying the hard-seed limited their wide scale impact (Michalk and Revell, 1994; Nutt, 1994a,b). The availability of seed of most of these cultivars faded from commerce soon after release.

An early maturing cultivar of *O. sativus*, Cadiz, was developed for Australian conditions and released commercially in 1996 (Anon., 1997a; Nutt and Paterson, 1997). It has achieved considerable success in the Mediterranean environment of south-western Australia. On average, 270 tonne of certified Cadiz seed (in the pod) is produced in Western Australia each year (data provided by AGWEST Seed Laboratories). Before the release of Cadiz the greatest quantity of *Ornithopus* certified in Western Australia was less than 40 tonne in a single year (mostly *O. compressus* cultivar Madeira). Like most forms of *O. sativus* cultivated around the world, cv. Cadiz is soft-seeded. Utilization of this cultivar is limited to systems where it is frequently re-sown (Dear *et al.*, 2002).
During the evaluation of cv. Cadiz, rare hard-seeds were observed during germination testing. Upon growing these hard-seeds to mature plants, some were found to produce high proportions of hard-seed. These observations led to the development and commercial release of two hard-seeded cultivars, Erica and Margurita (Anon., 2004a, b; Nutt and Loi, 2004). The possibility to develop these hard-seed producing genotypes from a largely soft-seeded population raised a number of questions which inspired this study. What are the genetic and environmental influences on the production and subsequent breakdown of hard-seed in *O. sativus*? Can diversity for this character exist within *O. sativus* populations? And what are the implications on management and further exploitation of *O. sativus* in farming systems?

The experimental chapters in this thesis have been prepared for publication, so there is some repetition of information in the Introduction and Materials and Methods sections of some chapters.
Chapter 2. Review of Literature.

2.1 The importance of nitrogen and the value of legumes to agriculture.

Agriculture exports nutrients in the form of plant and animal products, and this diminishes soil fertility if the exported nutrients are not replaced. The rate of exhaustion depends not only on gross exploitation, but also on the relative balance of different nutrients exported and the inherent fertility of the soil. Sustainable agriculture therefore requires management of plant nutrients in order to maintain productivity. One nutrient in high demand in most agricultural systems is nitrogen. As a result, global nitrogen turnover is estimated to have doubled in modern times due to agricultural activity (Vitousek et al., 1997).

Legumes are plants that can acquire nitrogen through a symbiotic relationship with nodule bacteria that fix nitrogen from the atmosphere (Postgate, 1998). Due to this ability, they can replace nitrogen exported by agricultural activity, or alternatively, grow successfully and competitively on soils with low (inherent or exhausted) levels of available nitrogen. Therefore, crop rotation involving legumes has been used as a management tool to maintain agricultural productivity from ancient times (Karlen and Sharpley, 1994; Karlen et al., 1994; Egerton 2001).
2.1.1 Historical use.

Evidence of the use of legumes is not only found in historical accounts, but also preserved pollen and seeds (or their remnants) have been recovered by archeologists in ancient domestic situations and tombs (Egerton 2001; Oybak-Donmez, 2006). The legumes that are recorded as cultivated in ancient times are mostly those species that yield large edible seeds, such as soybeans, chickpeas, cowpeas, phaseolus beans, peas, lupins and lentils (Zohary and Hopf, 1973). In modern times, these pulses are recognized to possess a number of subtle nutritional benefits, but the original incentive for cultivation would have been as a source of dietary protein and perhaps culinary and storage properties (Messina, 1999; Howieson et al., 2008). However, recognition of the agronomic benefits of rotating cereal and legume crops must have led to the eventual understanding that legumes could replace animal manure for soil amelioration when ploughed into the soil (Fred et al., 1932).

Small seeded legumes, which were not collected as human food (such as Trifolium, Medicago, Lotus and Ornithopus spp.), may also have played an important but less recognized role in ancient agriculture, as fodder producing plants. The rearing of animals to provide food, hide, and fiber, and their use as beasts of burden was a common feature of nomadic lifestyles. Shepherds and herdsmen would have sought pasture that provided good animal nutrition, and these are likely to have contained a significant legume component. The plant species utilized in good grazing land could then be spread by seed after being collected by their human companions for
livestock fodder, or in faeces after being directly grazed by animals. This would explain the presence of seeds of these species in ancient domestic settings (Oybak Donmez, 2006).

The importance of a pasture or forage legume in crop rotation was discovered, or rediscovered, during the European agricultural revolution of the late medieval period (Kjærgaard, 2003). Between 1750 and 1880, European agricultural output increased by an estimated 175%, and as much as two thirds of this increase was attributed to the cultivation of clover (Chorley, 1981). However the true nature of the benefits derived from legume cultivation was not fully understood until the phase of “scientific agriculture”, based on the use of inorganic soil amelioration, comparative experimentation, and plant breeding.

2.1.2 Nitrogen fixation.

Nitrogen is acquired by legumes through a symbiotic association with nitrogen fixing bacteria. These bacteria can survive as free saprophytes in the soil, but will infect compatible legumes to form nodules occupied by the bacteria, with the legumes supplying nutrition to the bacteria and in return obtain their requirements for nitrogen. Rhizobia is the generic term applied to the bacterial symbiotic partners, although eleven different genera have been distinguished so far; \textit{Allorhizobium}, \textit{Azorhizobium}, \textit{Blastobacter}, \textit{Bradyrhizobium}, \textit{Burkholderia}, \textit{Devosia}, \textit{Ensifer}, \textit{Mesorhizobium}, \textit{Ralstonia}, \textit{Rhizobium}, and \textit{Sinorhizobium} (Graham, 2008). For legumes to assimilate atmospheric nitrogen, effective strains of rhizobia need to be present in the
soil and the interaction between legume species/genotype with rhizobial genus, species and strain can be highly specific (Deaker et al., 2004).

In some situations, rhizobia have migrated along with their associated legume plants from their centres of origin. Where agriculture has required crop or forage legumes in regions lacking compatible indigenous or naturalised micro-symbionts, commercial inoculants have been required (Deaker et al., 2004; Howieson and Herridge, 2005). There may also be improvements in legume productivity through the introduction of additional rhizobial strains that are more effective on particular (contemporary) legume genotypes. Humans could, therefore, be considered a third party in the symbiosis, as agricultural activity spreads both the legume and associated nitrogen fixing micro-organisms.

Generally, nitrogen fixing rhizobia occupy nodules formed in the root cortex of legumes, although some tropical species also form nodules on their stems. The infection of the legume roots and stems by these bacteria follows a process of biochemical recognition signals. Facilitated by this chemical recognition, the bacteria invade the cortical tissue through cracks or via infection threads after encapsulation of the bacteria by induced curling of root hairs. The resulting infection of legume roots or stems by their appropriate rhizobia leads to either determinate nodules (i.e. of a limited size) or indeterminate nodules (i.e. that can continue to grow) (Sprent, 2001).
The chemical signaling between the legume plant and soil-borne bacteria, and the subsequent biochemical interactions, involve complex genetics on both sides of the symbiosis. The compatibility of these combined genetics will dictate how effectively nodulation occurs and nitrogen is assimilated. Added to this complexity is the influence of conditions, such as soil fertility, soil texture and soil pH reaction, that affect either the host legume or the rhizobial symbiotic partners (Postgate, 1998).

The combination of characteristics of bacterial growth on artificial media (e.g. metabolizable nutrients, physical appearance, and rate of growth) and effective associations with a range of test legume species (genotypes) were the key determinants of rhizobial taxonomy until the molecular era (Graham, 1976). More recently, molecular analysis, particularly of the \textit{nif} and \textit{nod} genes, and chromosomal genes such as 16S rRNA, has added to this knowledge to further the understanding of rhizobial phylogeny and evolutionary development (Martinez-Romero, 1994; Willems, 2006).

The \textit{Ornithopus} genus forms determinate nodules with \textit{Bradyrhizobium canariensis} (Stepkowski \textit{et al.}, 2005). This is an association tolerant of acidic soil conditions, and relative to many other legume-rhizobium associations, spontaneous nodulation in the field is reliably effective (Ballard, 1996; Howieson \textit{et al.}, 2008). Other legume genera nodulated by \textit{Bradyrhizobium canariensis} include \textit{Lupinus} and \textit{Chamaecytisus} and other species of \textit{Bradyrhizobium} nodulate members of the legume genera \textit{Lotus}, \textit{Lespedeza}, \textit{Medicago}, \textit{Melilotus} and \textit{Glycine max} (Willems, 2006).
2.1.3 Impact of industrial nitrogen fixation.

Industrial fixation of nitrogen through the Haber-Bosch process has reduced reliance on biological sources of nitrogen in agriculture over the past century (Peoples et al., 1995; Smil, 2001; Kjaergaard, 2003). This method of industrial nitrogen fixation is an energy demanding process and biological fixation remains an important, low input source of nitrogen in many agricultural systems (Sanginga, 2003). The rotation of legumes with cereals, and the use of legumes to improve pasture productivity remain a common feature of farming systems around the world (Howieson et al., 2008).

Grain and forage legumes are grown on some 180 million ha or 15% of the earth’s arable surface (Graham and Vance, 2003). This accounts for 33% of the global human dietary protein-based nitrogen. In crop rotation the stubbles remaining from these crops would play an important role in the nitrogen balance if they are returned to the soil. The nitrogen fixed by grazed legume-based pastures in Australia has been estimated to contribute between $800 million and $2 billion U.S. per year in terms of the input of nitrogen through atmospheric fixation (Howieson and Herridge, 2005). As we progress through the current environmental and political issues of our time (i.e. global warming, increasing cost of energy, and nitrification of aquatic ecosystems) there may be an even greater role for the legume symbiosis as a source of nitrogen fixation in the future.

In addition to the supply of nitrogen, the cultivation of legumes is encouraged by other pressures and opportunities within whole farming systems. Rotating
monocultures of legumes, brassicas and cereals between seasons provides a number of benefits through the complementation of activities to overall farm productivity (Puckridge and French, 1983; Robson, 1988). Different types of rotational crops provide a wider array of potentially integrated options for disease, insect and weed control (Karlen et al., 1994). The direct and indirect benefits of legume cultivation therefore continue to provide incentive for agronomy, plant breeding and microbiology to expand the use of legumes in agricultural systems.

2.2 The genus Ornithopus.

The genus *Ornithopus* L. belongs to family Leguminosae, sub-family Papilionoideae, tribe Loteae (Degtrareva et al., 2003; Sokoloff and Loch, 2005). It has also been classified under the synonyms Fabaceae (=Leguminosae), Faboideae (= Papilionoideae) and in the alternate tribe of Coronilleae (Polhill, 1981; International Plant Names Index, 2005; Germplasm Resources Information Network, 2007). *Ornithopus* species are collectively given the common names serradella or birds-foot (Gladstones and McKeown, 1977a; Frame et al., 1998).

European and North African species of *Ornithopus* are annual herbs that fill a ruderal ecological niche on acidic, relatively infertile, coarse textured and stony soils at low altitudes (Heywood and Ball, 1968; Gladstones, 1976; Hanf, 1983). One species (*O. sativus*), has been cultivated for fodder and green manure, and its seed has been collected and disseminated in Europe for hundreds of years (Griesinger and Klinkowski, 1939). This may explain...
the specific name *sativus* used by the naming taxonomist (Felix Avelar Brotero), as this Latin epithet translates to “that which is sown” or “cultivated”. The Iberian peninsular is believed to be the origin or first region of cultivation of *O. sativus*, and its use has spread through France and Belgium to northern and eastern Germany, Poland and western Russia (Schofield, 1950; Gladstones and Barrett-Lenard, 1964).

Species of *Ornithopus* other than *O. sativus* may also form an important component of grazed grasslands or meadow hay in their natural ranges (Gladstones and McKeown, 1977a; Hanf, 1983). Their spread has also been aided by intentional distribution (as pasture seed or seed contaminant), animal movement, and the use of fodder, to the extent that they have become naturalized in many regions around the world including South Africa, north and south America, New Zealand and Australia (Halliwell, 1960; Gladstones and McKeown, 1977b).

### 2.2.1 Morphological characteristics.

Species of *Ornithopus* grow initially as a rosette that develops sprawling, prostrate to ascending stems as flowering is initiated and internodes lengthen. They have alternate, imparipinnate leaves with small, membranous, linear stipules (Heywood and Ball, 1968; Chamberlain, 1970). Flowers are produced in an umbel borne on a long auxiliary peduncle of two sections separated by a node that may have an associated leafy pseudo-bract (Degtrareva *et al.*, 2003). The flowers are composed of small inconspicuous bracts, a tubular or campanulate calyx with five equal teeth, a
relatively large standard petal, two smaller wing petals and keel of two petals fused along the lower edges and positioned between the wings. The pistil and stamens are tightly bound by the keel petals and are exposed only with significant manipulation. Small nectaries are found at the base of the wing petals.

Seed is formed in indehiscent jointed lomentose pods, constricted to varying degrees between the segments, where there is a conspicuous inter-segment wall and suture. The pods are compressed or terete in cross section and strongly reticulate on the surface (Chamberlain, 1970; Polhill, 1981). Pods range from straight to a more or less single, hollow helical coil and the style remains as a rigid hook (beak) of various sizes and shapes (Figure 2.1). The whole umbel with several developed pods resembles a jointed “birds foot” with claws, the likely origin of the common name for the genus (Gladstones and McKoewn, 1977a; Michalk and Revell, 1994).

The morphological characters used to distinguish the different *Ornithopus* species, and ecotypes within the species, include presence or absence of small hairs on the stems, petioles, leaves, peduncles and pods; presence or absence of a small imparipinnate pseudo-bract subtending the flower umbel at the peduncle node; flower colour; and pod size and shape (Table 2.1, Figure 2.1) (Maire and Samuelsson, 1939; Griesinger and Klinkowski, 1939; Hanf, 1983; Fu *et al.*, 1994a, 1994b; Snowball, 1996).
2.2.2 Distribution.

The genus has a wide natural distribution with a northern boundary of Denmark, Sweden and United Kingdom; an eastern boundary of Georgia and the Levant; a southern boundary of Libya, Tunisia, Algeria and Morocco: a western boundary of Portugal and Spain, including the Canary Islands, Madeira and the Azores (Figure 2.2) (Griesinger and Klinkowski, 1939; Ball, 1968; Chamberlain, 1970; Hanf, 1983; Gladstones, 1976). *O. micranthus* is an isolated species, found only in Argentina, Uruguay and southern Brazil.

The three most common and widespread species *O. compressus*, *O. pinnatus*, and *O. perpusillus*, also have the most clear taxonomic classification, although some synonyms have been used in the past (Table 2.1). Besides *O. micranthus*, all other species and speculated hybrids occur within the boundaries of these species.

*O. compressus* and *O. pinnatus* share a similar natural geographical distribution around the Mediterranean region, although the latter is confined to lower altitudes and extends into the south-western United Kingdom (Hanf, 1983; Gladstones and McKeown, 1977a). *O. perpusillus* has a more northerly, continental distribution and overlaps the previous two species only in the Iberian peninsular, south-western France, Italy, and with *O. pinnatus*, in the southern United Kingdom. It has also been observed rarely in Morocco and Algeria (Gladstones, 1976). Of these three species only *O. compressus* has been noted as having an agricultural use as cut fodder in their natural
ranges, although all three may be present in grazed grasslands (Griesinger and Klinkowski, 1939; Gladstones and McKeown, 1977a).

Figure 2.1. Pods of the genus *Ornithopus*. A. *O. sativus* cv. Cadiz. B. (left to right) *O. sativus* var *roseus*, *O. perpusillus*, *O. sativus* var *isthmocarpus* and *O. sativus* var *sativus*. C. Variation in pods of *O. isthmocarpus*. D. Variation in pods of *O. compressus*.

*O. uncinatus* Maire & Sam., which has been recorded only rarely in the lower Atlas Mountains of Morocco, is distinguished from the other species in being very hirsute; it has white/cream coloured flowers; and very woody short, squat pods of not more than 5 seeds. *O. micranthus* is relatively unique in
the genus, not only because of its distribution, but also production of adventitious roots from nodes on the stem, occurring on waterlogged soils and having no subtending leafy bracts (Degtrareva et al., 2003). It is also unique in the genus for the production of both a secondary branch and an inflorescence at the same node. All other species of *Ornithopus* only produce an inflorescence at nodes past the first flowering node produced by that stem.

Classification of the pink flowered *O. sativus* Brot. complex is difficult due to the lack of descriptive characters that can clearly delineate groupings. The presence of subspecies and hybridization within these and other *Ornithopus* species may explain the continuum. The classification used in Table 2.1 is currently recognized by the International Legume Database and Information Service (2008), Germplasm Resources Information Network (2005) and the International Plant Names Index (2005). This system will be adopted in this thesis and the shortened form of *O. sativus* refers to *O. sativus* subsp. *sativus*.

The distribution of *O. sativus* subsp. *isthmocarpus* is predominantly in Morocco, but it also occurs occasionally in southern Spain and Portugal (Griesinger and Klinkowski, 1939; Gladstones, 1976; Gladstones and McKeown, 1977a; Aser, 1987). *O. sativus* subsp. *isthmocarpus*, in the most extreme form, produces a large standard petal relative to the wing petals, and pods with long narrow constrictions between the pod segments and a large hooked beak (woody remnant stigma). At the other extreme *O. sativus*
subsp. *sativus* produces a smaller standard petal, straight segmented pods and a small, straight beak (Figure 2.1). *O. sativus* subsp. *sativus* is considered a native of the western Iberian peninsular however it is also cultivated in Germany, Poland and Russia. The characters of *O. sativus* subsp. *macrorrhynchus* pods fall between these two sub-species and its distribution is limited to where their natural ranges overlap in the central Iberian peninsular (Figure 2.2). *O. sativus* subsp. *macrorrhynchus* has been proposed to be a natural hybrid between these sub-species, although artificial hybridization has been unsuccessful (Griesinger and Klinkowski, 1939).

Morphological and molecular data suggest the closest relatives to *Ornithopus* within the tribe *Loteae* are in the genus *Hosakia* Steud (Degtareva *et al.*, 2003). The genus *Hosakia* is comprised of both annual and perennial species and occurs only in North America. These factors were used to suggest that the South American species, *O. micranthus*, has a basal position in the phylogeny of *Ornithopus* and is native to South America rather than the result of transport during human migration (Degtareva *et al.*, 2003). It is possible that the genus was widely distributed in the past, including North America. However, the distribution of the Old World species strongly suggests the Iberian peninsular or north-west Africa as a centre of origin for the genus *Ornithopus*. 
### Table 2.1. Recognised species of *Ornithopus*, synonyms and distinctive characteristics.

<table>
<thead>
<tr>
<th>Species</th>
<th>Synonyms</th>
<th>English common names</th>
<th>Distinctive characters</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. compressus</em> L.</td>
<td><em>Artrobolium micranthum</em> Benth.</td>
<td>Yellow serradella,</td>
<td>Pubescent. Yellow flowers. Pinnate, leafy, subtending pseudo-bract. Pod only faintly</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yellow birdsfoot.</td>
<td>transversely constricted and usually compressed (Ball, 1968; Chamberlain, 1970; Hanf,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1983).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Slender serradella,</td>
<td>secondary branching and inflorescence development can occur at the same node (Deglare</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Orange birdsfoot.</td>
<td>a et al., 2003).</td>
</tr>
<tr>
<td><em>O. perpusillus</em> L.</td>
<td><em>O. pusillus</em> Lepech.</td>
<td>Birdsfoot, Common birdsfoot.</td>
<td>Pubescent. Pink/yellow or white/red flowers, corolla 3-4 mm (not more than 5 mm).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudo-bract much longer than flowers. Pods distinctly transversely constricted (Ball,</td>
</tr>
<tr>
<td><em>O. pinnatus</em> Druce</td>
<td><em>O. ebracteatus</em> Brot.</td>
<td>Slender serradella,</td>
<td>Glabrous. No pseudo-bract. Small cylindrical, slender pods, not constricted between</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Orange birdsfoot.</td>
<td>segments (Ball, 1968; Chamberlain, 1970; Hanf, 1983).</td>
</tr>
</tbody>
</table>
Table 2.1. Recognised species of *Ornithopus*, previously used synonyms and distinctive characteristics (cont.).

<table>
<thead>
<tr>
<th>Distinctive characters</th>
<th>Synonyms</th>
<th>English common names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pods curved with long narrow constriction between segments, beak 10mm or more (Ball, 1968; Chamberlain, 1970).</td>
<td><em>O. ishmocarpus</em> Coss.</td>
<td>French serradella.</td>
</tr>
<tr>
<td>Pods almost straight, beak not more than 5mm (Ball, 1968; Chamberlain, 1970).</td>
<td><em>O. salivus</em> subsp. <em>sativus</em> Alsina</td>
<td>Pink serradella.</td>
</tr>
<tr>
<td>Pods slightly curved, beak 5-8mm (Griesinger and Klinkowski, 1939).</td>
<td><em>O. macrorrhynchus</em> Klink. &amp; Schwartz.</td>
<td>- subsp. <em>ismoricarpus</em> Dostal.</td>
</tr>
<tr>
<td>Pods slightly curved, beak 5-8mm (Griesinger and Klinkowski, 1939).</td>
<td><em>O. isthmocarpus x O. sativus</em> Klink. &amp; Schwartz.</td>
<td>- subsp. <em>ismoricarpus</em> Alsina.</td>
</tr>
</tbody>
</table>
Figure 2.2. Distribution of European and North African *Ornithopus* species (adapted from Griesinger and Klinkowski, 1939; Ball, 1968; Chamberlain, 1970; Hanf, 1983; Gladstones, 1976).
2.2.3 Sources of conserved germplasm.

The main sources of conserved *Ornithopus* germplasm are the Australian Trifolium Genetic Resource Centre (ATGRC), Perth, Western Australia, the Agresearch Margot-Forde Genetic Resource Centre, New Zealand, Vavilov Genetic Resource Centre, St. Petersburg, Russia, and the Polish Genetic Resource Centre, Krakow, Poland.

2.3 Ecological importance of seed dormancy and implications for the use of legumes in agriculture.

Plant domestication as an evolutionary process leads to a number of changes to match the requirements for cultivation (Heiser, 1988). These include:

1. Loss of natural dispersal mechanisms.
2. Even and rapid seed germination.
3. Larger propagules.
4. Simultaneous ripening of fruits or seed.
5. Loss of mechanical protection (such as spines).
6. Particular fruit or seed colour.
7. Loss of toxic or bitter properties.

Often the characters selected in a cultivated environment are not adapted to a natural environment, resulting in genetic drift away from wild progenitors (Lester, 1989). Seed dormancy is one of these important “ecological fitness” characters that are often absent or reduced in cultivated plants (Baskin and Baskin, 2001).
2.3.1 Seed dormancy.

Dormancy, as applied to a seed, is the inability of the embryo to germinate. It is imposed by the physiology or morphology of the seeds themselves, surrounding structures or by environmental influences (Nicholaeva, 1977; Bewley and Black, 1994). As a survival strategy and a behavior subject to selective evolution, dormancy provides embryo protection during adverse conditions and may synchronise germination with conditions that lead to the further reproduction of the species and particular genotype. As such, seed dormancy is an important aspect in the ecology of a plant species, along with mortality patterns, reproductive effort, seed dispersal, rate of vegetative growth, and conditions required for germination and plant establishment (O’Connor, 1991).

Given the diversity of flowering plants, and the many environments in which they survive as self-perpetuating populations, a number of different mechanisms of seed dormancy have developed (Van Staden et al., 1989). At a broad level three descriptions have been applied to these different mechanisms (Nikolaeva, 1969; Nikolaeva, 1977; Bradbeer, 1988; Bewley and Black, 1994);

1. Exogenous or coat imposed dormancy, where tissues enclosing the embryo (testa, endosperm, pericarp or other floral structures) constrain germination.
2. Endogenous or embryo dormancy where control of germination is imposed by the embryo itself.
3. Combined dormancy where both of these mechanisms occur together.
These broad categories can be further subdivided according to the cause of dormancy and conditions under which the dormant state is broken (Table 2.2). Seeds have also been described as having primary or innate dormancy, when dispersed from the maternal plant in a dormant state. Both exogenous and endogenous dormancy may operate at a primary level. Alternatively, dormancy may be imposed or enforced by environmental conditions on an otherwise non-dormant seed. This may be a response to anaerobic conditions, darkness, light, water stress or temperatures either above or below an optimum range. Further, if seeds subject to enforced dormancy fail to germinate once returned to optimal conditions, they are considered to be in a state of secondary dormancy (Bewley and Black, 1994). The relationships between these states of dormancy as they relate to the dynamics of a seed bank have been illustrated as a flow diagram by Bradbeer (1988) (Figure 2.3). The application of this schematic to O. sativus will be discussed further in this thesis.

Figure 2.3. The dynamics of a soil seed bank (Bradbeer, 1988).
Table 2.2. Classification scheme of organic seed dormancy types (Baskin and Baskin, 2001).

<table>
<thead>
<tr>
<th>Type</th>
<th>Cause</th>
<th>Broken by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous dormancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Physical</td>
<td>Seed coat impermeable to water</td>
<td>Opening of specialized structures, abrasion</td>
</tr>
<tr>
<td>- Chemical</td>
<td>Germination inhibitors</td>
<td>Leaching</td>
</tr>
<tr>
<td>- Mechanical</td>
<td>Woody structures restrict growth</td>
<td>Warm and/or cold stratification</td>
</tr>
<tr>
<td>Endogenous dormancy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Physiological</td>
<td>Physiological inhibiting mechanism (PIM) of germination</td>
<td>Warm and/or cold stratification</td>
</tr>
<tr>
<td>- Morphological</td>
<td>Underdeveloped embryo</td>
<td>Appropriate conditions for embryo growth/germination</td>
</tr>
<tr>
<td>- Morphophysiological</td>
<td>PIM of germination and underdeveloped embryo</td>
<td>Warm and/or cold stratification</td>
</tr>
</tbody>
</table>

2.3.2 Seed dormancy, survival, and dispersal.

In the *Papilionoideae* family of legumes, both physical (impermeable seed coat referred to as hard-seededness) and physiological (embryo) dormancy have been described (Quinlivan, 1971; Baskin and Baskin, 2004). Physiological dormancy (which is primary and endogenous) in legumes is usually short lived, protecting the embryo from untimely germination during (and for a short period after) the final stages of seed development. Hard-seed dormancy (which is exogenous and could be considered secondary) is more persistent, and seed subject to this type of dormancy can remain quiescent in the soil for a number of years.

The relative importance of short-term physiological and hard-seed dormancy to the survival of a species could be expected to change depending on
climate. For late maturing *Trifolium subterraneum* L. genotypes from long-season, temperate environments, embryo dormancy protects against germination after rainfall late in the growing season but before the onset of seed coat impermeability (Morley, 1958; Rossiter, 1966). However for *T. subterraneum* and other annual legumes in Mediterranean climates, the production of hard-seed (and the release from this dormancy) is considered the most important type of dormancy leading to long term persistence (Quinlivan, 1971; Russi *et al*., 1992a; Norman *et al*., 1998).

There are a number of ecological advantages of the hard-seed dormancy strategy. These include;

1. Greater opportunity for temporal dispersal of reproductive effort to reduce risk (Norman *et al*., 2005).
3. Reduced competition between parents and offspring and/or between siblings (Ellner, 1986; Kobayashi and Yamamura, 2000).

All of these aspects of hard-seed dormancy have both short and long term involvement in competitive ability and overall survival.

Hard-seed dormancy as a survival strategy will also be interlinked with other plant characters and particular environmental conditions. How these characters, and interaction between characters, contribute to the survival of a genotype is largely driven by the balance between risk of failed reproduction
in the short term and long term (via offspring). Therefore no one character, behavior or combination is a guarantee for success, but rather need to be considered holistically within the conditions where the species successfully persists. Characters important for success in natural ecosystems may be different from agro-climatic systems, where the species is expected to contribute to farm productivity.

Within an annual cycle, seedlings can arise from germination of new non-dormant seeds in soil and from seeds that germinate after the breakdown in dormancy (Bewley and Black, 1985). The ratio of both will be dependent on the likelihood of both unseasonable rainfall (rainfall outside of the expected wet season) and sufficient follow up rain to allow germination and survival to reach reproduction within a season. Seed that germinates from a dormant pool may have been produced in previous seasons. In this way an established population can be composed of relatives that are several generations removed. Depending on the longevity of seed dormancy this could create genetic inertia or stability under an allogamous breeding system.

In the Mediterranean climate of south-western Australia, analysis of long term meteorological records show that a false-break event (unseasonal rainfall occurring in late summer to mid-autumn) occurs approximately 2 years in every 3 (Chapman and Asseng, 2001). The greatest risk period was early autumn (mid-March to mid-April) in the study area. If hard-seed breakdown has occurred by this time, significant loss of plant density can
occur and this is cited as a factor in the seasonal failure of *T. subterraneum* in this region (Taylor, 2005; Norman *et al.*, 2006). Delaying the breakdown of dormancy past this high risk period may provide better species persistence. Balanced against this, is a loss of competitive ability if germination occurs after that of potential competitors for the same ecological niche.

Accumulation of a persistent reserve of dormant seed minimises the impact of poor seasons, and allows a species to take advantage of good seasons (Brown and Venable, 1986). The requirement for this type of dormancy increases as the likelihood of favorable seasons decreases. This concept is supported by both conceptual modeling and field studies (Cohen, 1966; Venable, 1985; Brown and Venable, 1986; Silvertown, 1988). In most of these studies a poor season is usually judged by seasonal rainfall/moisture conditions, however it can also be expanded to other conditions that may lead to failed or reduced reproduction (Jansen, 1969). Fire, severe disease and insect predation, over-grazing and cultivation or herbicide use in farming systems can seriously reduce seed production regardless of seasonal conditions.

Hard-seed dormancy and seed coat impermeability may (in association with seed size) be an important strategy to survive animal predation and ingestion. This would also provide a means of seed dispersal (Malo and Suarez, 1995; Malo and Suarez, 1997). Seeds with permeable coats have a high probability of either being digested or suffer considerable damage when ingested by a grazing animal (Edward *et al.*, 1998). The likelihood of
passage of hard-seed is negatively correlated with seed size, although this may be complicated when the seed is tightly bound within woody pods or lomentums, such as in *Medicago* and *Ornithopus* spp. (Thomson *et al.*, 1990; Russi *et al.*, 1992b; Edward *et al.*, 1998).

The role of seed dormancy in reducing competition between parent plants and their offspring, as predicted by conceptual modeling, pertains only to perennial species. It may be important to both annuals and perennials in terms of sibling competition (Ellner, 1986; Philippi, 1993). However, for an annual species that has only one chance of reproduction, and potentially high levels of multiplication, this would be a minor influence in comparison to the other drivers for the development of seed dormancy and particular patterns of release from dormancy.

While seed dormancy is an important aspect of the ecology of plants in natural settings, it does present problems to the cultivation of some plants in agriculture. For efficient cultivation of grain and fodder crops, seed germination must predictably and reliably result in established plants. Absence of seed dormancy provides guaranteed seed germination and plant establishment. A residue of dormant seed may also become a weed or contaminant in future crops, and a potential source of seed borne disease. Therefore, active selection against dormancy may have taken place in the past, and remains a part of many modern crop plant breeding programs (Cowling, 1998). The constraints and problems of seed dormancy are
emphasized in plants with annual or very short life cycles because of their reliance on reproduction and cultivation by seed.

In addition to active selection against seed dormancy for agronomic considerations, the act of seed propagation itself creates significant selection pressure against seed dormancy. Under repeated cycles of sowing, seed harvesting and storage under mild conditions, only seeds that germinate will reproduce and contribute to the harvested population. If this management regime does not result in the release of seed dormancy, genotypes that do not produce dormant seeds will come to dominate the cultivated population.

Hard-seed dormancy is usually absent in cultivated legumes due to the selective pressures and requirements for efficient cultivation outlined above. However, it can be a useful trait in certain situations. Seed longevity is negatively related to relative humidity and temperature under storage (Ellis and Roberts, 1980; Dickie et al., 1990). Therefore, pulse crops such as cowpea with cultivars that do not form an impermeable seed coat can lose viability under storage in humid climates (Kilen and Hartwig, 1978; Singh and Ram, 1986). The development of hard-seeded forms of these crops has been investigated as a means of overcoming this problem. However, this solution would also require a means to overcome the hard-seed dormancy when the seed was required for sowing.

The spontaneous regeneration of a legume species from a persistent soil reserve of hard-seed is attractive to low-input systems for the production of
fodder and/or for atmospheric nitrogen assimilation. This principle has been the basis of the pasture-ley farming systems of southern Australia (Puckridge and French, 1983). These systems have relied largely on *T. subterraneum* and *Medicago truncatula* Gaertn., however other hard-seeded annual legumes are increasingly being adopted (Howieson *et al.*, 2000; Nichols *et al.*, 2007). The benefit to low-input systems is that the hard-seeded legumes require expenditure on only the initial introduction. Subsequent re-establishment occurs from the accumulated seed reserve in the soil. However, even in these systems the high proportion of hard-seed has presented problems for obtaining seed with high levels of germination for the initial introduction of these species (Aitken, 1939).

The challenges that hard-seed dormancy presents to efficient cultivation can be overcome with modern agricultural techniques and mechanization, although additional cost or processes are necessary. Firstly, the weed potential of a hard-seeded species in subsequent crops due to germination from a seed reserve in the soil can be controlled with herbicides. Secondly, germination of hard-seed can be enhanced with scarification that breaches the impermeable seed coat (see section 2.5). Clearly, seeds need to be accessible for scarification to be effective and this can be problematic in species in which the seed is tightly bound in a persistent and non-dehiscent pod.

Legume genera that may have agricultural value but their use is constrained by seed coverings hindering access to the seed coat include *Ornithopus,*
Medicago, Hedysarum, Onobrychis, Scorpiurus, Bituminosa and Hippocrepis (Gintzburger et al., 1990; Frame et al., 1998; Abbate et al., 1999). Removal of seed coverings may require specialist equipment (and may result in seed damage) and any additional processing requirements can be a barrier to adoption because of higher costs and restricted seed availability (Rolston, 2003).

2.4 **Seed coat structure and hard-seed dormancy of family Leguminosae, sub-family Papilionoideae legumes.**

The legumes of the sub-family Papilionoideae have similar seed anatomy. They are bilaterally asymmetrical, with bent (campylotropous) embryos and the chalaza and hilum structures close together (Figure 2.4) (Manning and van Staden, 1987). The hilum is a distinctive round or oval abscission scar with a central longitudinal fissure. Usually abscission occurs cleanly at the funicule-ovule junction although in some genera remnants of the funicule can sometimes obscure the hilum (Cormer, 1951; Gunn, 1981).

The lens (also refered to as the strophiole by some authors) appears as a raised dome along the raphe. Beneath the hilum is a longitudinal bar of tracheids that may continue to the region below the lens (Revell, 1997). The micropyle is generally close to the hilum, at the opposite side to the lens, and close to the radicular lobe.
2.4.1. The legume seed coat.

The surface of the general part of the seed coat usually appears smooth to the naked eye but sculptured, pitted and perhaps cracked under high magnification (Gunn, 1981; Manning and van Staden 1987; Serrato Valenti et al., 1989). In cross section, the seed coat is composed of a number of layers formed from two ovule integuments (diploid maternal tissue). The outer ovule integument develops into the protective layers of the seed coat, while the remnants of the inner ovule integument appear as a layer of crushed cells that have little or no protective role (Corner, 1976; van Staden et al., 1989). From the outside to the inside the outer integument comprises an outer cuticle; a sub-cuticular layer; a rigid palisade of radially elongated
macrosclereid cells; a layer of hourglass osteosclereid cells; the nutrient or parenchyma layer; and an inner epidermis (Figure 2.5) (Rolston, 1978; Gunn 1981; Serrato Valenti et al., 1989).

The outer cuticule is a non-cellular deposit of wax and cutin that covers the entire seed surface except the hilum (Cavanagh, 1981). Ballard (1973) has provided the best evidence so far that this layer does not play a major role in seed coat impermeability in legumes. Its removal with solvents does not make seeds permeable; it is stained by aqueous dyes; and water can move through this layer from remote punctures through the seed coat. However, the thickness of this layer has been associated with the degree of seed coat impermeability in Ononis sicula (Gutterman and Heydecker, 1973).

Figure 2.5. Stylized longitudinal section showing cellular layers of a T. subterraneum seed coat (from Bhalla and Slattery, 1984).
The sub-cuticular layer has been reported to contain pectin, cutin, suberin, cellulose, hemicellulose and/or callose in various legume species (Hamly, 1932; Aitken, 1939; Watson, 1948). The caps of the underlying macrosclereid cells appear imbedded into this layer in Trigonella caerulea L., Medicago lupulina L., M. sativa L., and T. subterraneum. However it appears as a separate layer in Rhynchosia minima (L.) DC. and Lupinus angustifolius L. (Rangaswamy and Nandakumar, 1985; Serrato-Valenti et al., 1989). The sub-cuticular layer was not regarded as being responsible for water impermeability of seed coats in Melilotus alba Des., T. subterraneum, and Coronilla varia L. (Hamly, 1932; Aitken, 1939; Brant et al., 1971). However, it was found to be impermeable in Rhynchosia minima. In Pisum, the sub-cuticular layer appeared to harden with dehydration in hard-seeded but not soft-seeded species (Werker et al., 1979).

The palisade layer of macrosclereid cells has a number of distinctive features and is the layer most likely associated with seed coat impermeability. This is the thickest layer formed from the cells of the outer ovule integument. Its cells are radial elongated, and tightly compressed as polyhedrons with domed caps (Manning and van Staden, 1987). The cell walls are principally composed of cellulose and have conspicuous secondary thickening. The cells have a lumen which is narrow at the top and widens towards the inner end.

In most reports on the subject of seed coat permeability many authors have concluded that it is water-impermeable substances (perhaps suberin)
deposited in the outer ends of the macrosclereids that is the main barrier to water entry into the seed (Hamly, 1932; Aitken, 1939, Watson, 1948; Spurney 1964; Manning and van Staden, 1987; Riggio-Bevilacqua et al., 1989). The impermeability of this layer has also been chemically associated with deposits of calose (T. subterraneum), phenolics (Pisum spp.) and lipids (annual Trifolium spp.) (Werker et al., 1979; Slattery et al., 1982; Zeng et al., 2005). The development of Impermeability has also been associated with the metabolism of phenolic compounds and activity of peridoxase in the palisade layer, during the final stages of seed development (Marbach and Mayer, 1974; Egley et al., 1983).

Another distinctive feature of the palisade layer is the “light line”, which appears usually in the upper part of the cells in the Papilionoideae subfamily. This line is considered not to have any structural significance but rather to be an optical phenomenon generated by the boundary between suberised and non-suberised regions of the cell (Hamly, 1935; Tran and Cavanagh, 1984). Alternatively, this line is explained as the boundary between regions of transverse and longitudinal microfibrils (Werker et al., 1973). The light line is often disregarded as a feature involved in water impermeability as it is visible in both hard and soft-seeds (Werker, 1980).

The hourglass-shaped osteosclereid cells that lie under the palisade layer are formed from the inner hypodermis of the outer integument. They have cell walls composed of cellulose and show some secondary thickening around the centre. These cells appear to be compressed as the embryo
expands in the developing seed, with the inner and outer regions of the cell expanding while the centre is restricted (Revell, 1997). This results in the separation of the cell walls in the central region to form large air spaces and the hourglass shape (Harris, 1984, 1987). The large intercellular spaces have been considered to play a role in seed dehydration and/or seed aeration (Corner, 1951; Manning and van Staden, 1985). The hourglass cells in *Lupinus. cosentinii* L. were found to be relatively longer in hard-seeded lines but shorter and rounded in soft-seeded lines (Miao *et al.*, 2001). Other than this association, the hourglass layer has not been reported to be linked to seed coat impermeability.

The nutrient layer is composed of non-thickened cells that become crushed by the expanding embryo (Manning and van Staden, 1987; Revell, 1997). They are composed of cellulose, pectin and callose. Large amounts of callose were found in this layer in hard-seeds but not soft-seeds of *T. subterraneum* (Bhalla and Slattery, 1984). However, the soft/hard-seeds were identified by imbibition in this study, which may have induced this difference. When moisture can reach the nutrient layer, it has been observed to greatly expand, perhaps due to the pectin content, but the expansion is reversible with subsequent dehydration (Aitken, 1939).

### 2.4.2 Establishment and breakdown of hard-seed dormancy in family Leguminoseae, sub-family Papilionoideae legumes.

Hard-seed dormancy imposed by an impermeable seed coat, common in the family Leguminoseae, is established in the final stages of ripening and seed
drying (Hyde, 1954). Initially seed dehydration occurs by moisture loss through the entire seed coat. Once the seed dries to below 14% moisture, relative to dry matter, the seed coat enters a state of semi-impermeability (Quinlivan, 1971). From this point, further moisture is lost from the seed through the specialized hilum structure, which effectively functions as a one way valve, opening when exposed to a dry atmosphere (relative to seed moisture) and closing under moist atmosphere (Hyde, 1954).

With further drying, the seed coat becomes irreversibly impermeable below a critical seed moisture content that may vary for different species and perhaps genotypes within a species (Quinlivan, 1971, Standifer et al., 1989). Reported critical levels for irreversible impermeability include 8-9% for *L. cosentini*, 5% for *T. subterraneum*, 5-7% for *T. cherleri* L. and *Medicago scutellata* (L.) Mill. Above these critical moisture contents, seeds can slowly take up moisture, even to the point where germination can be initiated. Below the critical moisture content, the seed is expected only to become drier, with seed moisture content at any particular time reflecting the driest atmosphere to which the seed has been previously exposed (Revell et al., 1999). Once the seed has dehydrated to below the critical moisture content, it will remain dormant even if placed in moist conditions.

Hard-seed dormancy can be considered a form of the secondary dormancy defined by Bewley and Black (1994) due to the role of seed moisture as seeds are unlikely to reach the critical moisture content until after they have dispersed from the maternal plant.
Once impermeability is established, the seed coat of a hard-seed will remain impermeable until the responsible layer/s of the seed coat is breached. This can occur with damage anywhere on the seed coat, through the action of abrasion, piercing, boiling, or acid digestion (Burns, 1959; Brant et al., 1971; Fu et al., 1996). Scratching the seed coat with abrasive surfaces is the most common technique for overcoming hard-seed dormancy in legumes at commercial scales (Townsend and McGuinnies, 1972).

Other techniques that can break hard-seed dormancy are impaction, freezing, and exposure to high temperatures (Hamly, 1932; Brant et al., 1971; Ballard, 1976; Mott, 1979). These treatments result in the breach occurring through specialized areas of the seed coat. Many of the studies on the effect of freezing or high temperatures acknowledge that the change in temperature may be as important for inducing the loss of impermeability as exposure to the extreme condition per se.

In natural settings, hard-seed breakdown is usually associated with particular seasons and functions as both a long term survival strategy and control on the timing of germination (Baskin and Baskin, 2001; Taylor, 2004). Most studies into hard-seed behavior have involved pasture and crop species of commercial interest. The greatest understanding of hard-seed breakdown has been developed through the study of Mediterranean and temperate species that are used for pasture improvement in southern Australian ley farming systems (Baskin and Baskin, 2001; Taylor, 2004).
In the legume species from Mediterranean and temperate climates, hard-seed breakdown usually occurs over the summer and autumn period (Quinlivan, 1966, 1971). It can also be induced under controlled conditions that simulate the temperature fluctuations expected during these seasons (Quinlivan, 1961a). However, the pattern and rate of hard-seed breakdown and seedling emergence observed in the field appears different for different species and even genotypes within a species (Smith et al., 1996; Revell et al., 1998; Norman et al., 2002; Norman et al., 2006).

*T. subterraneum, L. cosentinii* and *M. truncatula* hard-seeds have been shown to become permeable with diurnal temperature fluctuations over the range 15-70°C (Quinlivan, 1966; Quinlivan, 1968a,b). A regime of 15 to 60°C results in the greatest rate of hard-seed break down for these species and has been used to rank genotypes for this character (Norman et al., 2006). However, this temperature regime does not accurately predict the rate of hard-seed breakdown in the field when applied to other small-seeded pasture legume species (Taylor, 1993, 1996; Revell, 1997; Norman et al., 1998). The study of seeds of these species also revealed that for hard-seeds to breakdown in response to these temperatures they require a period of prior exposure to high temperatures. This important observation

Some genotypes of *Medicago polymorpha* appear to require a fluctuation of 10 to 35°C, and *O. compressus* 15 to 48°C for hard-seed breakdown to represent that observed in seed exposed to field conditions (Taylor, 1993, 1996; Revell, 1997). The study of seeds of these species also revealed that for hard-seeds to breakdown in response to these temperatures they require a period of prior exposure to high temperatures. This important observation
has lead to the proposal that different patterns of hard-seed breakdown across species and genotypes could be explained by a two-stage seed conditioning phenomenon (Taylor, 1981).

According to Taylor’s model, a first stage pre-conditions the seed and requires exposure to high temperatures (>50°C). Hard-seed breakdown then occurs as the pre-conditioned seeds are exposed to the second stage of diurnal temperature fluctuation. With this concept, differences in the rate or pattern of hard-seed breakdown across species or genotypes can be generated by differences in the heat sum required for pre-conditioning (first stage) and/or the parameters of the temperature fluctuation (second stage) required to induce seed softening (Taylor, 2004). It is unclear what physiological changes the pre-conditioning phase induces in the seed or seed coat to create this vulnerability to fluctuating temperature. The loss of lipids has been associated with the loss of permeability and this may be driven by exposure to the high temperature (Zeng et al., 2005).

Although the two stage model has contributed greatly to the understanding of hard-seed breakdown, there are other observations that are not easily accommodated within this model. These include;

1. A requirement for the fluctuating temperatures to occur under dark conditions for hard-seed breakdown to occur in yellow serradella seed (Revell et al., 1998; Taylor and Revell, 1999).
2. Loss of the pre-conditioned state under mild, stable conditions (Revell, 1997; Taylor and Revell, 1999).
3. Reversal of induced seed permeability when subsequently stored at low relative humidity (Hagon and Ballard, 1970; Janson and Ison, 1994; Smith *et al.*, 1996).

Further, delayed imbibition has been observed in some species, and this has led to the hypothesis that exposure to moist conditions is a possible third stage in the hard-seed breakdown model (Taylor, 2004, 2005).

The site of water entry into hard-seed that have been naturally induced to soften is generally thought to occur at the lens structure (Ballard, 1973). Presumably this is a point of weakness, and the shrinking and swelling associated with temperature fluctuation might result in the rupture of the longitudinal cleft on the lens. However, the development of cracks on the general seed coat as the seeds age in the field has also been implicated (Zeng *et al.*, 2005).

### 2.5 Genetic and environmental influences on hard-seeded production and breakdown.

The inheritance of hard-seed or soft-seed characters has been studied in some species through hybridisation. In *L. luteus*, *L. angustifolius* and *L. cosentinii*, soft-seed production is conferred through a recessive allele at a single locus (Serrato-Valenti *et al.*, 1989; Cowling *et al.*, 1998). In *L. angustifolius* this gene is identified as *mollis* and has been mapped in the genome with the use of molecular markers (Nelson *et al.*, 2006).
In some legume crops, their long histories of cultivation, combined with the selective pressures of cultivation, have led to speciation away from their wild progenitors. The study of the inheritance of hard-seed production in these genera, for instance *Vicia* and *Lens*, has required hybridization between cultivated and related wild species (Elkins *et al*., 1966; Donelly, 1971; Donelly *et al*., 1972; Ladizinski, 1985). For these species, soft-seed behavior has been associated with two or more genes.

The rate of breakdown of hard-seed is also considered heritable and heritability has been estimated (in the broad sense) at 55% for *T. subterraneum* and 90-96% for *T. michelianum* Savi. (Nair *et al*., 2004). Added to this, there are genotypic differences for the rate of hard-seed breakdown reported for *O. compressus*, *Stylosanthes* spp., *M. polymorpha* and *T. subterraneum* (Donald, 1959; Quinlivan, 1965, 1966; Gladstones, 1967; Bolland 1985; Taylor, 1996; Revell, 1997).

The role of genotype and environment on the initial level of hard-seed and its subsequent breakdown is difficult to separate. Within a species there will be potential interaction with environment due to different maturities, vegetative growth and seed production (Salisbury and Halloran, 1983). Annual legumes are indeterminate plants that can produce seed over a long period of time, and therefore over seasonal changes in weather. In many legumes, a pod may contain multiple seeds with different sizes and longevity of hard-seededness (McComb and Andrews, 1974).
The effect of the production environment on the breakdown of hard-seed is in dispute. Locations considered more favorable for seed development have been associated with higher levels of hard-seededness (Aitken, 1939). Likewise, more stressful conditions during seed development have been argued to reduce the longevity of hard-seededness in T. subterraneum, M. polymorpha, and M. truncatula (Quinlivan, 1965, 1966, 1971; Collins, 1981; Taylor et al., 1984; Taylor and Ewing, 1992; Taylor, 1996).

The opposite reaction to stress during seed development has also been argued. Moisture stress imposed on glasshouse grown T. subterraneum showed little influence on hard-seed level (Taylor and Palmer, 1979). Several Medicago spp., subjected to moisture stress during seed production in both the field and when grown in a glasshouse reduced the rate of softening of hard-seed (Revell, 1997). In addition, O. compressus hard-seed breakdown in the field was found to be more rapid in seeds that were formed during a protracted spring, simulated by irrigation (Revell, 1997; Revell et al., 1999).

An added complication is the effect of excess moisture during, and soon after, the last stages of seed development. If seed of T. subterraneum is exposed to moisture prior to the on-set of irreversible impermeability (i.e. has not dried below the critical moisture content), imbibition may occur that results in irreparable damage to the seed coat (Collins and Quinlivan, 1980; Smith, 1988; Archer, 1990). The timing of this may coincide with the presence of embryo dormancy, with the result that the seeds swell but do not
germinate, although the capacity to develop hard-seededness has been compromised. This may also be the cause of the reduced hard-seed content of *Glycine max* when produced under moist conditions, rather than as a consequence of (over) expansion of the cotyledons during seed development as claimed (Hill *et al.*, 1986a,b).

### 2.7 Annual legumes in farming systems in Mediterranean and temperate climates.

The serradella species, in particular *O. sativus* and *O. compressus* are interesting examples of the development of agricultural plants. Many plant species can provide potential increases in productivity within farming systems. However, this potential will not be realised in commercial practice if the productivity increase does not warrant the cost of implementation. A stalemate can develop if commercial availability of the new species requires specialized harvesting or processing techniques not available with existing infrastructure (Rolston, 2003). This is often overlooked by researchers who are usually dealing with small quantities of seed and where intensive processing is not an issue.

If seed production limitations are overcome, the value and adoption of a species or mixture of species in a farming system may not be purely based on productivity and cost of implementation. Adoption may also be influenced by the complexity of management required to sustain the species. For instance, the requirement for intensive pest management or application of particular rotational management may reduce potential adoption. An
increasing consideration when determining the positive and negative effects of a new species is the sustainability of the environment and farming system.

2.7.1 Development of the cereal-ley farming system of southern Australia.

The development and changes in the farming systems of southern Australian agriculture have been driven by a combination of technical innovation and world commodity prices. Change has also been influenced by the need to adapt the inherited European style agriculture to the environmental and social conditions of a low output form of agriculture, expansive areas and a sparse labour force. The introduction of subterranean clover and the application of super-phosphate fertilizer are two examples of innovations that have enabled Australian agriculture to be economically competitive.

Many species of annual legumes have become naturalized in Australia following European colonization and agricultural activity. Initially their introduction was passive, as contaminants in stock feed and crop seeds (Davidson and Davidson, 1993). In 1889 the value of one of these species (\(T.\) subterraneum) to improve pasture productivity was recognized by A.W. Howard, who started to harvest and spread the seed of a naturalized strain found on his Mt. Barker property. This strain was initially known as “Howard’s clover” and was later registered and commercially traded as the cultivar Mount Barker (Anon, 1990a). The success of \(T.\) subterraneum, particularly when combined with the application of superphosphate fertilizer, dramatically increased sheep stocking rates and inspired further development of this
species (Davidson and Davidson, 1993; Henzel, 2007). This was also assisted by long periods of high demand and prices for wool.

Since the first seed crops of *T. subterraneum* cv. Mount Barker were certified in 1934, 38 cultivars of *T. subterranean* have been developed and released to commercial seed production in Australia. In Western Australia alone, more than 100,000 tonnes of seed of *T. subterranean* clover cultivars has been certified by authorities between 1934 and 2006 (data supplied by AGWEST Plant Laboratories). Production peaked during the 1960’s due to high demand following the clearing of new land for agriculture, the release of better adapted cultivars, and high wool prices (Figure 2.6).

Two particularly important cultivars, accounting for 34% of the total seed certified in Western Australia are Dwalganup, available as “First early strain” from 1929 and registered in 1934, and Geraldton, registered in 1959 (Anon, 1990b, c). The main incentive for the adoption of both Dwalganup and Geraldton was an early maturity that provided adaption to areas with short growing seasons by allowing reliable and sufficient seed production for long term persistence (Millington, 1960).

Several limitations to the use of subterranean clover in southern Australia have been identified. These include;

1. Poor seed production on soils with hard-setting surfaces that hinder burr burial (Quinlivan and Francis, 1971; Collins *et al.*, 1976).
2. Potential for oestrogenic activity in ruminants (Davies and Dudzinski, 1965; Francis, 1968).

3. Susceptibility to fungal disease (Chatel and Francis, 1974).

4. Susceptibility to insect predation (Ridsdill-Smith, 1995).

5. Within season hard-seed breakdown that is vulnerable to untimely or false break rainfall (Chapman and Asseng, 2001).

6. A rapid between season breakdown of hard-seed that is unsuited to persistence under intensive crop rotation (Taylor et al., 1991).

7. Environmentally destructive and time consuming seed harvesting procedure (Boyce, 1997; Nutt and Loi, 1999).


Some of these limitations of subterranean clover have been overcome through an iterative process involving field observation and research into agronomy, breeding and rhizobiology. Alternative annual clover species, with a similar ruderal behaviour were also assessed to overcome some constraints, for example *T. hirtum* L. was developed on the basis of low phyto-oestrogenic activity compared to *T. subterraneum* (Francis et al., 1967).

In a similar developmental process to that of subterranean clover, farmers recognized the value of naturalized forms of *M. truncatula*. This species was very successful on soils with higher pH and generally with finer textures (and hard-setting surfaces), however their wider use was limited due to the interference by acidic soils on their symbiosis (Robson, 1969). This is reflected in the certified seed statistics recorded for annual medic species.
relative to subterranean clover in Western Australia, with its predominance of acidic soils (Figure 2.6).

Figure 2.6. Certified seed production of all annual pasture legumes (solid line) and *T. subterraneum* (broken line) cultivars in Western Australia, 1935 to 2005. Source – AGWEST Plant Laboratories, Certified Seed Statistics. Note – Main alternative species produced in different periods are shown in text.

Other alternative species that experienced some success were *T. hirtum* (Rose clover) and at a later stage *M. polymorpha* (burr medic), however the amount of certified seed of these species did not approach that of subterranean clover (Figure 2.6). *T. hirtum* had some history of being utilized as forage in the U.S.A. and several cultivars were developed from accessions obtained from this country or from Mediterranean germplasm collection missions (Anon., 1990d). The spike in *M. polymorpha* adoption in the late 1980s was the result of a high wool price combined with the development of spineless cultivars and improved rhizobial compatibility. The
improved rhizobial matching also had good saprophytic competence and symbiotic effectiveness in acid soils (Howieson and Ewing, 1986).

2.7.2 Serradella use in southern Australian farming systems.

*O. sativus* (French serradella) was noted 200 years ago (in a German agricultural journal in 1798, referred to by Griesinger and Klinkowski, 1939) as a valuable nitrogen source when grown in rotation with wheat on sandy acid soils. As mentioned above, French serradella was agronomically investigated on several occasions in Australia, however the European cultivars did not suit Australian conditions or farming practices (Gladstones and McKeown, 1977a, 1977b). One of these early importations may have contained seeds of *O. compressus* (yellow serradella) which became naturalized in the south-west of Western Australia.

As with subterranean clover and annual medic, a producer recognized the potential of the naturalized yellow serradella on sandy soils on his farm at Waroona, Western Australia. After the development of cv. Pitman, *O. compressus* was considered the preferred species of serradella for development in Australia due to the hardiness of the species, and an ability to self-regenerate from a persistent seed reserve accumulated through the production of hard-seed (Gladstone and McKweon, 1977b).

The potential of *Ornithopus* species relative to other pasture legume species on acidic, deep, coarse textured soils is reflected in seed yields from nursery rows (Nutt, 1998). This elevated potential is driven by a deeper rooting
system (Ozanne et al., 1965, Hamblin and Hamblin, 1985), acid tolerant symbiosis (Howieson et al., 2008), and efficient potassium and phosphorous utilization (Ozanne et al., 1965; Paynter, 1992) relative to other annual pasture legumes. Kemp and Michalk (2005) refer to more than 60 million hectares of suitable soils in Australian agricultural areas. Even if the potential of serradella is realized on only a small proportion of this area, its potential remains extensive and this has driven research into the development and use of *Ornithopus* species for almost fifty years, despite limited commercial impact. Kemp and Michalk (2005) estimated only 1.2 million hectares had been sown to serradella in Australia by 1998.

The *O. compressus* cultivar Pitman proved valuable for sandy soils in high rainfall areas, but its late maturity did not allow sufficient seed production below 800 mm of annual rainfall (Davidson and Davidson, 1993). Further, the high levels of hard-seed, enclosed in a tough woody pod, proved difficult to deal with in commercial quantities. Some of the methods developed to enhance germination included immersing the pod in boiling water or exposing the pod over summer in black plastic bags (Barrett-Lenard and Gladstones, 1964). Both are inconvenient in large quantities. Sowing of untreated pod resulted in insufficient established plant numbers to ensure successful persistence or rapid realization of the possible benefits (Bolland, 1985).

Initial attempts to improve upon cv. Pitman, targeting earlier maturity, involved EMS (ethylmethane sulphonate) mutation and resulted in the
cultivars Uniserra, released in 1970, and Eneabba, released in 1988 (Gladstones and Devitt, 1971; Anon, 1990e, f). However, it was in situ collections within the natural range of yellow serradella in the Mediterranean basin that was to have the biggest impact on access to adapted germplasm and cultivar release (Table 2.3).

Table 2.3. Cultivars of *Ornithopus* species, year of registration/commercial release, and commercial availability (> 5 t of seed) in Western Australia. Source Register of herbage plant cultivars (1990) and AGWEST Plant Laboratories.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cultivar</th>
<th>Origin</th>
<th>Year of registration or release</th>
<th>Commercial seed available 2000-2007</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. compressus</em></td>
<td>Pitman</td>
<td>Naturalised genotype</td>
<td>1966</td>
<td>No</td>
</tr>
<tr>
<td>Uniserra</td>
<td></td>
<td>Induced mutation in Pitman</td>
<td>1970</td>
<td>No</td>
</tr>
<tr>
<td>Tauro</td>
<td>Italy</td>
<td>1988</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Avila</td>
<td>Spain</td>
<td>1988</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Eneabba</td>
<td>Madeira Island, Portugal</td>
<td>1988</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Madeira</td>
<td></td>
<td>1988</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Elgara</td>
<td>Spain</td>
<td>1988</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Paros</td>
<td>Paros Island, Greece</td>
<td>1989</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Santorini</td>
<td>Santorini Island, Greece</td>
<td>1995</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Charano</td>
<td>Mykonos Island, Greece</td>
<td>1997</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>King</td>
<td>Santorini Island, Greece</td>
<td>1998</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Yelbini</td>
<td>Santorini Island, Greece</td>
<td>2000</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><em>O. pinnatus</em></td>
<td>Jebala</td>
<td>Morrocco</td>
<td>1988</td>
<td>No</td>
</tr>
<tr>
<td><em>O. perpusillus</em></td>
<td>Mamora</td>
<td>Morrocco</td>
<td>1988</td>
<td>No</td>
</tr>
<tr>
<td><em>O. sativus</em></td>
<td>Cadiz</td>
<td>South Africa</td>
<td>1996</td>
<td>Yes</td>
</tr>
<tr>
<td>Erica</td>
<td>Selection within Cadiz</td>
<td>2002</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Margurita</td>
<td>Selection within Cadiz</td>
<td>2002</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><em>O. sativus x O. compressus hybrid</em></td>
<td>Spectra</td>
<td>Manual hybridisation</td>
<td>1998</td>
<td>No</td>
</tr>
</tbody>
</table>

Aware of the need to enhance the range of germplasm available, collectors targeted yellow serradella (as well as *T. subterraneum*, annual *Medicago* or *Lupinus* species) in the Mediterranean region (Francis, 1979; Francis and Gillespie, 1981; Ewing and Howieson, 1987). The increased availability of
germplasm prompted a national field evaluation program that commenced in 1982. This involved 100 ecotypes assembled from a number of serradella species (Bolland and Gladstones, 1987). The result was the release of a number of cultivars based entirely on their superior agronomic performance; principally dry matter production and persistence. These were all hard-seeded and although valued when successfully established, without the availability of technology to produce commercial quantities of seed suitable for further propagation (with sufficient levels of germination), certified seed production statistics show that these cultivars failed to have any lasting impact (Figure 2.7).

Figure 2.7. Certified seed production of *O. sativus* (solid line) and *O. compressus* (dashed line) cultivars in Western Australia. (Source – AGWEST Plant Laboratories, Certified Seed Statistics).

2.7.3 *Constraints to the use of O.compressus.*
The difficulties of dealing with a combination of the high levels of hard-seed, and the seed being enclosed in a tough woody pod, drove the disparity between potential and actual impact of *O. compressus*. The seed in the pod, or pod segments, proved difficult to be separated from trash (plant material and small stones) because of large variation in particle size, shape and weight (density). Added to this, artificial breakdown of hard-seed dormancy involves seed extraction from the pod followed by mechanical scratching of the seed coat. In *O. compressus*, this extraction is difficult to achieve without damage to the seeds, because of the tightly bound, tough woody pod.

Alternative techniques to induce hard-seed breakdown, such as exposure of seed in pod form to fluctuating temperatures (in plastic bags placed in the open) did not suit large scale production systems. Notwithstanding this, seed production is an additional limitation with *O. compressus* and the other “wild” species of *Ornithopus*. The pods of the wild *Ornithopus* species readily detach from the vines upon senescence, and this hinders access to the seed for mechanical harvesting. Also, the fish hook shape of their pods (Figure 2.1) can make many genotypes difficult to process. They do not move well in bulk handling equipment such as augers and elevators, as their clustering into clumps produces a choking effect that can even damage equipment (Nutt and Loi, 1999).

Several machines have been designed to dehull serradella seed (Weeldenburg and Smith, 1969; Nutt, 1994b; Sanders, 1996a). In most, the mechanism of action is based on passing the pod between two parallel
abrasive plates with the bottom plate spinning to provide centrifugal force while the pod is fed through a central hole in the stationary upper plate. While this technique is satisfactory at an experimental and small scale, the slow throughput limits application to large scale production. In the report by Weeldenburg and Smith (1969) the output was only 60 lb (28 kg) of seed per hour. With sowing rates of 3 kg/ha a whole day's processing would be sufficient for less than 100 ha.

An innovative design for dehulling *O. compressus* was developed in 1992. This involved the use of two downward sloping plates which both rotated in the same direction but at different speeds to create a shearing force (Sanders, 1996b). The bottom plate was channeled and the top plate was toothed. This design improved throughput to a point that was feasible for commercial application on some pod types, but not for the cultivars developed to that time.

Experimental dehulling, in threshers used for preparing experimental seed lots, indicated ecotypes (or genotypes) could be selected with improved dehulling potential (Nutt, 1994b). A selection process, involving ease and efficiency of harvesting and dehulling, along with adequate productivity and persistence in the field lead to the release of the *O. compressus* cultivars Santorini (1995), Charano (1997) and Yelbini (2001) (Anon., 1997b,c, 2004c). The release of these cultivars combined with the use of Sanders dehuller (Sanders, 1996b) resulted in a significant increase in yellow serradella seed production and adoption (Figure 2.7).
2.7.4. *Ornithopus sativus*.

As with many agricultural plants, initial attempts to introduce *O. sativus* into Australian farming systems were based on European cultivars and these genotypes were unsuccessful (Gladstones and Barrett-Lennard, 1964). The late maturity of these genotypes and their lack of hard-seed dormancy were perceived as severe shortcomings for their use in southern Australian farming systems. Presumably, similar attempts to utilise *O. sativus* in South Africa were more successful, as a landrace cultivar Emena was developed and it remains commercially available.

During a collection mission by D.J. Gillespie in 1989, seeking annual medics, a very early maturing plant of *O. sativus* was recognized at a location near Darling, Cape Province, South Africa (D.J. Gillespie pers. com.). Seed collected from this plant possessed an early maturity when grown in Perth, Australia (Latitude $37^\circ$S). This germplasm was evaluated for merit and commercially released as cv. Cadiz in 1996 (Anon, 1997a).

Cadiz proved popular and was quickly adopted by Western Australian farmers, as demonstrated by the quantities of *O. sativus* seed certified since its release (Figure 2.7). These statistics can be deceptive, but there are significant trends. *Ornithopus* species in Australia prior to the mid-1990s could be certified in the pod or as naked seed, with no minimum acceptable germination level. After 1996, most of the certified seed of yellow serradella relates to dehulled seed while the majority of French serradella relates to
podded seed. These statistics indicate strong regional uses despite the view that soft-seeded *O. sativus* is limited in the farming systems and climates where it can play a productive agricultural role (Dear *et al.*, 2002).

Alternative approaches to develop the potential of hard-seeded *Ornithopus* have included inter-specific hybridisation and selection within *O. sativus* populations for hard-seed;. The hybridisation approach combined the domesticated characters of *O. sativus* with the persistence characters of *O. compressus*. After screening of the resulting hybrids for the desired combination of domestic and wild traits, the cultivar Spectra was developed (Williams *et al.*, 1987). Unfortunately, the maturity of cv. Spectra was too late for low input, mixed farming systems in low to medium rainfall regions.

Selection within the adapted *O. sativus* cultivar Cadiz resulted in the cultivars Erica and Margurita (Anon, 2002a, b). While they are hard-seeded, they are also easier to dehull and have a much higher seed to pod ratio than *O. compressus* cultivars. They have been well received commercially, however, their release has been too recent to evaluate success in the long term, particularly given the history of *Ornithopus* in commercial practice.
2.8 Conclusions.

*O. sativus* is a legume species whose cultivation can provide a number of clear benefits in southern Australian farming systems (or other regions of the world with Mediterranean or temperate climates). It is better adapted to low fertility and acid soil conditions than many other crop or pasture legumes, including the highly successful *T. subterraneum*. It has a reliable, acid tolerant symbiotic association with root nodule bacteria, it has its own source of nitrogen and through this ability can increase soil fertility. Also due to this association, the foliage produced by *O. sativus* has a high nutritional value to grazing animals and its use in this way is not compromised by anti-nutritional properties (Frame *et al.*, 1998).

Most importantly, *O. sativus* has a high capacity for seed production and the seed (pod) is easily collected (harvested), and processed, using machinery that is applied to many agricultural crops (Nutt and Loi, 1999). This allows *O. sativus* to be adopted as a fodder or soil manure at a relatively low cost and as a technology it is readily available. It is also relatively unique among annual Mediterranean-type annual pasture legumes by having genotypes that produce either soft-seed (e.g. Cadiz and Emena) or hard-seeded (e.g. Margurita and Erica).

Agricultural demand for annual pasture legumes in southern Australian farming systems has been historically based on their potential for improving stock carrying capacity, principally sheep for wool production. Periodically, wool prices have seen times of boom and crash, however a severe down
turn in wool prices occurred in the late 1980’s, from which this industry is yet to recover. Relative commodity prices of agricultural produce have changed to favour grain production. At the same time, the efficiency of labour in cropping activities has dramatically increased through the use of larger scale machinery. The result of this combination has been a dramatic intensification of cropping in southern Australian agricultural farming systems. Added to this, the widespread use of nitrogenous fertiliser and the development of feasible alternative types of rotational crops have reduced reliance on pasture legumes.

The successful wheat:lupin rotation that has emerged since the first cultivars of low alkaloid, non-shattering *L. angustifolius* (narrow-leaf lupin) were bred in the early 1970’s is perhaps the best example of system development in southern Australia over this time (Perry *et al.*, 1998; Pannell, 1998). However, intensive crop rotation is not profitable in all situations, such as on infertile soils that require high inputs relative to potential yield. Continuous cropping systems also develop their own set of limitations, including the promotion of herbicide resistant weed populations and due to their high input costs, increased exposure to financial loss in poor seasons.

Even though crop legumes may provide some of the rotational benefits of pasture legumes in mixed farming systems, powerful drivers still remain for the inclusion (or adoption) of a pasture-ley based on legumes. It is now recognized by many agronomists that the implementation of a pasture phase to interrupt continuous sequences of cropping is a valuable tool in the
Chapter 2

integrated control of herbicide resistant weeds (Doole, 2008). This has been highlighted by the rapid and large scale adoption of the soft-seeded *O. sativus* cultivar Cadiz in Western Australia. It can be applied with relatively low costs, and immediate benefits. Further, it can be applied tactically in response to seasonal conditions. In seasons where the opening rains to a growing season are too late to produce cost effective grain crops, Cadiz can be sown to provide livestock fodder, nitrogen input and diverse weed control options. Because of its seed harvesting and storage potential, this can be done without consideration for the long term persistence of the pasture legume.

The use of Cadiz contrasts with the traditional application of annual legume based pastures (such as *T. subterraneum*). In these systems, there is a high upfront investment in seed, but this investment provides long term benefits if the persistence of the legume is managed. There are many pastures in the wheatbelt of Western Australia that continue to contain *T. subterraneum* that was introduced in the 1960’s.

While the cultivar Cadiz has been widely adopted in Western Australian farming systems, the potential of *O. sativus* could be further expanded through investigating and exploiting the diversity of key agronomic characters within the species. These would include hard-seed production, pattern of hard-seed breakdown and maturity. The requirement for early maturity in pasture legumes is demonstrated by the rapid and large production of the early maturing *T. subterraneum* cultivar Geraldton upon its release to
agriculture in 1959 which became the dominant subterranean clover cultivar with certified seed production in the 1960s (Figure 2.6).

2.8.1 Aims of thesis

The primary aim of this thesis is to understand the inheritance of hard or soft-seed in *O. sativus* (Chapters 3 and 5). In other legume species when this dimorphic character is present, it is subject to relatively simple inheritance, and it is possible that this is also the case in *O. sativus*. If so, the relative frequencies of alleles associated with soft or hard-seededness in a mixed population are likely to be subject to selection under particular management. Therefore, this study will quantify factors that may influence a selective difference between the two characters in an *O. sativus* population. This includes the incidence of cross pollination (Chapter 4) and *in situ* changes in germination over time in soft and hard-seeds exposed on, or slightly buried, in the soil (Chapter 6).

Secondary aims are to examine the response to selection in *O. sativus* for early maturity, the interaction between maturity and hard-seed production, and the underlying physical differences between soft and hard-seeds. A greater understanding of seed germination, timing of flowering and the breeding system of *O. sativus* will enable more effective breeding and use of this species in Mediterranean and temperate climate agricultural systems.
Chapter 3. Selection for hard-seed dormancy and early flowering in *Ornithopus sativus* Brot.

### 3.1 Introduction

Control of seed germination and timing of flowering are important survival traits for flowering plants in natural ecosystems (Aitken, 1974; Grime, 1977; Baskin and Baskin, 2001). An understanding and subsequent exploitation of these characters is also a requirement for optimal production from most cultivated plants. Consequently, the timing of flowering and seed germination behavior are often targets for manipulation in plant breeding programs, studies of climate adaptation and germplasm characterization (Fu *et al.*, 1994a; Fu *et al.*, 1994b; Snowball, 1996; Norman *et al.*, 2002; Nair *et al.*, 2004). In the Papilionoideae family, initiation and longevity of flowering is controlled by permutations of photoperiod, temperature and vernalisation (Aitken, 1974).

In most species of the Papilionoideae family, timing and extent of seed germination is primarily controlled by physical seed dormancy induced by the development of an impermeable testa or seed coat (Quinlivan, 1971; Baskin and Baskin, 2001; Taylor, 2005). This type of dormancy is often referred to as “hard-seed” or being “hard-seeded”. The dormant hard-seed state is released following a breach through the impermeable
layer/s of the seed coat, either through physical damage or in response to other environmental triggers.

Hard-seed dormancy, as a survival strategy, has a number of elements. It protects seed from untimely germination and, in an evolutionary process, synchronises germination with conditions most favorable for an eventual reproductive outcome. In short lived species, or situations of limited reproductive opportunity, it is insurance against failed or poor performance in one or more consecutive seasons or reproductive cycles (Bewley and Black, 1985). A small, hard-seed is also more likely to pass through a grazing animal’s gut and disperse with animal movement than a seed that will readily imbibe moisture (Russi et al., 1992b; Edwards et al., 1998).

Conversely, the presence of dormant seed is normally undesirable in the cultivation of short-lived crops for the purpose of grain or seed production. Usually fast and reliable germination is required in these types of cultivated plants to optimize yield and any residual dormant seed can potentially become a weed or contaminant in subsequent crops. There is also considerable selective pressure towards non-dormant seed when seed is harvested upon ripening, stored under mild conditions and then sown into moist soil for further seed production. Under this regime, seed may not be exposed to dormancy releasing conditions, and only seeds that readily germinate will reproduce, be harvested in that season.
and contribute to newly sown populations (Harlen et al., 1973; Copeland and McDonald, 1995; Ladizinski, 1998).

The genus *Ornithopus* L., commonly referred to as serradella, is consistent with this pattern of hard-seeded wild forms and soft-seeded cultivated forms. There are six recognised species of *Ornithopus*. *O. compressus* L., *O. pinnatus* Druce, *O. perpusillus* L., and *O. uncinatus* Maire & Sam., are annual legumes that have natural distributions across Europe and north Africa. *O. microanthus* Arechav occurs only in south America. These are wild species with a ruderal ecology that normally produce high levels of hard-seed. *O. sativus* Brot. is divided by some taxonomies into the sub-species *sativus*, *isthmocarpus*, and *macrorrhynchus*. In others spp. *isthmocarpus* is classified as a separate species of the same name. With the exception of ssp. *sativus* these are hard-seeded. *O. sativus* spp. *sativus* is considered the greatest value to agriculture and has the greatest history of cultivation (Klinkowski and Griesinger, 1939; Gladstone and McKeown, 1977a, b; Frame et al., 1998).

*O. sativus* spp. *sativus* is used as legume forage and as a soil ameliorant or green manure in Europe, Southern Africa, United States of America, Australia and New Zealand (Frame et al., 1998). A critical aspect in the adoption of this species for agriculture is its ease of seed collection and handling. This requires a combination of pod retention on the stems after senescence and ready segmentation of pods into single seeded units that
are amenable to cleaning (to remove non-crop material and seeds) and bulk handling (Nutt and Loi, 1999). Almost all commercial cultivars are soft-seeded and fresh seed has high levels of germination while still encased in the woody pod.

The lack of hard-seeded cultivated forms of *O. sativus*, however, limits the farming systems and perhaps climatic zones where this species can be successfully exploited (Bolland and Gladstones, 1987; Dear et al. 2002). Cereal-ley farming systems of Southern Australia, for example, are reliant on the ability of pasture legumes to self-regenerate from a persistent seed reserve after one or more seasons of cereal cropping (Puckridge and French, 1983). Therefore, both the production of hard-seed and its subsequent breakdown are considered essential selection criteria for the development of pasture legumes for these systems. This includes *Ornithopus* species which is one of the few useful legume options for sandy, acid soils which abound in southern Australia (Williams and De Lautour, 1975; Freebairn, 1993).

The value of *O. sativus* (referring to the ssp. *sativus*) in Southern Australian farming systems would be greatly enhanced by the availability of cultivars with early maturity and hard-seed production. These two key agronomic traits appear to be rare in conserved germplasm collections of this species (Gladstones and McKeown, 1977a; Bolland, 1984; Fu et al., 1994a, b).
This study aims to test the hypotheses that, although rare, genes for heritable hard-seed production and early maturity are present within cultivated *O. sativus* populations and individuals with these traits may be isolated by intense selection.

### 3.2 Materials and methods

#### 3.2.1 Mass selection for the production of hard seed.

Two genotypes of *O. sativus* were examined for hard-seed production. Ecotype 97ZAF5sat was originally collected from Darling, Western Cape Province, South Africa (latitude 33° 22’ S, longitude 18° 21’ E, altitude 200m). It was first grown for characterization by the Australian *Trifolium* Genetic Resource Centre (ATGRC), Perth, Western Australia in 1998 and the seed used in this experiment was sourced from that material. It was chosen as a base population for its early maturity, pod retention to the stems and a capacity for high seed yield compared to available germplasm. The second genotype, cv. Emena is a landrace cultivar popular in Western Cape Province, Republic of South Africa and the seed used in this selection was sourced from commercial South African supplies.

Samples of 500 g of pod (containing approximately 100,000 seeds) of the two *O. sativus* accessions were dried at 40°C for 3 days then placed on moist paper at 15°C for 28 days to germinate. Any remaining un-germinated hard-seeds were recovered and 40 from each source were de-hulled, scarified and grown as single, spaced plants at Medina Research Station in 2001 (latitude 32° 13’S, longitude 115° 48’E). They formed the basis of first
selection (referred to as \( S_1 \) meaning selection cycle 1). Offspring, the second generation from initial selection (\( S_2 \)), of a subset of plants from each population was grown in 2002. These included plants which produced more than 90% hard-seed and 2 plants which produced no hard-seed from the 97ZAF5sat population and 10 plants ranging from 0 to 85% hard-seed from the cv. Emena population. For each of these plants, 30 offspring were grown as fully randomized spaced plants. Not all 30 offspring of each selection were harvested, as strong winds at senescence detached some plants from the ground so they could not be confidently identified.

The single spaced plants, in both 2001 and 2002, were cultured by placing de-hulled, scarified seed into small peat pots filled with commercial potting mix at the beginning of May. The seedlings were grown in a covered plastic house for 3 weeks, hardened for 2 weeks uncovered, then transplanted into cultivated soil, covered with plastic film mulch, at a minimum of 1.5m between plants. A basal fertilizer of 300 kg/ha of NPK was applied prior to the laying of the plastic film. The area was irrigated (at evaporation plus 10%) every 2\(^{nd}\) day between transplanting and the middle of November. In mid-December, after all plants had senesced, they were individually harvested by hand stripping the pods from the vines. No shed pods (on the ground) were collected to ensure identity. The pod sample was dried for 3 days at 40\(^\circ\)C, gently threshed and then cleaned by aspiration to remove leaf and stem material.
In both generations (S₁ in 2001 and S₂ in 2002) maturity was measured as days from germination to the first open flower, and production of hard-seed was determined by a germination test. The germination tests on the 2002 generation were conducted over a two month period with the samples stored under laboratory conditions until required.

In this experiment, all plants were grown in an open field where cross-pollination could occur but was unexpected from reports in the literature about the breeding system of *O. sativus* (Frame *et al.* 1998; Fu *et al.*, 1994b)

### 3.2.2 Mass selection from the cultivar Cadiz for early maturity.

Several commercial seed lots of cv. Cadiz were used as the source population for the selection of early maturity. This cultivar was chosen as a base population for selection because it is a successful cultivar in the south-west of Western Australia. The first 13 of approximately 6000 plants to flower after a mid May, 2002, sowing were isolated by transplanting into pots in a sealed glasshouse when the first open flower appeared, and then grown to maturity for seed production. Isolation was undertaken to ensure self pollination with other plants and each other.

Thirty single-spaced plants from each of these individuals were grown in blocks at Medina Research Station in 2003. A control plot of 59 Cadiz plants from the original seed lot was grown approximately 200m away to avoid contamination due to possible cross pollination through bee foraging. The
method of plant culture was similar to that described in 3.2.1. The number of days from germination to the production of the first open flower was recorded for each individual plant. After plant senescence, the pods were hand stripped from the vines, dried and the seed germination assessed. A bulk, single row of each of the five families that showed the greatest and most stable change to early maturity in 2003 were grown in 2004, at the same location, with maturity measured as days from germination to the first open flowers.

3.2.3 Determination of flowering response in O. sativus.

Three single plants were used as seed sources for monthly sequential plantings in a glasshouse to examine changes in the timing of flowering. Genotypes A1.1 (from 97ZAF5sat) and B1.2 (from cultivar Emena) are two hard-seed producing selections identified in 3.2.1. The third single plant, D1, was the earliest flowering selection from cultivar Cadiz identified in 3.2.2. Cultivar Cadiz was included as the fourth treatment.

At the monthly sowings between March, 2004 and February, 2005, several seeds (dehulled and scarified for Genotypes A1.1 and B1.2) were planted into 50 mm square pots filled with commercial potting mix. Five replicate pots were prepared for each treatment, with each pot thinned to a single plant at the single true leaf stage. The plants were grown in a glasshouse with the temperature controlled at a 15/20°C night/day regime. All pots were fertilized with an all purpose liquid fertilizer, and spatially re-randomized on a fortnightly basis.
Days to first open flower from germination, and average position of the first flowering node on the three primary stems was recorded. Accumulated daylight-hours to form the first open flower were calculated from the photoperiod profile at 32° South latitude, based on average monthly hours of daylight (Figure 3.1).

Figure 3.1. Seasonal photoperiod at 32° latitude at South Perth, Western Australia from March, 2004, until July, 2005 (Bureau of Metoralogy).

3.2.4 Germination tests.

Germination tests were conducted on seed contained in segmented pods. These were placed in rolls of moist filter paper stored at 15°C. Two replicates of approximately 100 segments were assessed for each
individual plant, and each replicate was undertaken at the same time. Germinated seeds (normal seedlings), viable imbibed seeds, abnormal seedlings, dead seeds and residual hard-seeds were recorded over a 21 day period. Seed was considered dead if it imbibed and began to decay. Any germinated seeds, dead seeds and abnormal seedlings were removed after 7 and 14 days to minimize movement of un-germinated seeds by the growing seedlings and microbial decay.

In all cases percentage hard-seed level was calculated as;

\[
\text{Proportion hard seed (\%)} = \frac{\text{Hard seed residue} \times 100}{\text{Hard seed residue} + \text{germinated seed} + \text{viable imbibed seed}}
\]

Dead seed or abnormal seedlings were infrequent and are not reported.

**3.2.5 Statistical analysis.**

To normalize the data for graphical description, and analysis of variance, proportion hard-seed was transformed in the following form;

Hard-seed score = arcsin\((\text{Proportion hard seed (\%)} - 50)/50)\).

The second generation of selections from cv. Emena (in 3.2.1), Cluster B1, were compared by analysis of variance using maternal parent and germination test sample (two per plant) as factors and days to first open flower as a covariant. Differences in time to produce an open flower between offspring of the early flowering selections and cv. Cadiz (in
3.2.2), were compared using a two-sided Student’s T-test. Time to form an open flower, first flowering node and accumulated daylight to flowering (in 3.2.3), were compared using analysis of variance with genotype, sowing time and replicate as factors. All analysis of variance, correlations and regressions were conducted using Genstat – Ninth Edition Version 9.2.0.152 (VSN International Limited).

### 3.3 Results

#### 3.3.1 Selection for hard-seededness.

Hard-seed was produced by some of the $S_1$ plants (grown from mass selected hard-seeds) from both the 97ZAF5sat and cultivar Emena populations. However, not all plants selected by this method produced hard-seed and the distribution of individual plants for the percentage of hard-seed differed between the two populations (Figure 3.2). 97ZAF5sat $S_1$ selections produced either a high proportion of hard-seed or no hard-seed. The $S_1$ selections from cultivar Emena produced a broad distribution in the proportion of hard-seed produced and a few individuals producing no hard-seed. Plants grown from mass selected hard-seeds from accession 97ZAF5sat were consistently earlier flowering than those from cultivar Emena, averages being $115 \pm 1$ and $137 \pm 1$ days after sowing respectively (Figure 3.2).

The $S_1$ plants could be clustered into four groups according to source population, maturity, and hard-seed score (Figure 3.3). These are;
Cluster A1 from accession 97ZAF5sat, which is formed of individuals producing high levels of hard-seed (average 95%) with no apparent relationship with maturity ($R^2 = 0.06$).

Cluster A2 from accession 97ZAF5sat, with individuals producing no hard-seed.

Cluster B1 from cultivar Emena, which produced low to high proportions of hard-seed (average 49%) and a significant positive relationship with maturity (corr. = 0.83, $R^2 = 0.68$, $P \leq 0.05$).

Cluster B2 from cultivar Emena, which produced no hard-seed.

The $S_2$ generation (offspring from a subset of plants) from Clusters A1, A2 and B1, and a single plant from Cluster B2, produced a similar distribution of hard-seed level, flowering time and source population clusters (Figure 3.4). The mean proportion of hard-seed produced by the individual $S_2$ plants was correlated to that of their individual $S_1$ maternal parents, but differences to the parent expression were observed in some offspring (Figure 3.5).

In the 97ZAF5sat selections, when variance in the proportion of hard-seed produced by a $S_2$ plant from the maternal parent occurred, it was a discrete segregation. Some of the offspring from two of the five 97ZAF5sat sourced selections from Cluster A1 produced soft-seed even though their maternal plants produced high proportions of hard-seed (Table 3.1). Conversely, the two individuals from 97ZAF5sat that
produced soft-seed, from Cluster A2, segregated in the S₂ generation, with some offspring producing high proportions of hard-seed.

Figure 3.2. Distribution of *O. sativus* S₁ plants (40 individuals for each source population) grown from hard-seeds selected from accession 97ZAF5sat (open bars) and Emena, (solid bars), according to - A. Proportion of hard-seed produced, and B. Maturity (measured as days to first open flowers from a late autumn sowing).
Figure 3.3. Clustering of individual S\textsubscript{1} plants grown in 2001 from hard-seed selected from accession 97ZAF5sat (○ groups A1 and A2) or cv. Emena (● groups B1 and B2), according to maturity and hard-seed score (40 plants for each source population).

Figure 3.4. Clustering of the first generation of offspring (S\textsubscript{2}) in 2002 from plants grown from hard-seed selected from accession 97ZAF5sat, 200 plants (○) or cv. Emena, 245 plants (●), according to maturity and hard-seed score.
Table 3.1. Hard-seed level over two generations of plants grown initially from hard-seeds selected from *O. sativus* accession 97ZAF5sat (denoted as A) and cultivar Emena (denoted as B).

<table>
<thead>
<tr>
<th>Cluster and S₁ plant</th>
<th>Parent (S₁) hard-seed (%)</th>
<th>Offspring (S₂) classified as</th>
<th>Mean hard-seed (%)</th>
<th>Variance</th>
<th>Number of S₂ plants assessed</th>
<th>Mean hard-seed (%)</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hard-seeded (&gt;1% hard seeds)</td>
<td>Number of S₂ plants assessed</td>
<td></td>
<td>Soft-seeded (≤ 1% hard seeds)</td>
<td>Number of S₂ plants assessed</td>
<td></td>
</tr>
<tr>
<td>A1.1</td>
<td>97</td>
<td>29</td>
<td>98.6</td>
<td>1.9</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A1.2</td>
<td>96</td>
<td>24</td>
<td>98.9</td>
<td>6.7</td>
<td>1</td>
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</tr>
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<td>88</td>
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<td>75.2</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>B1.2</td>
<td>81</td>
<td>28</td>
<td>83.8</td>
<td>330.0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1.3</td>
<td>72</td>
<td>21</td>
<td>83.9</td>
<td>123.6</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1.4</td>
<td>70</td>
<td>18</td>
<td>41.9</td>
<td>453.9</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1.5</td>
<td>26</td>
<td>15</td>
<td>9.6</td>
<td>239.3</td>
<td>6</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>B1.6</td>
<td>22</td>
<td>28</td>
<td>10.0</td>
<td>102.5</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>B1.7</td>
<td>14</td>
<td>30</td>
<td>16.6</td>
<td>246.4</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1.8</td>
<td>6</td>
<td>29</td>
<td>14.6</td>
<td>157.4</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1.9</td>
<td>4</td>
<td>24</td>
<td>8.3</td>
<td>138.4</td>
<td>6</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>B2.1</td>
<td>0.5</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>26</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Segregation for hard-seed production was less distinct among the subset of plants selected from the cultivar Emena. Offspring from plants in Cluster B1 (9 plants) reproduced the wide distribution for the proportion of hard-seed observed in the S_1 generation. In general, within this Cluster B1, parent and offspring hard-seed score was highly correlated (0.85) and maternal plant accounted for 63% of the total variation.

The significant (positive) interaction with maturity observed in Cluster B1 was repeated in the S_2 plants selected from this group (corr. 0.75, R^2 = 0.56, P ≤ 0.05). Using flowering time as a covariant in the analysis of variance to account for this interaction, the parent-offspring correlation for the proportion of hard-seed produced by S_2 plants was increased to 89% (Table 3.2). Even so, some offspring of the Cluster B1 maternal parents had considerably higher and lower hard-seed scores compared to the mean of their maternal siblings. All offspring grown from the single maternal plant from Cluster B2 produced no or very few hard-seeds (Table 3.1).

Considering both source populations together, time taken to flower in offspring was highly correlated to that expressed in the maternal parent (corr.= 0.95, R^2 = 0.91). Separately, however, the parent-offspring correlation was less distinct with as much variation in offspring maturity within individual parents as between parents (Figure 3.6).
Figure 3.5. Relationship between parent (S₁) hard-seed score and offspring (S₂) hard-seed score in *O. sativus* selections from 97ZAF5sat (○) and Emena (●) populations.

Figure 3.6. Relationship between parent (S₁) and offspring (S₂) maturity measured as days to first open flower in *O. sativus* selections from 97ZAF5sat (○) and Emena (●) populations.
Table 3.2. Analysis of variance for offspring hard-seed score (arcsin transformation) from the B1 cluster of hard-seed producing parents selected from the cv. Emena population using days to the first open flower as a covariate. Two germination tests (each on 100 seeds) were conducted on each offspring plant.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>cov.ef.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 plant</td>
<td>8</td>
<td>88.0948</td>
<td>11.0118</td>
<td>83.39</td>
<td>0.79</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Germ. test</td>
<td>1</td>
<td>0.0065</td>
<td>0.0065</td>
<td>0.05</td>
<td>1.00</td>
<td>0.824</td>
</tr>
<tr>
<td>Covariate</td>
<td>1</td>
<td>2.7862</td>
<td>2.7862</td>
<td>21.10</td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Residual</td>
<td>420</td>
<td>55.4650</td>
<td>0.1321</td>
<td></td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>430</td>
<td>310.9879</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.2 Selection for early flowering from cultivar Cadiz.

Offspring of the isolated early flowering selections produced open flowers on average 7 days earlier than the parent Cadiz population with the same time of sowing ($p \leq 0.01$). One full-sib family, selection D1, began flowering 12 days earlier than Cadiz (Table 3.3). Within each early flowering selection there was little variation in the timing of flowering particularly considering the variation in the source cv. Cadiz population. The difference from Cadiz and the early selections was confirmed in a third generation grown as a single bulk row; however this cannot be compared statistically. All of the early flowering selections produced soft-seed at senescence.
Table 3.3. Response in offspring maturity to selection for early flowering parents from *O. sativus* cv. Cadiz, with a late autumn sowing. D1 is the earliest of 12 selections from cv. Cadiz.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>S2 offspring days to first open flower</th>
<th></th>
<th></th>
<th>S3 offspring days to first open flower</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. tested</td>
<td>Mean</td>
<td>Variance</td>
<td></td>
</tr>
<tr>
<td>Earliest selection (D1)</td>
<td>30</td>
<td>92.0</td>
<td>12.4</td>
<td>95</td>
</tr>
<tr>
<td>All selections (12)</td>
<td>59</td>
<td>103.7</td>
<td>89.5</td>
<td>110</td>
</tr>
<tr>
<td>Cadiz</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.3.3 Effect of photoperiod on the time taken to flower.

Sequential monthly planting showed a significant interaction ($P \leq 0.05$) between date of sowing and genotype in three measures of maturity; days to form an open flower (Figure 3.7); first flowering node on the main stems (Figure 3.8); and accumulated hours of daylight to the first open flower (Figure 3.9). The longest period to form flowers and the greatest difference between genotypes occurred with autumn and late summer sowings. Flowering commenced earlier and converged to a point where there was little difference between genotypes with spring and early summer sowing. This response was also reflected in the position of the first open flower on the main stems.

Time to form an open flower showed reduced seasonality when expressed as accumulated daylight (based on the seasonal changes in photoperiod at 32° latitude, Figure 3.1). Even so, there was up to 200
hours (eight to nine 12 hour days) difference between sowing times in the early flowering selection (D1), and to up to 600 hours (fifty 12 hour days) difference in the latest flowering genotype (B1.2) (Figure 3.9). Plants of selection A1.1 and the cultivar Cadiz showed a similar pattern that fell between D1 and B1.2

Figure 3.7. Days from germination to the first open flower of *O. sativus* with sequential monthly sowing and grown in a glasshouse with 20/15°C controlled temperature. A. hard-seeded selections A1.1 (dashed line) and B1.2 (solid line). B. Cadiz (dash-dot line) and an early flowering selection, D1 (dotted line). Standard error shown by bars.
Figure 3.8. First flowering node (mean of the three primary stems) of *O. sativus* with sequential monthly sowing and grown in a glasshouse with 20/15°C controlled temperature, at 32°S latitude. A. hard-seeded selections A1.1 (dashed line) and B1.2 (solid line). B. Cadiz (dash-dot line) and an early flowering selection, D1 (dotted line). Standard error shown by bars.
Figure 3.9. Estimated accumulated hours of daylight (using photoperiods at 32° S, Figure 3.1) by the time of the first open flower from germination of *O. sativus* with sequential monthly sowing, grown in a glasshouse with 20/15°C day/night controlled temperature. A. Hard-seeded selections A1.1 (dashed line) and B1.2 (solid line). B. Cadiz (dash-dot line) and an early flowering selection, D1 (dotted line). Standard error shown by bars.
Chapter 3

Plotting the days required to produce a first open flower against the date that the first flower was observed emphasises the difference in seasonal response. This is shown in Figure 3.10 for plants of D1 and B1.2, the two genotypes which produced the greatest change in maturity with different times of sowing.

Figure 3.10. Days to form an open flower from germination according to when the flower is observed during the year in O. sativus selections B1.2 (●) and D1 (○) when grown in a glasshouse with 20/15°C day/night controlled temperature and at 32°S latitude.

3.4 Discussion.

Intense selection in O. sativus for early flowering and hard-seed production successfully isolated individual plants with heritable traits for these characters. Plants grown from hard-seeds in an earlier maturing accession 97ZAF5sat yielded discrete, extreme expressions in hard-seed production, while selection within the cultivar Emena produced a broad range of low to moderately high levels of hard-seed (Figure 3.2). The
discrete inheritance of hard-seed production in the 97ZAF5sat selections appears similar to that described in *L. angustifolius* and *L. hispanicus* (syn. *luteus*) (Forbes and Wells, 1968; Serrato Valenti et al., 1989; Cowling et al., 1998; Nelson et al., 2006). However, the more continuous expression in the proportion of hard-seed produced by the cv. Emena population is similar to that described in *Trifolium incarnatum* L. (Bennett, 1959).

The hard-seed trait appears to be under relatively simple inheritance when isolated from the *O. sativus* accession 97ZAF5sat, and more complicated when sourced from the *O. sativus* cultivar Emena. In both examples, the relative dominance of soft or hard-seededness could not be determined by selection and progeny testing alone. Discrete segregation away from the parent expression occurred in both directions with the 97ZAF5sat selection (hard-seeded parents produced some offspring that were soft-seeded and *visa versa*), and the broad range in the proportion of hard-seed produced by the Emena selections suggested either quantitative trait inheritance or environmental interaction.

The apparent segregation for hard-seed production evident in both populations could be explained by contamination due to cross pollination. However, the genus *Ornithopus* (including *O. sativus*) is considered autogamous, with anthesis occurring prior to the opening of the flower in a process classified as preanthesis cleistogamy (Lord, 1981; Wojciechowska, 1971). Cross pollination, even at low levels, would
necessitate self-pollinated line breeding for several generations to produce stable parents for hybridisation and the study of inheritance. The incidence and quantification of cross pollination in *O. sativus* will be further explored in Chapter 4.

Plants that provide a high probability of being homozygous for the hard-seed trait were identified in these experiments. All thirty offspring grown of the individual plant A1.1 (from accession 97ZAF5sat) produced high levels of hard-seed with little variability. Likewise, all 28 offspring grown of the plant B1.2 (from cultivar Em ena) produced hard-seed, although there was a greater variance in the level of hard-seed among these individual offspring. In order to avoid the time required to line breed these hard-seeded genotypes to homozygosis, these plants will be utilized as parents to further elicit inheritance of the hard-seed/soft-seed trait through hybridisation with soft-seeded genotypes (Chapter 5).

Selection of a very early flowering individual from the population within the cultivar Cadiz produced a genotype with significantly reduced time to form open flowers. Offspring from this plant consistently flowered before the cultivar Cadiz when sown throughout the year. However, the difference contracted with spring to early summer sowing, which coincides with growth under conditions of increasing and long photoperiod. The effect of selection for early flowering in *O. sativus*, from cv. Cadiz, therefore resulted in a reduction of the modifying influence of photoperiod on flowering time, rather than early maturity *per se*. These
results suggest that flower initiation in *O. sativus*, at least within the maturity range assessed in this study, occurs through a quantitative long-day response.

The relative stability of the timing of flowering in the early flowering selection D1, and the convergence of all lines studied under long photoperiod, suggests further reduction in maturity is unlikely in this species. Given the low level of incidence of hard-seed and early flowering traits in *O. sativus*, combining these traits may be best approached by separate selection processes and subsequent hybridisation. In this way the intensity of initial selection is reduced and the opportunity for further selection on other agronomic characters is maximized.

The hypothesis that heritable hard-seed production and early maturity are present within populations of *O. sativus* is supported by evidence presented in this study. The presence of variation in these traits also suggests the occurrence of cross pollination in *O. sativus* and perhaps other species of *Ornithopus*. 
Chapter 4. The breeding system of *Ornithopus sativus* Brot.

### 4.1 Introduction

Plants are usually cultivated for a particular trait or combination of traits, and agronomic management aims at reliable expression of these traits. Variability in expression of some traits can be induced through interaction with environmental conditions. Where it occurs, sexual reproduction and the combination and recombination of traits generated can also result in considerable variability. Therefore, an understanding of the breeding system or combination of breeding systems operating within a species is necessary when developing plant improvement programs. Without this understanding it is difficult to distinguish between the genetic and environmental influences that are generating diversity in any particular phenotype.

Many species in the Papilionioidae family (legumes) have commercial value as animal forage, human food and sources of pharmaceutical compounds. As such, they are common subjects of plant improvement programs which exploit genetic variation and particular genotype by environment interactions. Not only are agronomic traits such as maturity or pest resistance examined, but also product quality and safety, such as specific biochemical profiles or the absence of anti-nutritional compounds (Francis and Millington, 1965; Francis, 1968; Walton and Francis, 1975; Barbetti *et al.*, 1991; Berlandier, 1996). Understanding the breeding system of these species is necessary to
determine appropriate conditions to either minimize, or maximize, cross pollination. The production of genetically modified crops, such as canola (*Brassica napus* L.), is a modern situation where this knowledge is essential (Reiger *et al.*, 2002).

All species of the Papilioniodeae family have hermaphroditic flowers and they multiply primarily through sexual reproduction. Some perennial species may reproduce through vegetative stolon or rhizome succession, although this is usually combined with a potential for sexual reproduction. Where sexual reproduction does occur, the breeding systems in this family include the full spectrum from autogamy, describing self-fertilization within the same flower, to xenogamy, describing enforced cross-fertilization with an unrelated individual (Richards, 1986).

Among legumes the most extreme examples of autogamy are the subterranean flowers of amphicarpic species that never open, such as *Vicia sativa* L. subsp. *amphicarpa* (Dorthes) Asch and *Trifolium polymorphum* Poir. (Christiansen *et al.*, 1996; Real *et al.*, 2007). At the other extreme, xenogamy is usually the result of self incompatibility resulting from interference with ovule fertilization with pollen from the same or closely related individuals (Richards, 1986). However, mixed breeding systems can also occur when the genotypes are self-compatible, but there is a requirement for stimulation for fertilization (flower tripping). Nectar feeding animals are common vectors for cross pollination in these types of flowers (Richards, 1986; Free, 1993).
Insects are the most common pollen vectors in cross-pollinated Papilionoideae species, particularly honeybees (*Aphis mellifera* L.), leaf cutter bees (*Megachile rotunda* Fab.) and bumblebees (*Bombus* spp.) (Free, 1993). Seed production is often improved by high pollinator presence and hives of appropriate bee species are introduced into fields of commercial seed crops to maximize yield (Fairey *et al*., 1997).

Self-compatibility and autogamy can be confirmed in plant species or genotypes by successful reproduction when individuals or flowers are isolated, from each other and from pollinating vectors. Likewise, xenogamy can be confirmed by the failure of flowers to set seed in isolation, provided a requirement for stimulation or tripping has been taken into account (Real *et al*., 2004; Nair *et al*., 2006). Estimating the level of cross-pollination in a field situation can be more complicated if there is no barrier to self-pollination and the opportunity for cross-pollination exists.

The breeding system of genus *Ornithopus* L. is generally considered to be autogamous self-pollination (Frame *et al*., 1998). This is based on studies of flower pollination and ovule fertilisation which concluded that the flower structure and timing of gamete development made autogamy most likely (Wojciechowska, 1972). As well, fecundity remained high and there was no evidence of reproductive depression when plants were grown in isolation. However, this study also concluded that as the flowers do open, there is an opportunity for cross pollination in swards with access to insect pollinators.
Cross-pollination in *O. sativus* Brot. has been predicted (Klinkowski, 1942; Pfeffer, 1963). In these studies, offspring segregated away from maternal flower colour or to intermediate states of expression. This implied heterogeneity for flower colour in some of the individuals studied, which was most likely generated by cross-pollination. However, precise estimation of cross-pollination could not be determined by these studies. The tendency to cross-pollinate in *O. sativus* may also be influenced by climatic conditions affecting pollinator activity and thereby introducing regional considerations (Pfeffer, 1963). The incidence of cross-pollination in *O. sativus* was also implied from the data in the selection for a hard-seed trait reported in Chapter 3.

The flower structure of *O. sativus* is typical of many legumes. Flowers have a relatively large standard petal, two smaller wing petals and an inconspicuous keel composed of the two smallest petals fused along the ventral side. The wing petals have basal nectaries that attract insects, particularly bees. The keel completely encloses the carpel, although access through the dorsal split can be achieved through manipulation. In most genotypes of *O. sativus*, the standard and wing petals appear light pink, but this is the result of red or dark pink venation on a white background. However, accessions with pure white, burgundy, yellow and yellow/pink flowers are held by genetic resource centers in both pure and mixed forms. Some of this germplasm may have similar origins to the flower colours used by Klinkowski (1942) and Pfeffer (1963) in their cross pollination studies.
This chapter aimed to confirm and quantify cross pollination in *O. sativus* under conditions of a spaced plant selection nursery in an open field situation. The traits examined were flower and seedling pigmentation with the expectation that these would be simply inherited. Outcomes of this study will help to determine appropriate management of *O. sativus*.

### 4.2 Materials and Methods.

#### 4.2.1 Production of a white-flowered genotype.

A white-flowered genotype was produced by recurrent selection for the white colour over three generations from accession 4715 (held at the ATGRC) (Figure 4.1). The base population prior to selection was a mixture of approximately 1/3 pink-flowered and 2/3 white-flowered individuals. In each generation the plants were grown as spaced plants and any pink-flowered individuals were removed as flower colour became evident. Under this selection pressure all plants by the third generation were white-flowered.

#### 4.2.2 Hybridisation to determine inheritance of flower colour (white and pink).

Two white-flowered plants (the result of recurrent selection in 4.2.1) were hybridised with two pink-flowered plants (cv. Cadiz) by the technique discussed below. The parent plants were grown in a controlled environment glasshouse (free of pollinating insects) under a 20/15 °C day/night regime in 30 cm plastic pots in commercial potting mix.
The success of the recurrent selection described above and the lack of segregation once isolated, suggested that the white flower colour was recessive to the pink flower colour. This created a check for hybridisation as the F1 plants should show the dominant flower colour. Therefore white-flowering plants were used as the maternal parent in all controlled pollinations. Flowers were selected for cross pollination when the corolla was level with the calyx teeth, approximately 2 days prior to anthesis. All other flowers on the umbel were removed and the selected flower was opened by removal of the keel petals and emasculated using fine jewellers forceps (Figure 4.2).

Donor pollen was selected from a flower that was beginning to unfold and open. At this stage the anthers had ruptured and the clusters of pollen grains had a lustrous, pale yellow and granular appearance. The whole gynoecoeum was removed from these flowers and the attached pollen was wiped over the stigma of the maternal flower immediately after emasculation. The manually pollinated flowers were covered with a waxed paper bag for 7 days.

Seed formed from the manually pollinated flowers was collected when the pods were dry, about 60 days after pollination. Other flowers were left to autonomously pollinate with out manipulation and were presumed to be self pollinated in the sealed, insect-free glasshouse. The seed from self-pollinated flowers was collected when the majority of the plant had senesced. Forty seeds from cross-pollinated flowers and 40 seeds from self-pollinated flowers of both the maternal and paternal parents were grown over the winter
and spring of 2004 at Medina Research Station, Perth, Western Australia (latitude 32° 13’S, longitude 115° 48’E).

Figure 4.1. White-flowered and pink-flowered forms of *O. sativus* hybridized in this study.
Figure 4.2. Flower selection and steps used for emasculation in preparation for controlled pollination of *O. sativus* – maternal flower. **A.** Flower umbel at the end of the stem. **B.** Selection of a flower at the right stage (petals not emerged from calyx) and removal of all other flowers on umbel. **C.** Peeling back of the calyx (in this case removed). **D.** Removal of standard (top) petal. **E.** Removal of wing petals. **F.** Removal of fused keel petals exposing the cluster of 10 anthers. **G.** Exposed stigma after emasculation.
The resulting self and cross pollinated seeds were grown by first raising the seedlings in peat pots (filled with commercial potting mix) for 4 weeks and they were then transplanted into the field. They were arranged as spaced plants (1.5 m apart) in four randomized blocks of 10 plants each, planted into holes in plastic film covering the soil to minimize weed incursion. The plants were irrigated from the May sowing to November every 2\textsuperscript{nd} day at transpiration + 10\%. Seedling hypocotyl pigmentation, days from germination to the first open flower and flower colour were recorded for each plant. Plant diameter was measured on the 10\textsuperscript{th} of July (76 days after germination).

4.2.3 Cross pollination in a field situation.

This experiment was embedded into a spaced plant nursery that was composed of F2 hybrids, and self pollinated offspring of the respective parents, all of which were pink-flowered (described in Chapter 5). These pink-flowered plants were used as pollen donors by planting in 5 x 5 blocks (24 plants) with a white-flowered trap plant positioned in the centre of each block (Figure 4.4). The trap plants were propagated from self-pollinated seed harvested from the pure breeding white-flowered plants used as parents for hybridisation experiment described in 4.2.1.

Each 5 x 5 plant block was repeated 60 times in a 5 block x 12 block grid (60 m x 90 m) giving a total of 60 trap plants (Figure 4.4). The nearest neighboring trap plant was at least 7.5m away with 4 intervening pink-flowered pollen donor plants. The experiment was sited at Medina Research Station over winter/spring 2005, under the same conditions as described in
4.2.1. Although spaced at a minimum of 1.5 m between plant crowns, at flowering the distance between the ends of runners was reduced to about 50 cm and at the end of the growing period, adjacent plants were almost touching. Ten additional examples of the white-flowered trap plants were grown in isolation in a glasshouse over the same period to provide a check for segregation in self-pollinated offspring.

A sample of the first ripe pods was harvested from each individual trap plant prior to plant senescence. The remaining pod was combined into 6 bulk samples of 10 trap plants after plants senescence. Approximately 300 seeds from the first mature pods from individual trap plants were then grown in 2 replicate 2.5 m rows at South Perth, Western Australia over winter/spring of 2006. An additional 2 replicate 2.5 m rows were grown from seed of the 10 glasshouse grown white-flowered check plants, and the 6 bulk trap plant samples from Medina Research Station.

Figure 4.4. Planting layout with a minimum distance of 1.5 m between plant crowns. ⊗ White-flowered trap plants, • Pink-flowered pollen donor plants.
During this study an association between red hypocotyl pigmentation and flower colour became evident (Figure 4.5). Therefore the presence or absence of hypocotyl pigmentation was used as the principal discriminating character in this experiment. To confirm this association, one pigmented and one non-pigmented seedling (on the hypocotyls) were labeled in each row of one replicate and followed through to flowering.

4.2.4 Statistical analysis.

Descriptive statistics and analyses of variance were calculated using Genstat – Ninth Edition Version 9.2.0.152 (VSN International Limited). Spatial variations in offspring off-types were compared using ANOVA with columns and rows as factors.

Figure 4.5. Offspring from a white-flowered O. sativus trap plant showing seedlings with and without red-pigmentation on the hypocotyls.
4.3 Results.

4.3.1 Inheritance of flower colour.

Of the 40 putative F₁ hybrid plants produced from manual pollination between white-flowered and pink-flowered parents, 38 had distinct red pigmentation on seedling hypocotyls and produced pink flowers. The remaining two plants were without red pigmentation on seedling hypocotyls and produced white flowers. Both of these plants originated from different manually pollinated flowers that also produced offspring (siblings) from the same pods with red-pigmented seedlings and pink flowers.

4.3.2 Flowering time of hybrids.

The pink-flowered parent was the first to flower and the white-flowered parent the last. The hybrids were distributed between the two parents in mean and variability in time to flower but slightly skewed toward the early parent with a dominance of 0.5 (Table 4.1, Figure 4.6) (Falconer, 1999). The F₁ hybrids were significantly larger and more vigorous than either parent. The two white-flowered plants from manual pollination began to flower at a similar time to the self-pollinated offspring of the white-flowered parents. It was concluded that these were the results of contaminant self-pollinations.
Table 4.1. Mean days to first open flower, plant diameter at 76 days after germination, and flower colour in F1 hybrid and self-pollinated offspring of pink and white-flowered plants. The two white-flowered plants from manually pollinated flowers were determined to be non-hybrid and excluded from the analysis of variance.

<table>
<thead>
<tr>
<th>Seed source</th>
<th>Days to flower</th>
<th>Plant diameter (cm)</th>
<th>Flower colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 hybrid</td>
<td>123 ± 1</td>
<td>201 ± 7</td>
<td>38 pink (2 white)</td>
</tr>
<tr>
<td>Pink parent</td>
<td>113 ± 1</td>
<td>159 ± 6</td>
<td>All pink</td>
</tr>
<tr>
<td>White parent</td>
<td>153 ± 1</td>
<td>170 ± 5</td>
<td>All white</td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td>2.3</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.6. Histogram of the numbers of plants to form an open flower over time in *O. sativus* cultivar Cadiz (solid bars), a white flowered selection (open bars) and F1 hybrids between the two (shaded bars). Maturity of white flowered putative shown by arrows.
4.3.3 *Cross-pollination in a field situation.*

The pink-flowered plants (pollen donor) all flowered earlier and were in full bloom before the white-flowered trap plants. Honeybees (*Apis mellifera*) were present in high densities during the flowering period and, on fine days, up to six bees were observed working different flowers on the same plant at any one time. Figure 4.1b shows a honeybee working an *O. sativus* flower at the site. However, bee numbers were reduced on overcast days and absent on rainy days. Six trap plants failed to survive to harvest due to the action of birds.

Seed from the first pods formed by the white-flowered trap plants produced nearly 10,000 seedlings, of which 25.3% (± 0.8%) showed red hypocotyl pigmentation (Table 4.2). The proportion ranged from 16 to 39% of offspring from maternal plant on an individual basis. Six plants had a greater proportion of pigmented seedlings than the site mean (p < 0.05) however there was no obvious spatial pattern over the whole site. There was little correlation ($R^2 = 0.015$) between time to first open flower and the proportion of red pigmented offspring from the trap plants (Figure 4.7).

The bulk samples of seed, representing the whole flowering period of the trap plants, produced 14.5 ± 2.0% of seedlings with red hypocotyl pigmentation. This is significantly lower (p<0.01) than the estimate from the first pods formed on each individual plant. All seedlings from isolated white flowered plants grown in the glasshouse had green hypocotyls (and these were white-flowered). Seedlings with red or green hypocotyls grown to
maturity confirmed the association of red pigmentation in seedling hypocotyls and pink flower colour (100%).

Table 4.2. Proportion of with seedlings with red-pigmented hypocotyls from the first seed formed on each of the 54 trap plants (shown in the Table according to their position in the field - Figure 4.4). Proportions of red-pigmented seedlings are also shown from bulked samples (also shown in the Table according to position in the field of the plants that were bulked). NA = no plants recorded.

<table>
<thead>
<tr>
<th>Position in the field</th>
<th>3</th>
<th>8</th>
<th>13</th>
<th>18</th>
<th>23</th>
<th>28</th>
<th>33</th>
<th>38</th>
<th>43</th>
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<td>23</td>
<td>37</td>
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<td>25</td>
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<td>16</td>
</tr>
<tr>
<td>Row</td>
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<td>na</td>
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<td>21</td>
<td>25</td>
<td>30</td>
<td>27</td>
<td>17</td>
<td>18</td>
<td>25</td>
<td>29</td>
<td>18</td>
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<td>26</td>
<td>23</td>
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</tr>
<tr>
<td>Bulk sample</td>
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<td>20</td>
<td>16</td>
<td>7</td>
<td>12</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.7. Relationship between maturity and proportion of offspring with red-pigmented hypocotyls (off types) from 54 white-flowered trap plants after being grown as single spaced individuals surrounded by pink-flowered pollen donor plants in a field situation.
4.4 Discussion.

Hybridization between white-flowered and pink-flowered genotypes of *O. sativus* found the white flower colour to be inherited recessively to pink. The pink flower colour was always associated with red pigmentation on seedling hypocotyls. Similarly, green hypocotyls were always associated with white flowers. The states of expression in both traits appeared very discrete and were easily discriminated.

The absence of red pigmentation on seedling hypocotyls and flowers may be due to the same underlying mechanism. In other species, white-flower colour has been associated with interruptions in the biochemical pathway that results in anthocyanin synthesis. This is presumed to be the result of mutations in associated structural and regulatory genes (Martin and Gerats, 1991; Mol *et al.*, 1998; Nakatsuka *et al.*, 2005).

The pattern of inheritance of flower colour and the associated seedling hypocotyl colour provided a useful trait to estimate cross pollination in a field setting. The white-flowering genotypes were selected to be trap plants as they were known to be homozygous for this trait. Surrounding white-flowered trap plants at the commencement of flowering with homozygous pink-flowered donor plants in full bloom estimated xenogamic cross pollination, simply by examining hypocotyl (or flower) colour on the offspring of the trap plants. Offspring diverging from the maternal trap plant character, provided it is recessively inherited, in this situation can only occur through cross-breeding. The red hypocotyl pigmentation was particularly useful for this
purpose as a large sample number can be readily assessed without the requirement to grow all the plants to maturity.

This method resulted in an estimate of 25% xenogamous fertilization in *O. sativus*. This is based on the first pods formed on the plants, and when the pollen carried by pollinators would have been overwhelmingly pink-flowered in origin. As the flowering intensified on the trap plants, there would have been greater opportunity for bees to visit multiple flowers on the same plant. The decrease in the estimate of fertilisation by pink-flowered pollen in the bulk samples (16% versus 25%), was therefore most likely due to increased incidence of allogamic self-pollination (geitonogamy). On an individual plant basis, the estimate of xenogamous fertilisation based on the first seeds formed ranged from 16 to 39%. This wide range may be due to differences in abundance of flowering on the sampled plant at the time of fertilisation of the sampled seed.

The estimated fertilisation through xenogamous pollination that was calculated from the first seeds formed is more likely to represent a sward situation (i.e. where flowers from different plants are in close proximity). The upper estimate may also be possible where a rare genet is present in a population and the pollen population is dominated by different genotypes. For instance, one individual trap plant was assessed as having 39% cross-pollinated offspring (Table 4.2, position Row 8, Column 13).
The incidence of cross pollination in *O. sativus*, and other species with a similar breeding system, may be reduced in a spaced plant selection nursery by employing intervening plants of a different species. To be successful this would require the barrier plants to be in flower at the same time as the target species and their flowers would need to attract bee foraging. In species such as *O. sativus*, the level of cross pollination may vary over a season and with location due to differences in bee population and the influence of climatic conditions on bee foraging activity.

The level of cross pollination estimated by this study suggests that, for *O. sativus*, isolation from pollinating insects is required in situations where purity is a concern, such as on pedigree seed production and genetic resource conservation. Employing barrier plants may be a useful way to avoid this requirement, but this needs formal testing. Therefore the isolation distances required for certified seed production under the OECD seed scheme (200m) for cross-pollinated species is appropriate and should be applied to *O. sativus* cultivars.

The incidence of cross-pollination, even at the lower estimates produced by this study, is also an important consideration for *O. sativus* breeding programs. Isolation from pollinators would be necessary for efficient recurrent selection if the desired traits can not be achieved prior to flowering. Reproduction based characters, such as pod or seed characteristics, can only be selected after flowering and without isolation, cross-pollination will reduce the efficiency of the selection process.
The inheritance structure of the red pigmentation/no red pigmentation trait is also valuable for developing hybridisation techniques. The two green seedling hypocotyl/white-flowered plants among the progeny from the manual cross pollinations were clearly due to self-pollination from the maternal plant. This can be concluded with some confidence as their flowering time also matched that of the white-flowered maternal parent (Figure 4.6). These seeds were most likely the result of imperfect emasculation and the technique requires modification to ensure the production of true hybrids. In this study the anthers of the flowers for manual pollination were removed using forceps (a common technique with legume species), however the use of a vacuum delivered through a pipette may be more appropriate. This modification will be adopted for manual cross pollination in Chapter 5.

Earlier flowering appeared to have some dominance (d = 0.5) over later flowering in terms of mean and variance of time to anthesis. This may have been exaggerated by increased plant vigor and more rapid plant development in the hybrid material. The F1 hybrid plants were significantly larger in mid-winter than either parent genotype. Although no inbreeding depression has been reported in *O. sativus*, the improved vigour of the F1 hybrids relative to the two parent lines may be the result of heterosis.

The level of cross-pollination found in this study suggests *O. sativus* might be better described as having a mixed breeding system, rather than self-
pollinating or autogamous as suggested in previous studies (Wojciechowska, 1972; De Lautour and Rumball, 1986). There appeared to be no barrier to autogamous fertilisation, although allogamous cross-pollination obviously occurred in this study in the presence of pollinators. The pollinator activity resulting in allogamous fertilisation may result in more seed being formed by increasing the numbers of ovules fertilised. If there is no self-incompatibility, allogamous cross-pollination can be generated by pollen from different flowers on the same individual or a different individual of the same genotype (geitonogamy), or from an unrelated genotype (xenogamy). Seed production may therefore benefit from the encouragement of bee activity (by limiting the use of insecticides) and even introduction of hives into a seed production paddock.

The relative proportions of each type of pollination will be influenced by plant density, genotype representation in the population, relative maturity and the density of pollinators. Xenogamic cross pollination in *O. sativus* can therefore range from 0% in homozygous populations and in the absence of pollinators, to as high as 40% in a rare genotype that is late flowering relative to the rest of the population and subject to dense pollinator activity. Further studies using variants with matching maturity and other flower colours would be valuable to draw out this relationship and to ensure that the white flowered genotype is not more prone to cross-pollination than other *O. sativus*. The identification of discriminating molecular markers would greatly assist this type of studies.
The level of cross pollination estimated in *O. sativus* suggests significant shifts in a diverse population structure could occur in response to selective pressure. Therefore a limited number of generations from breeder’s seed should be considered for cultivar certification. Additional studies would be required to estimate the distance over which cross pollination could occur and therefore determine adequate isolation distances or pollination barriers. This information would be useful for designing selection nurseries or genetic resource conservation activities when isolation is impractical.
Chapter 5. Inheritance of hard-seed and early flowering in *Ornithopus sativus* Brot.

5.1. Introduction

The production of hard-seed is the principle seed dormancy mechanism in the Leguminoseae family of legumes (Baskin and Baskin, 2001). These are seeds that, when sufficiently dry, develop a seed coat that is impermeable to moisture. The exact layer in the seed coat that is responsible for moisture impermeability intrigues researchers, however its study can be difficult. Most studies conclude that the palisade layer is involved with or without interaction with the non-cellular sub-cuticular layer (Hamly, 1932; Aitken, 1939; Watson, 1948; Spurney 1964; Manning and van Staden, 1987; Riggio-Bevilacqua et al., 1989). The palisade layer is composed of macrosclereid cells that are radially elongated and tightly packed (Bhalla and Slattery, 1984). This layer is formed from maternal tissue developed from the cells of the outer ovule integument. The dormancy imposed upon the seed embryo by hard-seededness is maintained until the impermeable state is broken either through damage or changes in specialized structures of the seed coat (Baskin and Baskin, 2001).

In natural settings, the production of hard-seed is a survival strategy that can both synchronize germination with favorable environmental conditions and spread reproductive opportunity over time (Bewley and Black, 1985). However, because of the need for ready germination and selective pressures
associated with recurrent harvesting and sowing of seed, species that are cultivated for seed production generally lose this survival strategy in the course of domestication (Heiser, 1988).

A number of Leguminoseae sub-family Papilionoideae legumes are cultivated for their grain or fruits. The pressures brought by domestication are apparently against seed dormancy in these legumes and this has led to the development of genotypes of these species that do not form impermeable seed coats. When this occurs they are described as soft-seeded.

The inheritance of hard-seed or soft-seed production has been studied in some legume species through hybridisation. In *Lupinus luteus*, *L. angustifolius* L. and *L. cosentini* Guss., soft-seed production is conferred by a recessive allele at a single locus (Serrato-Valenti et al., 1989). In *L. angustifolius* this gene is identified as *mollis* and has been mapped in the genome with the use of molecular markers (Nelson et al., 2006).

In some legume crops, their long histories of cultivation, combined with the selective pressures of cultivation, have led to significant genetic drift away from their wild progenitors. The study of the inheritance of hard-seed production in these genera, for instance *Vicia* and *Lens*, has required second degree hybridisation between cultivated and related wild species (Elkins et al., 1966; Donelly, 1971; Donelly et al., 1972; Ladizinski, 1985). For these genera, soft-seed behavior has been associated with two or more genes.
The genus *Ornithopus* L. has several hard-seeded, wild species, and a soft-seeded cultivated species, *O. sativus* (spp. *sativus*). Hard-seed producing individuals have been isolated by mass selection in *O. sativus* (Chapter 3). However, two different types of hard-seed behavior were observed. From one population, Australian Trifolium Genetic Resource Centre (ATGRC) accession 97ZAF5sat, plants produced either soft-seed or high proportions of hard-seed (>80%). Offspring of these plants maintain the maternal expression in most cases, but some may segregate to the opposite expression. A second population, based on the landrace cultivar Emena, produced a range of hard-seed levels across individuals grown from hard-seeds. These selections did not show such extreme segregation in their offspring.

The inheritance of hard/soft-seed production appeared to be under relatively simple genetic control in at least one *O. sativus* population (97ZAF5sat) and recurrent selection for either hard-seed or soft seed expression is likely to be successful. However, a greater understanding of the inheritance would be essential to develop the most appropriate methods for efficient selection, particularly as *O. sativus* was found to be partially (approximately 25%) cross-pollinated in an open field situation in the south-west of Western Australia (Chapter 4).

This study aims to test the hypothesis that hard/soft seed production is inherited through the action of a single gene in a similar fashion to that reported in *Lupinus* species, and that soft-seed is associated with a
recessive allele. This will be tested by hybridisation between parent lines found to have reliably hard-seed producing offspring, and soft-seeded genotypes. Secondary aims are to examine the heritability of time to form open flowers and the interaction between timing of flowering and production of hard-seed. A better understanding of the control of hard or soft-seed expression will help explain the rarity of hard-seed production in *O.sativus* and this will be further explored in Chapter 8.

### 5.2 Materials and Methods.

**5.2.1 Hybridization of selected *O. sativus* plants.**

A flow diagram of the selection and hybridization procedure is shown in Figure 5.1. Plants were chosen from the 97ZAF5sat and cv. Emena populations as sources for early-flowering, hard-seeded (A1.1) and late-flowering, hard-seeded parents (B1.2) respectively (Chapter 3). They were chosen on the basis of consistent expression of maturity and hard-seed production in their progeny (Table 3.1). The earliest flowering 2002 selection from the cultivar Cadiz and Cadiz itself were used as early flowering, soft-seeded (D1) and late flowering, soft-seeded (C) parents respectively (Table 3.3).

Manual hybridization involved the selection of a maternal flower at the stage when the end of the corolla was just inferior to the tips of the calyx teeth (just prior to stage 3 according to Wojiechowska, 1971, and slightly earlier than shown in Figure 4.2b). All other flowers on the umbel and on the same runner were removed. The calyx was split and folded back to
allow the unfolding and removal of all petals. The anthers were removed using a vacuum delivered through a small plastic pipette tip.

Donor pollen was collected from an unfolding flower (stage 6 by Wojciechowska, 1971) by removing the whole gynoecium and gently wiping the attached pollen over the stigma immediately after emasculation. The calyx was then folded back to retain pollen and provide protection. The flower and the end of the stem were covered with a wax paper bag for three days to prevent desiccation of the exposed stigma.

A total of 97 crosses between the hard and soft-seeded parents were attempted (including reciprocal crosses) and putative hybrid seeds were harvested 60 days after pollination. In the insect proof glasshouse non-nanipulated flowers were presumed self-pollinated and this seed was collected from the parent plants once the majority of the plant had senesced. Seed from all cross pollinated flowers along with 8 representatives from the self-pollinated seed of the parents were grown in a glasshouse over summer (between November and March, 2004/05) (Figure 5.2). F₂ plants from the highest seed yielding F₁ plants were grown at Medina Research Station in 2005 as single spaced plants in a fully randomized design. Seeds were sown into peat pots at the beginning of May, and then transplanted into the field after 5 weeks (Figures 5.3 and 5.4).
**Figure 5.1. Flow diagram of parent selection, hybrid production and numbers of progeny tested (Maternal parent x Pollen source).**

**Source Populations:**
- 97ZAF5sat
- Emena
- Cadiz

**Character selected by screening of source population:**
- Hard seed production
- Early flowering
- Late flowering

**Parent cluster:**
- A1
  - Early flowering
  - Hard-seed Plant A1.1
- B1
  - Late Flowering
  - Hard-seed Plant B1.2
- D
  - Early flowering
  - Soft-seed Plant D1
- C
  - Late flowering
  - Soft-seed Plant C

**F₁ Hybrids generated:**
- B1.2 x A1.1: 4 plants
- A1.1 x D1: 19 plants & 17 plants
- D1 x B1.2: 1 plant
- A1.1 x C: 4 plants & 1 plant

**F₂ Hybrids grown:**
- 120 B1.2 x A1.1: from 3 F1s
- 240 A1.1 x D1: from 6 F1s & 240 D1 x A1.1: from 6 F1s
- 120 D1 x B1.2: from 1 F1
- 120 A1.1 x C: from 3 F1s & 120 C x A1.1: from 1 F1
The plants were harvested in the first weeks of December. Days to first open flower from sowing, germination and percentage hard-seeds were recorded for each individual in the F\textsubscript{1} and F\textsubscript{2} generations.

Figure 5.2. Self pollinated offspring from parent plants and F\textsubscript{1} hybrids growing in the glasshouse. The plants were tied to stakes to prevent inter-tangling of vines with neighboring plants and to minimise space.

5.2.2 Plant culture.

Plants grown in the glasshouse (free of pollinators) were sown into commercial potting mix and the temperature was controlled at a 15/20°C night/day regime. Parent plants for hybridization were grown in 250mm round pots. F\textsubscript{1} generation hybrids were grown in 50mm square pots and the stems were tied to a vertical stake to conserve space. All plants grown in the glasshouse received daily watering and a fortnightly application of general purpose liquid fertiliser.
Single spaced F₂ and selfed parental plants grown at Medina Research Station were cultured by placing scarified seed into small peat pots filled with commercial potting mix at the beginning of May. The seedlings were grown in a covered plastic house for 3 weeks, hardened for 2 weeks uncovered, then transplanted into the ground with plastic film mulch at a minimum of 1.5m between plants. A basal fertilizer of 300 kg/ha of NPK was applied prior to the laying of the plastic film. The area was irrigated (at evaporation plus 10%) every 2\textsuperscript{nd} day between transplanting and the middle of November. Plants were harvested in December, after full senescence, by stripping of pods from stems, drying for 3 days at 40\textdegree C, and cleaning the sample by removing leaf and stem material by hand.

Figure 5.3. Second generation self pollinated offspring and F₂ hybrids growing in potting mix in peat-pots prior to transplanting into the field.
Figure 5.4. Second generation self pollinated offspring and F$_2$ hybrids growing in the spaced plant field nursery at Medina, Western Australia.

5.2.4 Germination tests.

Germination tests were conducted on seed (in pod segments) placed in rolls of moist filter paper stored at 15°C. In the parent selection, progeny and F$_1$ tests, two replicates of approximately 100 pod segments per plant were used. Germinated seed (normal seedlings), viable imbibed seed, abnormal germinants, dead seed and residual hard-seed numbers were recorded over a 21 day period. Seed was considered dead if it imbibed and began to decay. An abnormal germinant was a seed which imbibed and germinated but would not produce a viable seedling due to cotyledon or radical fracturing.

Germinated seed, dead seed and abnormal seedlings were removed after 7 and 14 days to minimise decay. Because of the high numbers of
genotypes involved in the F2 generation, a single sample of 25 seeds per plant was assessed and germinated, viable imbibed and residual hard-seed, were counted after 14 days. In all cases proportion of hard-seed was calculated as;

\[
\text{Proportion hard-seed (\%)} = \frac{\text{Hard-seed residue} \times 100}{\text{Hard-seed residue} + \text{germinated seed} + \text{viable imbibed seed}}
\]

Dead seed or abnormal seedlings were infrequent and not included in any analyses. Progeny were grouped into hard-seed (> 1\%) and soft-seed (\leq 1\%).

The presence or absence of fine hairs on the pod surface was also recorded in parent and hybrid progeny as a further check for heterozygosis in the parent lines (Figure 5.5).

5.2.5 Statistical analysis.

Proportion hard seed was transformed to arcsin to normalize data for all analyses in the following form;

\[
\text{Hard-seed score} = \arcsin\left(\frac{\text{Proportion hard-seed (\%)} - 50}{50}\right)
\]

Parent and hybrid times to flower were compared and environmental variance estimated using a one way analysis of variance. Parent and segregating F2 populations were categorised for hard-seed score using group average cluster analysis. These groups were then compared using
a two way analysis of variance using hard-seed score group and parentage as independent factors. Segregating proportions were tested for goodness of fit to Hardy-Weinberg equilibrium by Chi-square (Hartl, 1999). All statistical analyses were conducted using Genstat 9.2.0.152 (VSN International Limited).

5.3 Results.

5.3.1 Self pollinated offspring states of expression.

The hard seed by flowering time relationship in the presumed self-pollinated offspring of the parental lines showed clustered distributions similar to those described in Chapter 3 (Figure 3.2 and Figure 5.5). Based on the behavior of the self-pollinated offspring (S2), the parental lines could be described as follows;

- A1.1 has stable production of high levels of hard-seed with consistent, relatively early flowering and glabrous pod surface. Selected from the 97ZAF5sat population.
- B1.2 has variable, low to moderate levels of hard-seed, with consistent, relatively late flowering and pilose pod surface. Selected from the cv. Emena population.
- D1 has no hard-seed production, with consistent very early flowering and glabrous pod surface. Selected from the cv. Cadiz population.
- C has no hard-seed production with variable, mid-ranged time to flower and had glabrous pod surface in the plant used for hybridisation, however self-pollinated offspring from this plant
segregated for the pilose or glabrous pod surface. Sourced, un-
selected, from the cv. Cadiz population.

Figure 5.5. Mean and standard deviation for flowering time and hard-seed
score in self pollinated S₂ generation offspring from parent O. sativus
plants used for hybridization.

There was no obvious segregation in time to form open flowers, hard-
seed production or pod surface in the self-pollinated S₁ or S₂ (grand-
offspring) generations of A1.1, B1.2 and D1, indicating homozygous
genotypes. The self-pollinated S₁ and S₂ generations from the un-
selected cv. Cadiz plant, Parent C, segregated for pilose or glabrous pod
surfaces (Figure 5.6). It also had a relatively broad distribution in time to
flower among the S₂ generation. However, both generations consistently
produced no hard-seed and appeared homozygous for this character.
When genotypes with glabrous pod surface were crossed with a pilose genotype, the resulting F₁ plants produce glabrous pod surfaces.

Figure 5.6. Pilose (A) and glabrous (B) pod surfaces in *O. sativus*.

5.3.2 *Hybrid states of expression and segregation.*

Of 97 attempted hybridizations, 14 were successful and resulted in 1 to 6 seeds per flower and 45 seeds in total. The germination type of all was according to the maternal parent phenotype of hard or soft-seed. The 45 putative F₁ hybrids and 8 self-pollinated offspring per parent were grown in a glasshouse over the summer months. The early summer sowing resulted in contracted flowering times, to the extent that there was very little difference between the genotypes in this character (Table 5.1).
Table 5.1. Time to flower and hard-seed score of self pollinated progeny of parent plants and F₁ hybrid plants in *O. sativus*. Plants were classified as hard-seeded when > 1% hard-seed and soft-seeded when ≤ 1% hard-seed were present in the germination test.

<table>
<thead>
<tr>
<th>Parentage</th>
<th>Days to 1&lt;sup&gt;st&lt;/sup&gt; open flower</th>
<th>Dormancy classification</th>
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<tr>
<td></td>
<td></td>
<td>Hard-seeded</td>
<td>Soft-seeded</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. plants</td>
<td>Hard seed (%)</td>
<td>No. plants</td>
</tr>
<tr>
<td>Self pollinated offspring</td>
<td></td>
<td>D1</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>A1.1 (self)</td>
<td></td>
<td>65</td>
<td>8</td>
<td>95</td>
</tr>
<tr>
<td>B1.2 (self)</td>
<td></td>
<td>64</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>C (self)</td>
<td></td>
<td>63</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Cross pollinated offspring (maternal x paternal)</td>
<td></td>
<td>A1.1 x D1</td>
<td>61</td>
<td>17</td>
</tr>
<tr>
<td>D1 x A1.1</td>
<td></td>
<td>58</td>
<td>19</td>
<td>98</td>
</tr>
<tr>
<td>A1.1 x C</td>
<td></td>
<td>61</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>C x A1.1</td>
<td></td>
<td>65</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>D1 x B1.2</td>
<td></td>
<td>74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B1.2 x A1.1</td>
<td></td>
<td>60</td>
<td>4</td>
<td>97</td>
</tr>
</tbody>
</table>

All F₁ hybrids involving the early flowering, hard-seeded Parent A1.1 produced high levels of hard-seed regardless of whether A1.1 was used as the maternal parent or the pollen source. Although parent B1.2 was selected for hard-seed production, its self pollinated and single F₁ hybrid with the early flowering, soft-seeded parent (D1) produced little or no hard-seed when grown in the glasshouse over summer. This was likely to be an artifact of insufficient seed drying to achieve the full expression of potential hard-seed (Chapter 7).

The time taken to flower of all F₂ hybrids grown under field conditions over winter and spring was normally distributed between the expression
in the self-pollinated offspring (S₂) of the parent genotypes and their
mean expression was close to the mid point between the parental means
(Table 5.2). The variance in time to flower was generally higher in the F₂
hybrids than the parent genotypes except for the S₂ generation of the cv.
Cadiz parent plant (C).

The pattern of segregation in F₂ hybrids for hard-seed production was
different for the two sources A1.1 (97ZAF5sat) and B1.2 (cv. Emena)
(Table 5.2). The early hard-seeded parent, A1.1, when hybridized with
both soft-seeded parents, C and D1, segregated in a bimodal pattern in
the F₂ generation, producing either high levels of hard-seed (>60%) or no
hard-seed. The proportion of plants producing either hard-seed or no
hard-seed with these parentages was very close to a ratio of 3:1 (Table
5.3). The late-flowering soft-seeded parent C generally produced F₂
hybrids with lower levels of hard-seed than the early flowering parent D1
(mean hard-seed score of A1.1 x C and C x A1.1 = 1.23 compared to
A1.1 x D1 and D1 x A1.1 = 1.51).

The F₂ hybrids involving the late flowering, hard-seeded parent B1.2 and
the early flowering parent D1 also produced a bimodal pattern of
segregation of either moderate to high levels of hard-seed, or no hard-
seed. However, with this parentage only 23% of plants fitted the hard-
seed category and the segregation was better represented by a 1:3 ratio
(Table 5.3).
Table 5.2. Mean and variance of time to flower and hard-seed score in the S\textsubscript{2} generation of self pollinated offspring from parent genotypes and F\textsubscript{2} hybrid plants. Plants were classified as hard-seeded when \( > 1\% \) hard-seed and soft-seeded when \( \leq 1\% \) hard-seed were present in the germination test.

<table>
<thead>
<tr>
<th>Parentage</th>
<th>No. plants</th>
<th>Mean</th>
<th>Var.</th>
<th>Hard seed score</th>
<th>No. plants</th>
<th>Mean</th>
<th>Var.</th>
<th>Hard seed score</th>
<th>LSD (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1.1</td>
<td>112</td>
<td>103.5</td>
<td>0.30</td>
<td>-1.48</td>
<td>0</td>
<td>0.04</td>
<td>0.00</td>
<td>-1.49</td>
<td>-</td>
</tr>
<tr>
<td>B1.2</td>
<td>77.2</td>
<td>127.3</td>
<td>0.29</td>
<td>-1.57</td>
<td>5</td>
<td>0.08</td>
<td>0.00</td>
<td>-1.57</td>
<td>0.00</td>
</tr>
<tr>
<td>C</td>
<td>100.0</td>
<td>35</td>
<td>0</td>
<td>-1.57</td>
<td>92</td>
<td>0.07</td>
<td>0.00</td>
<td>-1.57</td>
<td>0.00</td>
</tr>
<tr>
<td>D1</td>
<td>11.6</td>
<td>84.9</td>
<td>0.04</td>
<td>-1.57</td>
<td>98</td>
<td>0.08</td>
<td>0.00</td>
<td>-1.57</td>
<td>0.00</td>
</tr>
<tr>
<td>A1.1 x C</td>
<td>68</td>
<td>101.1</td>
<td>0.07</td>
<td>-1.57</td>
<td>30</td>
<td>0.07</td>
<td>0.00</td>
<td>-1.57</td>
<td>0.00</td>
</tr>
<tr>
<td>C x A1.1</td>
<td>73</td>
<td>103.5</td>
<td>0.08</td>
<td>-1.57</td>
<td>25</td>
<td>0.07</td>
<td>0.00</td>
<td>-1.57</td>
<td>0.00</td>
</tr>
<tr>
<td>A1.1 x D1</td>
<td>169</td>
<td>90.7</td>
<td>0.02</td>
<td>1.27</td>
<td>58</td>
<td>0.03</td>
<td>0.00</td>
<td>1.52</td>
<td>0.00</td>
</tr>
<tr>
<td>C x A1.1</td>
<td>166</td>
<td>91.0</td>
<td>0.03</td>
<td>1.19</td>
<td>55</td>
<td>0.03</td>
<td>0.00</td>
<td>1.50</td>
<td>0.00</td>
</tr>
<tr>
<td>D1 x A1.1</td>
<td>24</td>
<td>102.7</td>
<td>0.16</td>
<td>1.52</td>
<td>80</td>
<td>0.15</td>
<td>0.00</td>
<td>1.55</td>
<td>0.00</td>
</tr>
<tr>
<td>B1.2 x A1.1</td>
<td>95</td>
<td>105.6</td>
<td>0.14</td>
<td>1.50</td>
<td>134.5</td>
<td>0.14</td>
<td>0.00</td>
<td>1.55</td>
<td>0.01</td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td>-</td>
<td>-</td>
<td>2.2</td>
<td>1.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
<td>-</td>
</tr>
</tbody>
</table>
Hybrids between both hard-seeded parents (B1.2 x A1.1) produced F1 plants with high levels of hard-seed and an F2 generation with moderate to high levels of hard-seed. There was no obvious truncation point across this range on which to base a segregation ratio.

Hard-seed score was slightly reduced with increasing time to flower considering all hard seed producing F2 hybrids, with a negative correlation of 48%, however the relationship was poor ($R^2 = 0.23$).

Table 5.3. Chi squared test results (and probability of exceeding $X^2$) for segregating F2 hybrids between hard and soft-seed producing parents.

<table>
<thead>
<tr>
<th>Parentage</th>
<th>Ratio of hard:soft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3:1</td>
</tr>
<tr>
<td>A1.1 x C</td>
<td>1.57 ($p \leq 0.05$)</td>
</tr>
<tr>
<td>C x A1.1</td>
<td>0.01 ($p \leq 0.001$)</td>
</tr>
<tr>
<td>A1.1 x D1</td>
<td>0.04 ($p \leq 0.001$)</td>
</tr>
<tr>
<td>D1 x A1.1</td>
<td>0.00 ($p \leq 0.001$)</td>
</tr>
<tr>
<td>D1 x B1.2</td>
<td>123.25 (NS)</td>
</tr>
<tr>
<td>Total</td>
<td>124.88 (NS)</td>
</tr>
</tbody>
</table>

5.4 Discussion.

Mendelian-like segregation occurred in F2 families when hard-seed producing genotypes were hybridised with soft-seeded genotypes. Surprisingly, the pattern of segregation for hard-seed production was different when the trait was sourced from two different populations. The absolute and discrete hard-seed production by the 97ZAF5sat selection (A1.1) clearly showed dominant inheritance over soft-seed production.
The segregating proportions in F\(_2\) hybrids were highly suggestive of a single gene effect. However, the variable hard-seededness in the Emena selection (B1.2) was recessive to soft-seed, but also in a single gene distribution pattern.

These two observations explain the segregation that was evident in the S\(_2\) progeny of the mass selected hard-seeds from the two different source O. sativus populations (Chapter 3). The plants grown from hard-seeds in accession 97ZAF5sat that produced no hard-seed, arose from segregation of heterozygous hard-seed producing parents. It may also have been due to cross pollination of heterozygote hard-seeded genotypes with the predominant soft-seeded genotypes in the original populations. Then, subsequent segregation in these offspring away from the maternal expression of soft-seededness is likely to be due to cross pollination with hard-seeded phenotypes. The selection nursery for the parent lines was conducted in an open field with free access to honey bees. At the same location where this experiment was conducted, cross pollination in O. sativus was estimated to be close to 25% (Chapter 4).

The lower incidence of both soft-seeded plants from mass selected hard-seeds, and segregation away from hard-seed production, is due to the recessive expression of the hard-seed character from the cultivar Emena. Because of this recessive expression, these plants must be homozygous for this character in order to produce hard-seed. This hard-seed trait also appears to be influenced by the environment of seed ripening as the
seeds grown in the glasshouse expressed very low levels of hard-seed. This behaviour has the capacity to create misleading results. Along with the different pattern of inheritance, this behaviour supports a hypothesis that two different mechanisms of hard-seed may be occurring between the different source populations.

The intense selection for early flowering reduced the time to commencement of flowering and proved to be highly heritable, but in a more quantitative way than hard-seed production. The F₂ hybrids between parents of different maturity groups segregated in a normally distributed fashion and 70% of the variance could be attributed to parentage. The only exception was a broad variance in flowering time in the self-pollinated offspring of the cultivar Cadiz plant that was the C parent in the hybridisation studies. The self-pollinated and F₂ hybrid offspring of this parent plant also showed segregation for smooth and pilose pod surface. The variability in time to flower relative to the other parents and F₂ hybrids, and the segregation for texture of the pod surface suggests that this plant was in a heterozygous state, at least for these characters. This further supports the hypothesis that cross-pollination is occurring at some level in seed crops of *O. sativus*.

Sequential planting of the parental genotypes showed two primary controls over the timing of flowering (Chapter 3). Firstly, a general minimum number of days to flower, that is best expressed with spring and early summer sowing, and secondly, variation in the extent of
suppression of flowering under conditions of decreasing and short photoperiod. Given the interaction between these two characters it is not surprising that time to flower in the hybrids was normally distributed between parent expression and without clear segregation.

The pattern of inheritance of hard-seededness selected from the O. sativus accession 97ZAF5sat is similar to that reported in L. angustifolius. In this species a single gene, with two alleles Mollis and mollis, was found to be responsible for conferring seed coat behavior. The hard-seed character in this species was also found to be dominant and soft-seededness a recessive trait (Forbes and Wells, 1968; Gladstones, 1970, Cowling, 1998).

The recessive and highly variable hard-seed behavior from the cultivar Emena also has some similarities to hard-seed studies reported for other legume species (such as T. incarnatum in James, 1949 and Bennett, 1959). The observed inheritance of hard-seed in Vicia and Lens species has been attributed to two genes, one dominant and one recessive, possibly three major genes in Glycine max and classified only as “simple” but possibly multi-genic in Phaseolus vulgaris (Donnelly et al., 1972; Kilen and Hartwig, 1978; Lebedeff, 1947).

Recurrent selection for hard-seed in T. incarnatum L. was partially successful in two reported studies. The lack of response in the first was considered to be due to poor heritability or environmental interference
(James, 1949). The second study suggested high heritability (although it was not quantified), and moderate levels of hard-seed were achieved after several selection cycles (Bennett, 1959). The behavior of *T. incarnatum* may be due to a similar genetic make-up described here for the selections from the Emena populations. In this case there appears to be considerable genotype and seed ripening (or post harvest) environment interaction on the expression of initial hard-seed level.

The pattern of inheritance of a character that is strongly modified by the environment can be difficult to ascertain. A continuous distribution in a character due to environmental modification can be confused with complex multigenic control. When the distribution of expression is discontinuous, as in the 97ZAF5sat selections and *L. angustifolius*, segregating proportions are easily classified. However, when the distribution is more continuous the determination of classifications is more problematic. As all hybrid progeny between the two types of hard-seed expression in this study were hard-seeded, a third allele for the gene responsible for the development of hard-seed is proposed, as an alternative approach of a more complex genetic makeup. The interaction of two genes (or more) is possibility and is not discounted, however, this can not be confirmed by the results presented here and may require a molecular approach for a definitive answer.

The three alleles proposed, using S is an abbreviation of Seed Coat Permeability, are;
i) $S_1$ a dominant allele, conferring the extreme, high expression in hard-seed that is only mildly influenced by environment;

ii) $S_2$, which is recessive to $S_1$ and confers the absolute soft-seed state when homozygous. This expression may be due to mutation away from the wild state in some structural or biochemical way that disrupts the establishment of impermeability in the seed coat; and

iii) $S_3$, which is recessive to both $S_1$ and $S_2$, and confers a potentially hard-seed coat with the level of expression highly influenced by ripening or post harvest environment.

The disparity between the two patterns of hard-seed inheritance, and the proposed three allele model, may be understood further by examining the process by which the hard-seed state is established. The seed coat, or testa, of legumes becomes decreasingly permeable as the mature seed dries to low moisture content. After a critically low moisture level is reached, an impermeable state is achieved. This critical level has been estimated at 14% in *Trifolium repens* L., *T. pratense* L. and *Lupinus arboreus* Sims. Further loss of seed moisture, once general seed coat impermeability is attained, occurs through the valve-like hilum (Hyde, 1954).

Other studies on seeds of *Lupinus*, *Trifolium*, *Medicago*, *Astragalus*, *Melilotus* and *O. compressus* found the hard-seed state to be partially reversible at moisture contents below 14% but irreversible below 9 or
10% (Gladstones, 1958; Nakamura, 1962). The understanding of this process was further refined in *Lupinus cosentinii* (syn. *L. varius*) where seeds at moisture contents above 10% (dry weight basis) were conditionally hard, and imbibition could proceed slowly by moisture penetration at random sites across the testa. Absolute or irreversible hard-seed occurred in *L. cosentinii* at moisture contents below 8.5% (Quinlivan, 1968). The establishment of an impermeable seed coat is therefore a two stage process influenced by the environment and perhaps seed coat chemistry.

The absolute state of hard-seed in *Lupinus cosentinii* was broken down by exposure to high and fluctuating temperature (diurnally 15/60°C). This treatment resulted in the fracture of the seed coat at the lens (Quinlivan, 1961). The conditionally hard-seeds above the 10% moisture content level did not respond to this fluctuating temperature treatment. In *Trifolium subterraneum* L. fluctuating temperatures also lead to the rupture of the lens region of the seed. The lens is also where the first point of water entry into naturally softened seeds when they are exposed to moist conditions (Hagon, 1971; Aitken, 1939).

The variable and moderate amount of hard-seed found in the Emena selections and its F₂ hybrids may hypothetically be due to a different moisture binding property in the seed coat compared to the 97ZAF5sat selections and its hybrids. All seed was treated in a similar way (dried at 40°C for 3 days) and it was assumed that they were at similar moisture
content, of below 7% of dry weight. The critical moisture contents required to establish the absolute hard-seed state is possibly different for the two hard-seed characters (alleles S₁ and S₃). This influence of seed moisture and post harvest treatment on the expression of hard-seed and the sites of moisture entry into the seed will be further investigated in Chapter 7.

If hard-seed production is the natural condition, which appears likely considering the germination behavior of wild legume ecotypes, the soft-seed state may be the result of a mutation disrupting either structural or biochemical processes in the seed coat tissues. In other species, histological studies have been inconclusive and it is difficult to differentiate between inherent seed coat disruption and damage during the sample preparation and sectioning procedure (Revell, 1997). At a biochemical level, reduction in the permeability of the seed coat in the Pisum genus has been explained by the presence and oxidation of phenolic compounds in the seed coat as the seeds undergo drying (Marbach and Mayer, 1974; Werker et al., 1979). Soft-seeded forms of Pisum contained little catechol oxidase activity and have very low levels of phenolics compared to hard-seeded forms. Soft-seed production in O. sativus may be due to a similar biochemistry and the difference in the two hard-seed behaviors may be generated by this mechanism, and, if so, two totally different genes in a biosynthetic pathway.
The hypothesis that heritable hard-seed and early maturity are present within populations of *O. sativus* is supported by the evidence presented in this study. Given the low level of incidence of these characters across accessions, combining these traits may best be approached by separate selection processes and subsequent hybridisation. The manual pollination of *O. sativus* is difficult due to the small flower size, and there is a high rate of abortion when flowers are manipulated. Even so, a success rate of 14% in this study indicates hybridisation can be effectively undertaken to combine desirable characters in this species. This study has found that the pod surface can be used as a marker for true hybrids. For instance, when crosses involve a female with a pod surface with a pilose texture and pollen from a line with glabrous pod surface, a glabrous F1 plant is indicative of hybridization.

The hypothesis that timing of flowering and ability to produce hard-seed are independent is also supported at a genetic level by this study. However interaction between genotype and environment may result in these attributes appearing associated in terms of phenotypic expression.
Chapter 6. Changes in seed dormancy and germination of *Ornithopus sativus* Brot. and *Ornithopus compressus* L. with field exposure and under controlled conditions.

6.1 Introduction.

The introduction and maintenance of a legume component in pastures is an important management tool to maximise productivity in many different farming systems. The legumes produce quality forage for animal production and improve soil fertility through biological nitrogen fixation (Frame *et al.*, 1998). Legume based pastures also provide rotational benefits such as opportunities for integrated disease, pest and weed management (Karlen, 2004). In the Mediterranean and temperate climates of Southern Australia, the annual legumes *Trifolium subterraneum* L. and several annual *Medicago* L. species have played a key role in the development and success of cereal-ley farming systems because of these qualities (Puckridge and French, 1983; Robson, 1988; Davidson and Davidson, 1993; Kemp and Michalk, 2005).

In more recent times, a broader range of annual legumes have been examined for their potential to improve pasture productivity in cereal-ley farming systems. These include a number of *Ornithopus* L. and other *Trifolium* L. species, as well as *Biserrula pelecinus* L. and *Lotus ornithopodioides* L. (Norman *et al.*, 2000; Nichols *et al.*, 2007). The concept
that species diversity may provide greater system stability, and the limited success of *T. subterraneum* and annual *Medicago* species in some situations, have been drivers for this research (Howieson *et al.*, 2000; Norman *et al.*, 2002; Loi *et al.*, 2005).

A key characteristic relating to the success of annual legumes in cereal-ley farming systems is the development of a persistent seed pool in the soil from which the legumes can spontaneously self-regenerate (Puckridge and French, 1983; Taylor, 2004). This is achieved though the production of seeds that are held in a dormant state, imposed by an impermeable seed coat. This is a common seed dormancy mechanism in the Leguminosae family of plants (legumes) and when in this state they are described as hard-seeds. Release from the dormant seed pool occurs when the impermeable seed coat of a hard-seed is breached by damage or in response to environmental triggers (hard-seed breakdown) (Badbeer, 1988; Baskin and Baskin, 2001).

There are two aspects to the timing of hard-seed breakdown in the ecology of self-regenerating pasture legumes (Quinlivan, 1971; Russi *et al.*, 1992; Norman *et al.*, 1998). In the short term, the timing of hard-seed breakdown can provide synchrony of germination within a season with conditions favorable for germination, plant survival and eventual reproduction. In Mediterranean and temperate climates, summer and early autumn rainfall events can be common and germination on these “false breaks” may lead to seedling death due to inadequate follow up rain (Chapman and Asseng,
Being in a dormant, hard-seed state protects the seeds from germination on these unseasonal rainfall events.

The second component of hard-seed as a survival strategy is the timing and quantities of seed released from the dormant pool over a sequence of seasons. This distributes germination over a number of seasonal reproductive cycles and insures persistence against reproductive failure in one or more of these cycles. Reproductive failure may occur due to adverse climatic conditions, such as drought, or other imposed conditions such as animal grazing, disease, pest predation or agricultural cultivation (Jansen, 1969).

Ornithopus sativus Brot. is an annual legume species that has been used for forage and green manure for more than 300 years (Klinkowski 1939). It is the most common Ornithopus species cultivated in regions with Mediterranean and temperate climates (Duke, 2002). The cultivated forms of O. sativus do not produce impermeable seed coats, and therefore do not develop persistent seed reserves. This limits ability to self-regenerate in systems where annual seed production does not occur, for example due to rotational cropping (Dear et al., 2002). Use of these types of O. sativus is therefore reliant on frequent re-sowing (Frame et al., 1998).

As reported in Chapter 3, hard-seed producing genotypes can be isolated from the predominantly soft-seeded O. sativus populations by mass selection. However, the long term dynamics of the hard-seed dormancy of
these selected genotypes, and the effect of environmental interactions has not yet been described. This study aims to test the hypothesis that genotypes of *O. sativus* that do produce hard-seed, have sufficient longevity of seed dormancy to develop a persistent seed reserve. It will be undertaken by measuring changes in seed germination over time with field exposure, when seed is either on the surface or slightly buried in the soil (in this study at 1cm). It will also test the hypothesis that hard-seed breakdown in these genotypes can be induced in the laboratory by simulating the field temperature conditions recorded at the time of the greatest rate of breakdown.

### 6.2 Materials and Methods.

#### 6.2.1 Sites of seed production, sampling and preparation.

Four commercial cultivars of *Ornithopus* were examined for changes in seed germination with field exposure; soft-seeded Cadiz (*O. sativus*), hard-seeded Erica and Margurita (*O. sativus*) and hard-seeded Santorini (*O. compressus*). To produce the seed for this study, small swards, 1.2m x 10m, were sown at two sites in the south-western region of Australia, Mingenew (latitude 29° 10’ S, longitude 115° 14’ E) and Muresk (latitude 31° 43’ S, longitude 116° 44’ E). The seed was first inoculated with a commercial peat culture (Australian Group S, *Bradyrhizobium* strain WSM471) and then sown at 7 kg/ha of scarified seed (with greater than 75% germination) for the hard-seeded cultivars and at 15 kg/ha of podded seed for Cadiz (approximately 50% seed content). This seed was sourced from commercial suppliers.
The swards were fertilised at sowing with 140 kg/ha of superphosphate and 70 kg/ha of muriate of potash. Chemical analysis of the soil at each site was undertaken by a commercial soil analysis service (CSBP Soil Analysis Service). The soil provided for analysis was sampled prior to sowing (and fertilization) and consisted of an aggregate of 20 cores (5cm deep, 2cm diameter) across each site of production. Descriptions from the soil analysis, growing season rainfall and sowing and harvesting dates at each of the sites are provided in Table 6.1.

Table 6.1. Soil characteristics and seasonal rainfall at the two sites of seed production used in the seed germination studies.

<table>
<thead>
<tr>
<th></th>
<th>Muresk</th>
<th>Mingeneew</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texture</td>
<td>Sandy clay loam</td>
<td>Loamy sand</td>
</tr>
<tr>
<td>Depth to clay (m)</td>
<td>0.6 – 0.8</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>April to Dec rainfall (mm)</td>
<td>306</td>
<td>332</td>
</tr>
<tr>
<td>Nitrate N (mg/kg)</td>
<td>31</td>
<td>8</td>
</tr>
<tr>
<td>Amonium N (mg/kg)</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Phosphorus (Colwell mg/kg)</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>Potassium (mg/kg)</td>
<td>94</td>
<td>39</td>
</tr>
<tr>
<td>Organic C (%)</td>
<td>1.7</td>
<td>0.7</td>
</tr>
<tr>
<td>pH (1:5, 0.01M CaCl2)</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Sowing date</td>
<td>23/5/04</td>
<td>25/5/04</td>
</tr>
<tr>
<td>Harvest date</td>
<td>24/11/04</td>
<td>25/11/04</td>
</tr>
</tbody>
</table>

Prior to sowing, glyphosate was applied as a knockdown herbicide and bifenthrin (Talstar®) insecticide was applied immediately after sowing for the control of *Halotydeus destructor* (red-legged earthmite). Imazamox (Raptor®) was applied at about the 5 leaf stage for the control of broadleaved and grass weeds. Swards were allowed to mature naturally under rain-fed conditions, and achieved expected dry matter and pod production under these conditions. Pod samples were collected from 2 quadrats of 0.5m² and
bulkied together for each cultivar and site. The sample was processed by gentle threshing to remove stems, soil and to break up the pod material for sample splitting and treatment allocation.

6.2.2 Field exposure.

The clean pod samples were mixed thoroughly and split using an Encott two way sample divider and counted into 100 pod segment (seed) lots. These were sealed into plastic coated, fiberglass mesh (fly-wire) formed into strips of pockets (each 5cm x 3cm) with the use of a heat-sealer. Each strip of pockets contained a randomized replicate of all cultivar x site treatments. These were pinned to the soil surface or buried at 1cm of depth in the soil on the 9th of January, 2005, at Medina Research Station (latitude 32° 13’S, longitude 115° 48’E, Figure 6.1). The site of field exposure was an un-shaded area of loamy sand, 3 x 1.5m, which was kept free of plants or dry residue over the course of the experiment.

The mesh pockets of samples were recovered and referred to according to the schedule shown in Table 6.3. Before each germination test was conducted, the seeds were removed from the mesh pockets and cleaned of sand and debris. The seed pods gradually degraded over time so that mostly free seed was recovered by the winter 2 sampling time.
Table 6.2. Mean of minimum and maximum air temperature and relative humidity, rainfall and the range in relative humidity at Medina Research Station, the site of seed exposure over the course of the experiment.

<table>
<thead>
<tr>
<th>Year</th>
<th>Month</th>
<th>Mean min. air temp. (°C)</th>
<th>Mean max. air temp. (°C)</th>
<th>Mean min. RH (%)</th>
<th>Mean max. RH (%)</th>
<th>Total rain (mm)</th>
<th>Total rain days</th>
<th>Min. RH (%)</th>
<th>Max. RH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>December</td>
<td>15.9</td>
<td>29.1</td>
<td>33.5</td>
<td>78.5</td>
<td>4.8</td>
<td>3</td>
<td>13.7</td>
<td>95.4</td>
</tr>
<tr>
<td>2004</td>
<td>January</td>
<td>16.2</td>
<td>29.3</td>
<td>30.1</td>
<td>77.7</td>
<td>0.2</td>
<td>1</td>
<td>10.6</td>
<td>91.9</td>
</tr>
<tr>
<td>2004</td>
<td>February</td>
<td>16</td>
<td>29.4</td>
<td>33</td>
<td>77.8</td>
<td>0.8</td>
<td>2</td>
<td>8.5</td>
<td>92.1</td>
</tr>
<tr>
<td>2004</td>
<td>March</td>
<td>17.9</td>
<td>30.1</td>
<td>39.7</td>
<td>82.4</td>
<td>16.2</td>
<td>9</td>
<td>11.4</td>
<td>95.9</td>
</tr>
<tr>
<td>2004</td>
<td>April</td>
<td>12.1</td>
<td>24.1</td>
<td>44</td>
<td>88.3</td>
<td>37.6</td>
<td>8</td>
<td>18.8</td>
<td>96.8</td>
</tr>
<tr>
<td>2004</td>
<td>May</td>
<td>14.3</td>
<td>23</td>
<td>54.7</td>
<td>87.6</td>
<td>183.4</td>
<td>16</td>
<td>27.4</td>
<td>96.9</td>
</tr>
<tr>
<td>2004</td>
<td>June</td>
<td>9.6</td>
<td>17.8</td>
<td>58.8</td>
<td>91.9</td>
<td>216.6</td>
<td>19</td>
<td>33.4</td>
<td>99.3</td>
</tr>
<tr>
<td>2004</td>
<td>July</td>
<td>7.5</td>
<td>18</td>
<td>53.3</td>
<td>94.4</td>
<td>71.4</td>
<td>14</td>
<td>30.4</td>
<td>98.9</td>
</tr>
<tr>
<td>2004</td>
<td>August</td>
<td>8.2</td>
<td>17.9</td>
<td>54.2</td>
<td>93.4</td>
<td>115.8</td>
<td>15</td>
<td>27.1</td>
<td>98.7</td>
</tr>
<tr>
<td>2004</td>
<td>September</td>
<td>8.9</td>
<td>18.4</td>
<td>50.7</td>
<td>92.6</td>
<td>93.8</td>
<td>20</td>
<td>24.9</td>
<td>98.2</td>
</tr>
<tr>
<td>2004</td>
<td>October</td>
<td>9.3</td>
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<td>93.5</td>
<td>58.8</td>
<td>12</td>
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<td>24.1</td>
<td>39.1</td>
<td>85.5</td>
<td>15</td>
<td>6</td>
<td>21</td>
<td>97</td>
</tr>
<tr>
<td>2006</td>
<td>December</td>
<td>12.8</td>
<td>22.6</td>
<td>45.3</td>
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<td>12</td>
<td>7</td>
<td>27</td>
<td>94.9</td>
</tr>
<tr>
<td>2006</td>
<td>January</td>
<td>16.3</td>
<td>28.2</td>
<td>38.5</td>
<td>80</td>
<td>57.2</td>
<td>6</td>
<td>13.5</td>
<td>93</td>
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<tr>
<td>2006</td>
<td>February</td>
<td>17.4</td>
<td>30.4</td>
<td>34.1</td>
<td>78.1</td>
<td>2.6</td>
<td>1</td>
<td>17.8</td>
<td>94.3</td>
</tr>
<tr>
<td>2006</td>
<td>March</td>
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<td>30.1</td>
<td>31.2</td>
<td>75.6</td>
<td>11.4</td>
<td>3</td>
<td>12.9</td>
<td>94.8</td>
</tr>
<tr>
<td>2006</td>
<td>April</td>
<td>11</td>
<td>23.1</td>
<td>43.2</td>
<td>88.3</td>
<td>30.8</td>
<td>6</td>
<td>28</td>
<td>96.7</td>
</tr>
<tr>
<td>2006</td>
<td>May</td>
<td>9</td>
<td>22.2</td>
<td>42</td>
<td>88.8</td>
<td>48.2</td>
<td>10</td>
<td>16.6</td>
<td>97.6</td>
</tr>
<tr>
<td>2006</td>
<td>June</td>
<td>5.9</td>
<td>19.9</td>
<td>40.3</td>
<td>89.1</td>
<td>28.4</td>
<td>6</td>
<td>18.5</td>
<td>97.6</td>
</tr>
</tbody>
</table>

Table 6.3 Sampling regime of *Ornithopus* seed exposed on the soil surface at Medina, Western Australia.

<table>
<thead>
<tr>
<th>Date of sampling</th>
<th>Days of exposure</th>
<th>Season label</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>9/1/05</td>
<td>0</td>
<td>Summer 1</td>
<td>4</td>
</tr>
<tr>
<td>20/3/05</td>
<td>70</td>
<td>Autumn 1</td>
<td>4</td>
</tr>
<tr>
<td>24/6/05</td>
<td>166</td>
<td>Winter 1</td>
<td>4</td>
</tr>
<tr>
<td>27/9/05</td>
<td>261</td>
<td>Spring 1</td>
<td>4</td>
</tr>
<tr>
<td>9/1/06</td>
<td>365</td>
<td>Summer 2</td>
<td>4</td>
</tr>
<tr>
<td>10/3/06</td>
<td>425</td>
<td>Autumn 2</td>
<td>4</td>
</tr>
<tr>
<td>13/6/06</td>
<td>520</td>
<td>Winter 2</td>
<td>4</td>
</tr>
<tr>
<td>14/6/07</td>
<td>876</td>
<td>Winter 3</td>
<td>6</td>
</tr>
</tbody>
</table>
6.2.3 Germination tests and determination of moisture content on residual hard seed.

Initial germination and germination after exposure were determined by placing the seed/pod in rows on moist filter paper that was then folded and formed into a roll (Figure 6.2). These rolls were then placed in a sealed container and stored at 15°C. Normal and abnormal seedlings were counted and removed at 7, 14 and 21 days and any residual hard seeds retrieved and counted.

Residual hard seed was air dried for 24 hours at ambient temperatures and weighed. This seed was then further dried at 105°C for 3 days and re-
weighed. Seed moisture content was estimated from the change in weight between the air dried seed and oven dried seed and expressed relative to the oven dried seed weight (DW) (after Taylor 1987). The initial dry seed weight in the cultivar Cadiz was calculated from an additional four replicate samples of 100 seeds without prior germination.

Figure 6.2. Germination of *Ornithopus* seed/pod in rolls of moist filter paper.

6.2.4 *Simulated pre-conditioning and diurnal temperature fluctuations.*

The seed lots of cv. Erica (*O. sativus*) and cv. Santorini (*O. compressus*) from the Mingenew and Muresk sites of seed production were used in the simulated softening experiment. The pre-conditioning treatment involved placing seed in paper envelopes in an oven at 60°C for 2 days. Alternating temperature treatments were generated by placing seed in paper envelopes in an insulated chamber in a room cooled to a constant 15°C. The heating to produce the required temperature cycles inside the chamber was provided by two 25W light globes controlled by a step-less Yokogawa UP27e programmable controller.
The temperature inside the chamber was programmed for 2 hours at 15°C, a continuous increase to 50°C over 4 hours, 2 hours at 50°C and a continuous decrease to 15°C over 4 hours. Therefore, 2 cycles were completed in each 24 hours. Four replicate samples of 100 pod segments (seeds) each were exposed to 8 temperature cycles. The samples were then assessed for germination and un-germinated seed re-treated with a further 8 cycles (16 fluctuating temperature cycles in total).

Germination after the different seed treatments and residual hard-seed at the end of the germination test was determined by the same method described for the field exposure experiment (rolls of moist filter paper). Temperature and relative humidity in the controlled conditions were monitored every 10 minutes using a Hygrochron iButton datalogger. The temperature cycle was chosen after analysis of the results of hard-seed breakdown in the field, which demonstrated the greatest level of breakdown occurred at the temperature conditions measured at 1cm depth in the soil (see Results and Figure 6.4).

6.2.5 Statistical analysis.

The categories of seed behavior that were expressed as proportions of germinable, abnormal and hard-seed were compared by analysis of variance in Genstat 9.2.0.152 using cultivar and site of production as factors. The average germination rate (co-efficient of germination velocity after Kotowski, 1926) was calculated as;

$$C_V = \left( \frac{\Sigma G_n}{\Sigma(G_n \cdot D_n)} \right) \cdot 100$$
Where $G_n =$ number of seeds germinated by day $n$; $D_n =$ days from initial wetting.

The soft seeded cultivar Cadiz produced only a low number of hard seeds at any sampling time (<3%) and is considered separately from the hard seeded cultivars for the first field sampling and omitted in subsequent samplings.

Winter residual hard seed were compared for each cultivar separately using analysis of variance calculated in Genstat edition 9.2.0.152 (VSN International Limited), using site of production, seed placement and year as factors.

### 6.3 Results.

#### 6.3.1 Initial seed viability and germination.

The hard-seeded cultivars produced high levels of hard-seed, particularly cv. Santorini (*O. compressus*), while the soft-seeded cv. Cadiz produced almost no hard-seed (Table 6.4). Some of the germinated seeds showed damage by *Helicoverpa punctigera* (native budworm). This damage appeared as small, circular holes in the pod wall that continued through the seed coat and in some cases cotyledon tissue (Figure 6.3). It was more common in the *O. sativus* cultivars, including cv. Cadiz and may have contributed to the higher initial level of germination in cv. Margurita and cv. Erica relative to cv. Santorini.
Figure 6.3. An *O. sativus* seed that has been damaged by *Helicoverpa punctigera* (native budworm) predation, removing the imposed hard-seed dormancy.

There was little difference in most seed characters measured between the sites for each cultivar, and between Margurita and Erica. Hard-seeded cultivars had slightly higher seed moisture content from the Muresk site and with the exception of Erica, seeds tended to be slightly larger from the Mingenew site. The samples of cultivar Cadiz seeds returned a greater number of abnormal seedlings than the hard-seeded cultivars. The abnormality was usually detachment of the radicals in the plumule region of the seed embryo and was not associated with the insect damage. These abnormal seedlings would not be expected to develop into established plants. The majority of seeds of the *O. sativus* cultivars germinated within 7 days. Although low in numbers, seeds of the *O. compressus* cultivar Santorini were significantly slower (*p < 0.05* expressed as coefficient of germination velocity) to germinate (Table 6.4).
Table 6.4. Seed characteristics of *O. sativus* cultivars, Cadiz, Erica, and Margurita, and *O. compressus* cultivar Santorini grown at two locations, Mingenew and Muresk, Western Australia, 2004.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Coeff. of germinat' velocity (Cv)</th>
<th>Seed moisture content (%)</th>
<th>Seed dry wt (mg/seed)</th>
<th>Empty pod segm. (%)</th>
<th>Hard-seed (%)</th>
<th>Abnormal or dead (%)</th>
<th>Normal seedlings (%)</th>
<th>Lsd (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mingenew</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadiz</td>
<td>13.8</td>
<td>8.5</td>
<td>2.47</td>
<td>8.5</td>
<td>13.5</td>
<td>1.0</td>
<td>16.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Erica</td>
<td>11.9</td>
<td>6.7</td>
<td>1.83</td>
<td>6.2</td>
<td>15.5</td>
<td>2.2</td>
<td>15.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Margurita</td>
<td>13.4</td>
<td>6.3</td>
<td>2.16</td>
<td>6.2</td>
<td>6.2</td>
<td>1.5</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Santorini</td>
<td>5.7</td>
<td>6.3</td>
<td>2.48</td>
<td>0.7</td>
<td>92.8</td>
<td>1.5</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Muresk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadiz</td>
<td>13.9</td>
<td>8.4</td>
<td>2.40</td>
<td>4.0</td>
<td>16.5</td>
<td>0.8</td>
<td>14.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Erica</td>
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<td>7.1</td>
<td>1.95</td>
<td>8.5</td>
<td>7.0</td>
<td>2.0</td>
<td>16.8</td>
<td>5.4</td>
</tr>
<tr>
<td>Margurita</td>
<td>12.2</td>
<td>6.9</td>
<td>2.07</td>
<td>8.5</td>
<td>7.0</td>
<td>1.8</td>
<td>4.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Santorini</td>
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<td>7.5</td>
<td>2.33</td>
<td>1.5</td>
<td>93.8</td>
<td>0.8</td>
<td>4.0</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Lsd (p<0.05)
6.3.2 Changes in seed behavior in response to field exposure.

There was insufficient rain by the first field sampling for extensive germination/degradation and therefore the majority of seeds exposed for 70 days (between 9th January and 20th March) were recovered. Over this period the number of abnormal seedlings, dead or un-recovered seed increased in cv. Cadiz seeds exposed on the soil surface but not when covered by 1cm of soil (Table 6.5). Abnormal seedlings had detached radicals similar to the abnormalities seen in the initial germination test. The hard-seeded cultivars did not show the same trend. There were no or very few hard-seeds recovered from any field exposed samples of Cadiz seed.

Table 6.5. Seed characters of cv. Cadiz exposed to field conditions between 9th January and 20th March, 2005.

<table>
<thead>
<tr>
<th>Site of production</th>
<th>Seed placement</th>
<th>Normal seedlings (%)</th>
<th>Abnormal seedlings (%)</th>
<th>Hard-seed (%)</th>
<th>Un-recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mingenew</td>
<td>Surface</td>
<td>52.5</td>
<td>42.8</td>
<td>0.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Buried 1cm</td>
<td>81.5</td>
<td>18.0</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Muresk</td>
<td>Surface</td>
<td>52.0</td>
<td>21.5</td>
<td>1.8</td>
<td>24.7</td>
</tr>
<tr>
<td></td>
<td>Buried 1cm</td>
<td>78.2</td>
<td>9.5</td>
<td>3.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Lsd (p&lt;0.05)</td>
<td></td>
<td>5.8</td>
<td>6.7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Cultivar, site of production and position in the soil all influenced the level and pattern of hard-seed breakdown in cv. Erica, cv. Margurita and cv. Santorini (Figure 6.4). However, the major effects and interactions had different levels of influence between the three cultivars. The most consistent main effect was site of production. Seed produced at the Muresk site resulted in lower
numbers of hard-seed than the Mingenew site at most times of sample recovery after field exposure. Overlaid on this effect was the difference between samples exposed on the soil surface or buried at 1cm. Lower numbers of hard-seed were found in all buried samples of cv. Santorini seed compared to the surface samples from both sites of production. The buried samples of cv. Erica and cv. Margurita from the Muresk site of production also returned lower numbers of hard-seed than the surface samples. However, the difference was less pronounced in cv. Margurita, and reversed in cv. Erica seeds produced at the Mingenew site (Figure 6.5).

Seasonal sampling over the first 18 months showed, in general, a more protracted breakdown pattern in cv. Erica and cv. Santorini compared to cv. Margurita. Hard-seed breakdown for cv. Margurita was largely confined to the summer to autumn period (January to March) in both 2005 and 2006. However hard-seed breakdown in cv. Erica and cv. Santorini (when buried at 1cm) was spread over a larger part of the year with a decrease in hard-seed occurring from spring 1 to summer 2.

Winter seed numbers are perhaps the most appropriate measure of the long-term hard-seed breakdown particularly when adjusted for initial hard-seed level. This minimizes the influence of the different patterns of breakdown across treatments and the confounding effect of insect damage on initial hard-seed level. The winter hard-seed levels of cv. Erica were most influenced by site of production (p<0.001) however there was a significant interaction with placement of the seed (p<0.003) (Figure 6.5). The general
trend in cv. Erica seed was to lose 27%, 19% and 24% of the original hard-seed placed in the field by the first, second and third winter respectively, bearing in mind the site of production and seed placement interactions do result in variation around these means.

The levels of hard-seed in winter samples of cv. Margurita were influenced by site of production (p>0.001) and placement of the seed (p<0.001). Considering both sites of production and seed placement, cv. Margurita seed lost 30%, 25% and 27% of the original hard-seed by the first, second and third winter respectively. These rates of loss were around 7% higher than this average if the seed was buried at 1cm and 7% lower if the seed was placed on the soil surface (Figure 6.5).

There was a considerable influence of seed burial at 1cm on the loss of hard-seed measured at all three winter sampling times with the cultivar Santorini (p<0.001). However there was a significant interaction with site of location (p<0.035). Ignoring the site effect, the rate loss was 32%, 30% and 28% of the original hard-seed by the first, second and third winter respectively when placed at 1cm depth in the soil. This contrasted with the surface samples which lost only 8%, 5% and 11% of the original hard-seed by corresponding winter samples.
Figure 6.4. Hard-seed recovered in three *Ornithopus* cultivars relative to initial hard-seed when exposed in the field. Seed from Mingenew on the soil surface (□) or at 1cm depth (■). Seed from Muresk on the soil surface (○) or at 1cm depth (●).
Figure 6.5. Winter residual hard-seed of *Ornithopus* cultivars relative to initial hard-seed over three seasons, 2005 – 2007 (year 1, 2, and 3 at base of columns), when exposed in a field situation at Medina, Western Australia.
After germination the residual hard-seeds from buried samples had higher moisture contents compared to the seeds from samples on the soil surface (Figure 6.6). The estimated seed moisture content also fluctuated slightly over the course of exposure however this was not correlated with hard-seed breakdown.

Figure 6.6. Estimated moisture content of *Ornithopus* residual hard-seed after germination. Seed from Mingenew on the soil surface (□) or at 1cm depth (■). Seed from Muresk on the soil surface (○) or at 1cm depth (●) (data presented are the mean of cultivars Margurita, Erica and Santorini).

6.3.3 Hard seed breakdown of *O. sativus* cultivars Erica and *O. compressus* cultivar Santorini under controlled conditions.

Hard-seed breakdown of the three *Ornithopus* cultivars in the field occurred mostly over summer and autumn. During these seasons, daily temperature
at the soil surface ranged from around 15-20°C minimum and 35-60°C maximum (Figure 6.7). The diurnal temperature fluctuations were slightly buffered, particularly in the warmer months, when measured at 1cm soil depth compared to the soil surface.

Figure 6.7. Average daily maximum and minimum temperatures recorded on the soil surface (□ max., ○ min.) or at 1cm soil depth (■ max., ● min.) at the site of seed exposure, Medina, WA, in 2005.

Over the months where the greatest change in hard-seed was measured, the temperature at the soil surface increased over a 4 to 5 hour period at 5 to 8°C/hour on a daily basis, remained at the maximum for 2 hours then decreased over the afternoon for about 4 hours at around 5 to 6°C/hour. These diurnal temperature cycles were also associated with diurnal fluctuations in relative humidity of more than 40%, when measured by a datalogger at the soil surface (Figure 6.8). The maximum and minimum relative humidity measured by the official weather station suggests an even
greater range in this fluctuation (Table 6.2), with virtual saturation of the atmosphere occurring, presumably in the late night/early morning even in the middle of summer.

Figure 6.8. Average February (●) and March (x), 2006, diurnal fluctuation of temperature (solid line) and relative humidity (dashed line) measured at the soil surface at the site of seed exposure, Medina, WA.

The controlled environment was programmed to produce an 8°C/hr rate of change over 4 hours between a maximum of 55°C and a minimum of 15°C. The minimum and maximum temperatures were held for two hours per cycle so that each cycle was completed in 12 hours. The achieved profiles in temperature closely matched that measured in the field over the months of February and March, in amplitude and rate of change (Figure 6.9). However, the extremes were approximately 5°C lower in the cabinet. The relative humidity profiles were lower in both level and amplitude and do not match the averages measured in the field.
Figure 6.9. Temperature (solid line) and relative humidity (dashed line) profiles generated in a controlled temperature cabinet to stimulate hard-seed breakdown.

The alternating temperatures described in Figure 6.9 induced hard-seed breakdown in the *O. sativus* cultivar Erica, and to a lesser extent the *O. compressus* cultivar Santorini. The amount of breakdown was enhanced when the seeds were preconditioned by exposure to 60°C for 2 days prior to being subjected to the alternating temperature cycles (Figure 6.10). The seed produced at the Muresk site showed a greater breakdown of hard-seed compared to that produced at Mingenew. There was little breakdown of hard-seed of Santorini from both sites of production without the high temperature preconditioning.
Figure 6.10. Change in hard-seed level of *O. sativus* (cv. Erica) and *O. compressus* (cv. Santorini) induced by controlled temperature conditions (Figure 6.9). Seed from Mingenew with pre-conditioning of 60°C for 2 days (■) and without preconditioning (□). Seed from Muresk with pre-conditioning of 60°C for 2 days (●) and without preconditioning (○).

Seed stored at a constant 15°C for 6 months, returned similar levels of germination, hard-seeds, abnormal seedlings and dry seed weight of residual hard-seeds as in the initial germination test. The only significant change with storage was slightly lower moisture content in the residual hard-seeds after
the germination test. The change was a consistent 0.4% less moisture relative to seed dry weight in the residual hard-seed of the cultivars Margurita and Santorini from both sites of production. However, the residual hard-seed of the cultivar Erica had 0.6% and 0.2% less moisture from the Mingenew and Muresk sites, respectively, a difference sufficient to produce a significant site by cultivar interaction ($p \leq 0.001$).

6.4 Discussion.

Hard-seed breakdown in the *Ornithopus* cultivars when exposed to field conditions was strongly influenced by genotype, location of seed production and the position of exposure of the seeds in relation to the soil surface. Viable seeds of the hard-seeded *O. sativus* cultivars, Erica and Margurita, and the *O. compressus* cultivar Santorini, continued to be recovered after 36 months of field exposure. In contrast, viable seed of the soft-seeded *O. sativus* cultivar Cadiz were only recovered at the first sampling time, i.e. after 70 days of exposure without a significant rainfall event (Table 6.2).

The long term breakdown of hard-seed in the *O. sativus* cultivars, Erica and Margurita, was generally around 20 to 30% of the original hard-seed number per year. This means that the rate of hard-seed breakdown accelerated with age (when breakdown is expressed as a proportion of seed within a breakdown cycle). This hard-seed breakdown pattern could be expected to leave persistent, viable seeds in the soil through a year without seed production (such as during a crop rotation). As it appears to be a relatively constant quantity of seed that becomes capable of germination each season,
a good seed yield from a pasture year may provide sufficient seed to persist through two years of cropping.

There was considerable variation in hard-seed breakdown due to site of production, slight burial, and interactions between these factors, and this would require consideration when modeling seed germination over time. It also suggests that caution should be taken when comparing hard-seed behavior based on a single season or site of production. In this study the most extreme example was the *O. sativus* cultivar Margurita, where annual breakdown of hard-seed from the Muresk site of production was almost twice that from the Mingenew site (Figure 6.5). The effect of maternal site of production on hard-seed breakdown warrents further investigation which may elicite better procedures when studing hard-seed breakdown. The change in the proportion of hard-seed over time, when buried compared to on the soil surface, also demonstrates the importance of seed placement. In the extreme case of *O. compressus* cv. Santorini, greater than 70% of the seed remained after 36 months when exposed on the soil surface, however only 20% or less remained when buried at 1cm (Figure 6.5).

The differences in the rate of breakdown between the sites of production were most likely due to the rate of senescence. More stressful conditions during seed development have been observed to reduce the longevity of hard-seededness in *T. subterraneum*, *M. polymorpha*, and *M. truncatula* (Quinlivan, 1965, 1966, 1971; Collins, 1981; Taltyor *et al.*, 1984; Taylor and Ewing, 1992; Taylor, 1996). The Mingenew site has a deep profile of loamy
sand, while the Muresk site has a finer textured soil which overlays clay. Therefore, the senescence may have been more rapid at Muresk, due to less effective rooting depth and the water binding of the finer textured soil. A slower senescence at Mingenew may also explain the slightly larger seed size at this site.

Seed moisture content is considered critical in the establishment of an impermeable seed coat. The impermeable state is not imposed until a critical dry level is achieved, after which seed moisture content is expected to reflect the lowest relative humidity to which the seeds have been exposed (Hyde, 1954; Quinlivan, 1968; Baskin and Baskin, 2001; Taylor, 2004). However, estimates of the moisture content of residual hard-seeds in this study fluctuated over time (Figure 6.6). Furthermore, seed was considerably drier at each sampling when exposed on the soil surface compared to shallow burial. The enhanced breakdown of hard-seed when the seeds were slightly buried therefore suggests a role for seed moisture, and perhaps fluctuating seed moisture, in the breakdown process.

Hyde (1954) demonstrated moisture absorption by hard-seeds if there was a slow increase in relative humidity, although he suggested that this was unlikely to occur under field conditions. He also reported a small initial uptake of moisture by the counter palisade cells surrounding the hilar region, resulting in the closure of the hilar fissure. One or both of these functions could be operating in the breakdown of hard-seed and the observed changes in seed moisture content observed in this study. It may also help to explain
the two stage model for hard-seed breakdown proposed by Taylor (1981), where breakdown of hard-seed occurs after a period of preconditioning at high temperatures followed by exposure to cycles of fluctuating temperature. With this hypothesis, preconditioning and the parameters of the fluctuating temperatures may be specific to different species and perhaps genotypes (Taylor, 2004).

An inhibitory effect on hard-seed breakdown when pods or seeds are exposed to light has been proposed in O. compressus, and that this mechanism drives the increased rate of hard-seed breakdown when seed is covered with soil (Taylor and Revell, 1999). Burial was also observed to enhance hard-seed breakdown in this present study with the O. compressus cultivar Santorini and to a lesser extent with the O. sativus cultivars Margurita and Erica. As the buried seeds maintained higher moisture contents than those on the soil surface, the response could equally be explained by burial modifying the dynamics of seed moisture.

The breakdown of hard-seeds in response to exposure to fluctuating temperature in the O. sativus cultivar Erica and the O.compressus cultivar Santorini was confirmed in this study and supported the hypothesised two stage model. The level of hard-seed under stable conditions (15°C) did not change after 6 months, but as few as 8 cycles of fluctuating temperature (over 4 days) resulted in a significant inducement of germination and breakdown of hard-seed. Furthermore, this breakdown of hard-seed in response to these fluctuating temperatures required a preconditioning by
prior exposure to high temperature in Santorini and was accelerated by this preconditioning in Erica. This response could be explained by a lower preconditioning requirement in Erica hard-seed than cv. Santorini hard-seed, and was more rapidly satisfied by accrued exposure to high temperature during the cycles of temperature fluctuation.

The 15/50°C temperature fluctuations that were imposed on *Ornithopus* seeds in this study were condensed into a 12 hour period, resulting in 2 cycles per day. This was achieved by reducing the “cool” part of the diurnal temperature profile. As this did not reduce the effectiveness of the temperature fluctuations on hard-seed breakdown, it could be argued that this part of the diurnal profile does not play a major role in hard-seed breakdown.

Exposing seeds to a pre-conditioning period of high temperature followed by fluctuating temperatures successfully predicted the difference in propensity of hard-seed to soften under field conditions. This prediction is however, only applicable in a relative sense. This has been recognized in previous studies on other Mediterranean type annual legumes that compared observed hard-seed breakdown in the field to that which occurred under controlled conditions (Crawford, 1983; Bolland, 1985; Taylor, 1996a; Taylor 1996b, Smith et al., 1996; Norman et al., 1998; Revell et al., 1999). This is not surprising, given the variability in field conditions compared to treating seeds to a set regime of temperature and relative humidity. Added to this complexity is the effect of site of seed production and placement of the seed
relative to the soil surface observed in this study. Even so, hard-seed breakdown pattern under simulated conditions has been used to rank genotypes for field hard-seed breakdown in breeding and selection programs (Quinlivan, 1966; Quinlivan, 1968; Norman et al., 2006). The results obtained with the temperature fluctuations described in Figure 6.9 suggest that this approach could also be applied in breeding and selection programs for hard-seeded forms of *O. sativus*.

The seed of the soft-seeded *O. sativus* cultivar Cadiz that was recovered at the first sampling showed a considerable loss of viability when exposed on the soil surface (around 50%). However, seed of the hard-seeded cultivars did not show any loss of viability with field exposure. This may be related to the inability of soft-seeded genotypes to prevent reaction to cycles of moist and dry conditions. The same loss of viability did not occur when Cadiz seeds were buried at 1 cm, suggesting slight burial may provide a buffer against these fluctuating conditions. As the majority of freshly produced seed is likely to be on the soil surface through the first summer and autumn, loss of seed viability would be a critical factor when considering the fitness of soft-seeds relative to hard-seeds. It may also explain reports of poor regeneration of Cadiz in pastures in Western Australia, even when false break conditions are not experienced (per. com. C. Revell, pasture agronomist, Department of Agriculture and Food, Western Australia).
Chapter 7. Induced hard-seed breakdown, the dynamics of moisture content and the pathways of moisture entry in seeds of *O. sativus* Brot.

### 7.1 Introduction

The terms hard-seed, hard-seeded, and hard-seededness are applied to describe a type of seed dormancy that is common in species of the Leguminosae family (Bradbeer, 1988; Baskin and Baskin, 2001). It is a physical, exogenous dormancy, imposed by a seed coat that is impermeable to moisture. The dormant state develops as the seed dries, in a transition from having a seed coat that is initially permeable, to being conditionally (reversibly) impermeable, and finally to being irreversibly impermeable.

The critical points in the transition to impermeability are associated with particular levels of moisture in the whole seed (Quinlivan, 1971, Standifer et al., 1989). The general seed coat is referred to in the context of irreversible permeability, as the hilum and lens structures are specialized regions that do not allow the passage of moisture under particular conditions. The hilum is a one-way valve that, once the general seed coat becomes impermeable, allows further drying of the seed but not re-hydration (Hyde, 1954). The lens allows the uptake of moisture by the seed, but only after it has been affected...
by certain temperature cues. This process is referred to as hard-seed breakdown.

The breakdown of hard-seed dormancy in annual legume species including *Ornithopus compressus* L. and hard-seeded genotypes of *O. sativus* Brot., occurs over the summer and early autumn (Revell, 1998; Chapter 6). The timing of this breakdown coincides with exposure to large amplitude fluctuations in temperatures on or near the surface of bare soil (Quinlivan and Millington, 1962; Taylor and Rossiter, 1967; Taylor, 1984).

The association between temperature and hard-seed breakdown has been confirmed by subjecting legume seeds to controlled temperature conditions, and can be explained by a two stage process (Taylor, 1981). The first “preconditioning” stage, requires seed to be exposed to a period of high temperatures (>50°C). Hard-seed breakdown then occurs as preconditioned seeds are exposed to fluctuating temperature. Using this hypothesis, differences in the total heat sum required for sufficient preconditioning, or the parameters of the necessary temperature fluctuations could generate differences in hard-seed breakdown between species, genotypes or even location of seed production (Taylor, 2004). This hypothesis was supported by the hard-seed breakdown observed in *O. sativus* and *O. compressus* seeds with both field exposure and in controlled conditions that were reported in Chapter 6.
The formation of an impermeable seed coat upon drying has been shown to be under relatively simple Mendelian genetic control in a number of legume species, including *O. sativus* (Forbes and Wells, 1968; Donnelly *et al.*, 1972; Ladizinski, 1985; Chapter 3). Further, the rate of breakdown of hard-seededness has been shown to have a quantitative pattern of inheritance with medium to high levels of broad-sense heritability in *T. subterraneum* and *T. michelianum* Savi (Salisbury and Halloran, 1983; Nair *et al.*, 2004).

The ability to produce hard-seed in three different *O. sativus* genotypes was found to have three levels of expression and inheritance (Chapter 5, where SCP refers to Seed Coat Permeability);

SCP<sub>1</sub> - Consistent high levels of hard seed and dominant, which is observed in genotype A1.1 (and likely to be the expression found in the *O. sativus* cultivars Erica and Margurita).

SCP<sub>2</sub> - No hard seed and recessive to SCP<sub>1</sub>, which occurs in genotype D1 (and the expression observed in *O. sativus* cultivar Cadiz).

SCP<sub>3</sub> - Moderate and variable levels of hard seed, recessive to SCP<sub>1</sub> and SCP<sub>2</sub>, which is observed in genotype B1.2.

This pattern of inheritance could be explained by the action of different alleles at a single locus or by two (or more) genes that are closely linked. The ability to distinguish between these two alternatives is confounded by the dominance of the hard-seed expression of genotype A1.1 relative to genotype B1.2, and the variability of hard-seed expression by plants derived from genotype B1.2.
The aim of this study was to examine the seed moisture dynamics in seed lots of the three genotypes, A1.1, B1.2 and D1, and how this relates to changes in seed germination in response to temperature conditions. The pathways of moisture into these seeds after the various treatments will be examined through the use of an iron based dye. Changes in seed germination (and the pathways of moisture entry) will be tested after the temperature regimes that elicited different responses in hard-seed breakdown in the *O. sativus* cultivar Erica and the *O. compressus* cultivar Santorini in Chapter 6 (Figure 6.9, Figure 6.10). The same temperature regimes will also be applied to seed produced by F2 hybrids between the *O. sativus* genotypes to test the hypothesis that these treatments will enhance recognition of segregation within sibling groups.

### 7.2 Materials and Methods.

#### 7.2.1 Induced hard-seed breakdown.

The seed (in pods) of *O. sativus*, A1.1, B1.2 and D1, and F2 hybrids between these genotypes (Chapter 5) used in this experiment were produced in an open field as single spaced plants. The seed used to represent the parent genotypes was bulked from between 100 and 110 plants which were grown from self-pollinated seed of the actual parental plants. The seed produced by B1.2 x A1.1, A1.1 x D1, and D1 x B1.2 hybrids were based on individual F2 plants grown from seed harvested from self pollinated F1 hybrids.
Post harvest, the seeds were dried at 40°C for three days and then stored under dry, mild conditions for eight months until the experiments were conducted. For each genotype and treatment, a volumetric measure was used to sample between 100 and 200 pod segments (seeds). The pod samples were placed in small paper envelopes and were tested for germination and residual hard seed after either;
1. Storage under ambient laboratory temperature and relative humidity conditions (untreated).
2. Stored for 2 days at a constant 60°C.
3. Stored under conditions of fluctuating temperature (8 cycles over 4 days of alternating temperature between 15 and 50°C, Figure 6.9).
4. A combination of storage conditions; firstly 2 days at a constant 60°C, followed by the 8 cycles of alternating temperature.

Due to space and time limitations, a single sample of each F₂ plant was exposed to each storage treatment. The storage treatments were not also applied to each sibling group concurrently, but conducted 7 days apart. Four replicate samples from each of the three parent genotypes were treated with each run of storage treatments and each sibling group (providing a total of 12 replicates per parent and treatment).

After each storage treatment the seed was placed onto moist filter paper and stored at 15°C, with germination counted and seedlings removed 5, 10, and 15 days after wetting. The amount of hard-seed in each sample was based on the residual hard-seed after this 15 day germination test and expressed
as the number of residual hard seeds * 100 / (total germinated seeds + residual hard seeds).

7.2.2 Changes in seed moisture content.

Approximately 1000g of pod from the bulked sample of the parent genotypes A1.1, B1.2 and D1 was rubbed between ridged rubber matting to free the seed from the lomenta. The collected free seed was cleaned of debris and seed remaining in the pod, and the sample split using an Encott, 8 lane, 2-way sample divider into 24 representative lots per genotype, each measuring between 3 and 5 grams. All samples were weighed using an electronic balance with a precision of 1mg.

A subset of four samples was placed in an oven at 105°C and re-weighed periodically over 10 days. A hyperbolic relationship was fitted to the observed dehydration curve resulting from this treatment. The predicted equilibrium weight from the fitted equation was considered the dry weight of the seed samples and was used to estimate the dry matter and initial moisture content of the remaining samples.

Changes in moisture content of the seed samples after exposure to different storage conditions was calculated from the change in weight, relative to the estimated dry weight in the individual samples. In all cases, moisture content and change in moisture content is expressed as a percentage of seed dry weight. Dehydration curves were established by weighing the seed samples periodically after storage over dry silica gel in a sealed container at 20°C (5%
RH), and in ovens at constant temperatures of 40°C, 60°C, and 80°C. Hydration curves of the various seed samples were established by periodic weighing of the seed samples over a 14 day period while suspended over saturated saline solution in a sealed container (76% relative humidity). The samples used for observing seed hydration were previously stored at either ambient conditions (untreated) or at constant temperatures of 40°C, 60°C, and 80°C for 10 days.

In an alternative treatment, seed samples stored over silica gel for 10 days were split and stored for 14 days either over saline solution at 20°C or in an oven at 60°C. Germination tests on these samples were then conducted as described in 7.2.1, except the test was run for 60 days, with germination recorded and seedlings removed every 5 days.

7.2.3 Pathways of moisture entry into seeds of *O. sativus*.

Seed of Genotypes A1.1 and B1.2 were prepared by dehulling (in 7.2.2) and to ensure the samples contained only hard-seed, seeds were placed on moist filter paper for two weeks. The remaining, un-germinated seed was then air dried for five days. Seed of Genotype D1 was left untreated. The seed samples were split in two, with one set left as a control and the other exposed to 60°C for 2 days followed by exposure to eight cycles of 15°C/50°C (as in 7.2.1).

The pathway of moisture into the control and treated seed was observed through a binocular dissecting microscope while the seeds were soaking in a
0.003M ammonium ferrous sulphate hexahydrate solution. As a brown/black
coloured staining of the seed coat became evident, the stained seeds were
removed from the solution, rinsed in water and placed on filter paper to dry.
Images of the stained seeds were captured using a 6 megapixel digital
camera through the dissecting microscope. Internal staining was examined in
the same way after splitting the seed using a single sided razor blade.

7.2.4 Statistical analysis.
The hard-seed residue after germination for Genotypes A1.1, B1.2 and D1,
was compared by analysis of variance within each level of genotype and
seed treatment. The hard-seed residue of the F2 hybrid plants was compared
in an unbalanced analysis of variance (soft-seeded segregates were not
included in the analysis), with sibling group and seed treatment as factors.
The dry matter of the seed samples was estimated from the equilibrium
weight predicted by a hyperbolic relationship fitted by regression of seed
sample weight over time when stored at 105°C. The components of the
drying and re-hydration curves were estimated by linear and hyperbolic
regression of estimated seed moisture content against time. All analyses
were conducted using Genstat – Ninth Edition Version 9.2.0.152 (VSN
International Limited).
7.3 Results.

7.3.1 Changes in seed germination after high and fluctuating temperature treatments in three genotypes of O. sativus.

The three different temperature treatments, storage for 2 days at 60°C, 8 cycles of 15/50°C fluctuation, and the combined treatment, had no significant effect on the germination of the soft-seeded Genotype D1 (Table 7.1). However, storage at 60°C for 2 days increased the number of hard-seed (and decreased the proportion of germinated seed) compared to untreated seed in both Genotype A1.1 and B1.2. The change was slight and non-significant for Genotype A1.1 but the percentage of hard-seed for Genotype B1.2 almost doubled and was highly significant when compared to the untreated seed (Table 7.1). Alone, eight cycles of fluctuating temperature between 15 and 50°C also significantly increased the proportion of hard-seed in Genotype B1.2, but significantly reduced the hard-seed in Genotype A1.1.

When the high temperature treatment was followed by 8 cycles of fluctuating temperature there was a significant breakdown of hard-seed and an increase in germination of both Genotype A1.1 and B1.2 (Table 7.1). There was no significant difference (p<0.05) between any treatments or genotypes in germination behavior between the three different sets of samples undertaken 7 days apart.
Table 7.1. Mean residual hard-seed level (%) after various temperature treatments in three genotypes of *O. sativus*. Seeds were treated at 60°C for two days (preconditioned), given four days (8 cycles) of fluctuating temperature between 15 and 50°C (Figure 6.9), or given the preconditioning followed by fluctuating temperature treatments. Genotype D1 was not included in the analysis of variance across genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Temperature treatment</th>
<th>Lsd (p&lt;0.05) between treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>60°C</td>
</tr>
<tr>
<td>A1.1</td>
<td>89.8</td>
<td>91.2</td>
</tr>
<tr>
<td>B1.2</td>
<td>48.3</td>
<td>85.7</td>
</tr>
<tr>
<td>D1</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Lsd (p&lt;0.05)</td>
<td>3.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

7.3.2 *Dehydration and re-hydration in seed of three genotypes of O. sativus.*

Seed of the three genotypes, A1.1, B1.2, and D1, were at different levels of hydration at the time of assessment despite a similar history of post-harvest treatment and storage (Table 7.2). Even accounting for these initial differences, the rate of dehydration across all temperatures was greatest in Genotype D1 and slowest in Genotype A1.1. Seed dehydration in all cases was accurately predicted by a hyperbolic equation, with the seed moisture equilibrating with the storage atmosphere over time. This equilibrium weight was dependent on the severity of the drying conditions (Table 7.2, Figure 7.1).
Table 7.2. Initial and equilibrium seed moisture content (% of dry matter) of *O. sativus* genotypes predicted from hyperbolic drying curves observed over 10 days when stored in a dry atmosphere or at constant high temperature.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Initial (untreated)</th>
<th>Dry silica gel (20°C)</th>
<th>40°C</th>
<th>60°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1.1</td>
<td>6.6</td>
<td>4.5</td>
<td>4.2</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>B1.2</td>
<td>8.7</td>
<td>4.7</td>
<td>4.1</td>
<td>2.1</td>
<td>0.5</td>
</tr>
<tr>
<td>D1</td>
<td>9.2</td>
<td>4.5</td>
<td>4.5</td>
<td>2.7</td>
<td>0.4</td>
</tr>
<tr>
<td>LSD (p&lt;0.05)</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Hydration of seeds, over saturated saline solution (76% relative humidity), from the moisture contents resulting from the drying treatments were notably different in the three genotypes. Genotype D1 seed rapidly absorbed moisture regardless of the starting moisture content to reach between 12 and 15% on a dry weight basis within 10 days (Figure 7.2). The hydration of genotype A1.1 seeds showed two phases, the rapid absorption of moisture (within 2 hours), then a very slow linear increase with time. Genotype B1.2 seeds continued to absorb moisture when untreated in a similar pattern to Genotype D1, although it was considerably slower to reach equilibrium with the moist atmosphere. After the drying treatments, the pattern of hydration in genotype B1.2 seed was similar to genotype A1.1, showing a short period of rapid increase in moisture content followed by a slow rate of increase over time. However, the longer term absorption of moisture by seed of this genotype was more dependent on the extent of prior dehydration (severity of the drying treatment).
Figure 7.1. Seed moisture content of *O. sativus* Genotypes A1.1, B1.2 and D1 over time when stored over dry silica gel at 20 °C (x) or at constant 40°C (◊), 60°C (○) and 80°C (+) temperatures. Plotted points represent the mean of 4 replicate samples and the curves are the fitted hyperbolic relationship.
Figure 7.2. Seed moisture content (% of seed dry matter) of O. sativus Genotypes A1.1, B1.2 and D1 over time when exposed over saturated saline solution after storage under ambient conditions (x) or in ovens at 40°C (◊), 60°C (○) and 80°C (+). Plotted points represent the mean of 4 replicate samples and the curves the fitted hyperbolic relationship.
7.3.3 Change in germination with dehulling, seed drying and re-hydration in three genotypes of O. sativus.

The dehulling of seed to remove the woody pod was required to accurately measure changes in seed weight and estimate moisture content after the various temperature treatments. Unfortunately, this also appeared to have an effect on the level of seed germination in the hard-seeded Genotype A1.1. As untreated seed in the pods, the level of hard-seed was measured at 90% (untreated seed in Table 7.1). After removal from the pod, this level was reduced to, at best 54% (after storage over dry silica gel in Table 7.3). There was no significant change ($p \leq 0.05$) from this amount of hard-seed after the application of the drying or hydration treatments reported in Table 7.3. The germination of Genotype A1.1 seed, when it occurred, was rapid and mostly complete after 5 days of moist conditions (Table 7.3).

The high level of germination of the soft-seeded Genotype D1 did not change after dehulling or the different storage conditions (Table 7.4). In all samples, germination was mostly complete after 5 days of moist conditions and there were no residual hard-seeds. Unaccounted seeds were either due to rot or were abnormal seedlings.

The confounding effect of dehulling is more difficult to quantify for Genotype B1.2 seed. As untreated seed in the pod, hard-seed was measured at 48%, but this was enhanced by 2 days at 60°C to 86% (Table 7.1). These results are based on residual hard-seed after 15 days of moist conditions. In this experiment, untreated seed removed from the pod returned only 5% hard-
seed, however, germination continued to occur over a 60 day period (Table 7.3). The level of germination was reduced after drying these seeds, however, the protracted pattern of germination continued to be observed.

Table 7.3. Cumulative germination over time in dehulled seed samples of three genotypes of *O. sativus*, A1.1, B1.2 and D1, when stored under ambient conditions in a laboratory (untreated) or stored for 10 days over dry silica gel (20°C, 5% relative humidity). The samples stored over dry silica gel were then split and stored a further 14 days at either a constant 60°C or over saturated saline solution at 20°C in a sealed container (76% relative humidity).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Genotype</th>
<th>Seed moisture content after storage (% DM)</th>
<th>Cumulative germination after</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 days</td>
<td>15 days</td>
</tr>
<tr>
<td>Untreated (ambient)</td>
<td>A1.1</td>
<td>6.6</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>B1.2</td>
<td>8.7</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>9.2</td>
<td>96</td>
</tr>
<tr>
<td>Dried over silica gel</td>
<td>A1.1</td>
<td>4.5</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>B1.2</td>
<td>4.7</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>4.5</td>
<td>92</td>
</tr>
<tr>
<td>Stored over saline solution after drying over silica gel</td>
<td>A1.1</td>
<td>6.2</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>B1.2</td>
<td>6.6</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>15.9</td>
<td>89</td>
</tr>
<tr>
<td>Stored at 60°C after drying over silica gel</td>
<td>A1.1</td>
<td>2.5</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>B1.2</td>
<td>2.5</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>D1</td>
<td>2.5</td>
<td>90</td>
</tr>
</tbody>
</table>

7.3.4 *Pathways of moisture entry into seeds of O. sativus.*
Fresh, air dried seeds of the three O. sativus genotypes examined, A1.1, B1.2, and D1, differed slightly in colour and shape. Genotypes A1.1 and B1.2 produced pale beige coloured seed coats, while Genotype D1 produced slightly darker coloured seed coats. Some seeds of Genotype D1 had seed coats that were dark brown in colour, and had a cracked and stressed appearance. Even so, these seeds produced viable seedlings.

When fresh seeds of Genotype D1, were soaked in the Fe$^{++}$ solution, spots of dark staining were observed very rapidly (between 2 to 5 minutes), at points over the entire seed coat, but not at the hilum or lens regions (Figure 7.3). A few seeds also stained along cracks in the seed coat. Both of these types of staining occurred after all seed treatments, air dried, 2 days at 60$^\circ$ C and 2 days at 60$^\circ$ C followed by eight cycles of 15/50$^\circ$ C temperature fluctuation. The seeds of Genotype A1.1 did not stain with the Fe$^{++}$ solution when air dried or stored at 60$^\circ$C for two days. After 2 days at 60$^\circ$C followed by the eight cycles of temperature fluctuation, some seeds became stained at the lens region (Figure 7.3 and 7.4). This staining required at least 10 minutes of soaking before being observed.
Figure 7.3. Unstained (left) and stained (right) seed of Genotypes A1.1 stained at the lens (A), B1.2 stained at the lens and slightly over general seed coat (B), and D1 stained over the general seed coat (C). Seeds were soaked until staining was apparent in a 0.003M Fe++ solution after application of temperature treatments to induce hard-seed breakdown.
Figure 7.4. Longitudinally split unstained (left) and stained (right) seeds of Genotypes A1.1 (A), B1.2 (B), and D1 (C). Prior to splitting, stained seeds were soaked in 0.003 M Fe\(^{++}\) solution after application of temperature treatments to induce hard-seed breakdown.

Seeds of Genotype B1.2 showed a staining behavior between the two other genotypes. Air dried seeds developed staining at points randomly scattered over the general seed coat, apart from the lens and hilum regions (Figure 7.5). This staining required between 5 to 10 minutes of soaking to develop. In a similar way observed in Genotype D1, a few seeds rapidly developed
staining along splits in the seed coat. After storage at 60° C for 2 days, staining only occurred in seeds with split seed coats. However, after the combined treatment of storage at 60° C for 2 days followed by fluctuating temperature, seeds of Genotype B1.2 developed staining at the lens region in a similar way to Genotype A1.1 (Figure 7.3 and 7.4).

Figure 7.5. Unstained (top) and stained (bottom) seeds of Genotype B1.2 at approximately 8.7% moisture content. Seeds were stained by soaking in 0.003M Fe^{++} solution.
7.3.5 Changes in seed germination with high and fluctuating temperatures in F₂ hybrid progeny between three genotypes of O. sativus.

Confirming the results reported in Chapter 5, F₂ progeny with Genotype D₁ parentage (soft-seeded) segregated into 24 soft-seeded and 78 hard-seeded individuals when hybridized with Genotype A₁.₁, and 78 soft-seeded and 24 hard-seeded individuals when hybridised with Genotype B₁.₂. This pattern remained discernible after exposure of their seeds to constant high temperature or fluctuating temperature (Table 7.5).

There remained some hard-seed present in the samples of all the F₂ hybrid plants between Genotypes A₁.₁ and B₁.₂. After storage at 60°C, the numbers of hard-seeds in the samples of these hybrids increased if the untreated level of hard-seed was below 92%. Above this, the high temperature storage had a slightly negative effect on the amount of hard-seed.

Hard-seed producing A₁.₁ x D₁ F₂ plants had more than 87% hard-seed in the untreated sample (Table 7.5). However, some of the F₂ hybrids with Genotype B₁.₂ parentage, bred with either A₁.₁ or D₁, returned as little as 14% hard-seed in the untreated sample. F₂ plants with the different parentage were therefore not evenly distributed along the relationship between the amount of hard-seeds in untreated samples and those stored at 60°C. For the D₁ x B₁.₂ F₂ plants, exposure to fluctuating storage temperature also generated an increase in hard-seed (Table 7.5). However,
in F2 plants with A1.1 parentage, there was a general reduction in hard-seed in response to exposure to fluctuating temperature alone. Storing the seed samples at 60°C for two days, followed by eight cycles of fluctuating temperature produced a large range in the amount of hard-seed observed with all F2 hybrids, regardless of parentage (Table 7.5). The proportion of hard-seed in some of the hybrid plants with Genotype B1.2 parentage was higher after the combined treatment than in the untreated samples.

Table 7.5. Range and mean residual hard-seed level (%) after various temperature treatments of seed from hard-seeded F2 plants from hybridisation between three genotypes of O. sativus, A1.1, B1.2, and D1. Seeds were stored at 60°C for 2 days and the fluctuating temperature treatment (Figure 6.9) involved exposure of the seeds to 8 cycles between 15 and 50°C over 4 days. (Note soft-seeded F2 plants were omitted from the analysis of variance).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. F2 plants with hard-seed</th>
<th>Temperature treatment</th>
<th>Lsd (p&lt;0.05) between treats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Un-treated</td>
<td>60°C</td>
<td>Fluct. 15/50°C</td>
</tr>
<tr>
<td>B1.2 x A1.1</td>
<td>94</td>
<td>85.2 (21 - 99)</td>
<td>89.7 (73 – 99)</td>
</tr>
<tr>
<td>A1.1 x D1</td>
<td>78</td>
<td>94.4 (87 – 99)</td>
<td>93 (86 – 99)</td>
</tr>
<tr>
<td>D1 x B1.2</td>
<td>24</td>
<td>56.7 (14 – 87)</td>
<td>90.3 (77 – 96)</td>
</tr>
<tr>
<td>Lsd (p&lt;0.05)</td>
<td>between genotype</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lsd (p<0.05) between genotype
Expressing residual hard-seed after the combined treatment as a proportion of hard-seed after the high temperature treatment also produced broad distributions in all families (Figure 7.6). There was no relationship between the amounts of residual hard-seed and maturity measured as days to form an open flower in either the untreated samples or those receiving the combined temperature treatment (Figure 7.7).

Figure 7.6. Histogram of the proportion of plants returning different levels of residual hard-seed in $F_2$ hybrids between three $O. sativus$ genotypes, A1.1 x D1 (solid bars), B1.2 x A1.1 (shaded bars), and D1 x B1.2 (open bars) after the seed samples were stored at 60°C for two days followed by 8 cycles of fluctuating temperature (15-50°C) over 4 days.
Figure 7.7. Distribution of residual hard-seed in F$_2$ hybrids between three *O. sativus* genotypes, A1.1 x D1 (•), D1 x B1.2 (▲) and B1.2 x A1.1 (○), according to time to produce an open flower. A. Untreated samples. B. After the seed samples were stored at 60°C for two days followed by 8 cycles of fluctuating temperature (15-50°C) over 4 days.
7.4 Discussion.

The effect of imposing high and fluctuating temperature on the levels of hard-seed present in seed lots produced by the hard-seeded genotypes of *O. sativus*, A1.1 and B1.2, find close agreement to the two-stage model of hard-seed breakdown proposed by Taylor (1981). The dynamics of moisture exchange between these seeds and the environmental conditions to which they are exposed, specifically temperature and humidity, has provided further insight into the mechanism that may be behind these observations.

Recognizing a role for changes in the moisture content in seeds in the process of hard-seed breakdown may also explain some observations which otherwise are not easily accommodated within this two-stage model. These include reversal of high temperature pre-conditioning, re-establishment of the hard-seed state after induced breakdown, and greatly enhanced hard-seed breakdown when seed is slightly buried in the soil relative to being exposed on the soil surface (Hagon and Ballard, 1970; Janson and Ison, 1994; Smith *et al.*, 1996; Revell, 1997; Revell *et al.*, 1998; Taylor and Revell, 1999; Chapter 6).

According to the two-stage model, exposure to high temperature pre-conditions hard-seeds to be vulnerable to fluctuating temperatures, and in response to this combination seed coat impermeability is breached. In a field situation, pre-conditioning occurs through accumulated exposure to the high temperature period of diurnal fluctuations. Once sufficiently pre-conditioned, hard-seeds will then respond to appropriate diurnal fluctuations.
The two stage description matches the observations in this study using hard-seed produced by *O. sativus* genotype A1.1. The initial amount of hard-seed present in this seed sample was high (90%) and was not affected by two days of storage at 60°C (91%). However, eight cycles of temperature fluctuation between 15°C and 50°C significantly increased germination and reduced the number of residual hard-seeds to 82%. The high temperature pre-conditioning required by these seeds was accumulated during the 15°C to 50°C fluctuations, however this can be effectively substituted by a relatively short period of constant high temperature. Breakdown of hard-seed was greatly enhanced if the temperature fluctuation treatment was preceded by two days of storage at 60°C (Table 7.1).

Hard-seed of *O. sativus* Genotype B1.2 did not match the two-stage model in response to the temperature treatments (Table 7.1). The seed of this genotype showed moderate levels of hard-seed when untreated and elevated levels of hard-seed after two days at 60°C (48% compared to 86% respectively). Exposure to fluctuating temperatures alone also elevated the amount of hard-seed detected in the sample (80%). However, the combination of two days at 60°C followed by 8 cycles of fluctuating temperature significantly reduced residual hard-seed compared to all other treatments, including the untreated sample.

The results of this study produced some problems for interpretation and highlight the difficulty in conducting meaningful studies of seed dormancy.
What is the appropriate baseline level of hard-seed on which to relate changes in response to temperature treatments or exposure to field conditions? The level expressed after exposure to two days of 60°C may be most appropriate, as this may represent potential of the seed to become hard. Alternatively, seeds exposed in the field over summer may require frequent monitoring to capture increases in hard-seed content in response to high soil temperatures.

The discrepancy in response to the various treatments between the two different hard-seed producing genotypes could be explained by their respective seed moisture contents. Although the seed samples of the two had similar histories of post harvest storage, genotype B1.2 had higher initial moisture content than genotype A1.1. At the moisture content measured in genotype B1.2 (8.7% of seed dry matter), some of the seeds appeared in a state of reversible hard-seededness. Prolonged exposure to moisture in the germination test resulted in protracted germination, with the majority of the seed germinated after 60 days (Table 7.3). Likewise, these seeds continued to absorb moisture when stored in a moist atmosphere over a saturated saline solution and may even reach comparable levels with soft-seeds given time (Figure 7.2).

Observations on the sites of moisture penetration showed that the general seed coat in the Genotype B1.2 was not completely impermeable at the 8.7% moisture content (Figure 7.5). Storage in a mild dry environment (over dry silica gel) or at higher temperature reduced seed moisture content and
increased the amount of hard-seed detected in the seed samples. It can be concluded that, under these conditions, the seed dried past the critical moisture level for the imposition of “irreversible” hard-seed (for this genotype and seed lot) (Quinlivan, 1971).

The seeds in the untreated samples of Genotype A1.1 (at 6.6% moisture content), were presumably already below the particular critical level in moisture content for the establishment of irreversible hard-seed. Seed at this (and lower) moisture content only slowly absorbed moisture after a small rapid increase when placed in moist storage (Figure 7.2). These results confirm the concept of hard-seed irreversibility with further drying past this critical moisture content in this genotype. However, even the dried seeds of genotype B1.2 appear to absorb moisture when stored in a moist environment. Therefore, the concept of critical moisture content for the imposition of irreversible hard-seededness has some limitations when referring to genotype B1.2, and appears transitory compared to genotype A1.1.

The breakdown of hard-seed formed by genotype B1.2 appears to take two forms. Once dried (and pre-conditioned?) by the high temperatures that occur at the soil surface over the summer months, the established hard-seededness will breakdown in response to exposure to fluctuating temperature conditions. This is likely to be rapid, according to the response of this genotype’s seed samples to eight cycles of temperature fluctuation after high temperature pre-conditioning (Table 7.1). In seeds where the hard-
seed state has been established, but seeds are not exposed to or broken down by fluctuating temperature, hard-seedness may be reversed by slow re-absorption of moisture. This needs to be confirmed by further study, but conceptually this character could have agricultural application.

The results obtained in this study suggest that the pre-conditioning of hard-seeds with high temperature may be related to the drying of seeds to a moisture content that places them in a range that will then respond to fluctuating temperatures. The response to fluctuating temperature may also be related to the associated changes in relative humidity and small changes in seed moisture content.

Dry hard-seeds in this study showed a rapid increase in seed moisture content within two hours of being placed in a humid atmosphere. Hyde (1954) found the same observation when he elegantly demonstrated the “one-way” valve properties of the hilum structure of legume seeds. This structure was shown to open under dry conditions relative to the seed moisture content, and to close when placed in a moist environment. Hyde speculated that moisture uptake occurred in the cells of the hilum structure and the resulting expansion closed the valve. However, other specialized parts of the seed coat, particularly the lens structure, may absorb moisture during this process in preference to the seed as a whole unit. Repeated cycles of expansion and contraction of the cells under the lens palisade layer, due to this uptake and loss of moisture in a diurnal cycle, may create the pressure necessary to fracture and even open the central lens fissure.
During continued exposure to moist conditions, where the hilum is unlikely to open again, the differential in moisture content of the hilum and lens may slowly equilibrate with the rest of the seed whereby the preconditioned state or even hard-seed breakdown is reversed.

Moisture entry through structures other than the lens has been proposed to explain slow imbibition in *O. compressus* (Taylor, 2004). In a similar concept for *Sophora alopecuroides* L. seeds subjected to hard-seed breakdown treatments, it was concluded that moisture entry first occurred through the hilum (Hu *et al.*, 2008). This activated the lens, presumably through expansion, to form a breach allowing further moisture entry into the seed. Although these two proposals relate to moisture entry leading to imbibitions, the same mechanism could be operating to cause a facture through diurnal fluctuation in moisture.

The soft-seeded genotype of *O. sativus*, D1, produced seed that freely interacted with storage conditions and, at any given time, the moisture content of these seeds generally reflected the relative humidity of most recent storage. This property must be due to these seeds possessing a highly permeable seed coat and this permeability is not affected by storage at high temperatures or the state of seed hydration. Due to this seed coat permeability, the seeds of genotype D1 (and presumably all soft-seeded *O. sativus* genotypes) rapidly imbibe and germinate when the seeds are placed in moist conditions.
The expansion and contraction of the seed embryo associated with rapid changes in seed hydration may create stress on the embryo and thereby reduce seed viability over time. Seed of the soft-seeded cultivar Cadiz was found to develop abnormal seedlings when the seeds were exposed on the surface of soil in the field (Chapter 6). The abnormality was usually a separation of the cotyledons and the radical, and it may be caused by a fracture induced by the stress of changes in seed hydration.

Although $F_2$ hybrids with B1.2 parentage could show much greater hard-seed content in samples after storage at 60°C, all hybrids considered soft-seeded did not show any change in germination. This confirmed the segregation of hard and soft-seed production in $F_2$ hybrids between the three genotypes, A1.1 x D1, B1.2 x A1.1, and D1 x B1.2, reported in Chapter 5. Among the remaining hard-seed producing $F_2$ hybrids there were insufficient numbers, particularly of the D1 x B1.2, to form clear groups according to their respective changes in the amount of hard-seed in untreated or temperature treated samples (two days at 60°C, eight cycles of 15/50°C or two days at 60°C followed by eight cycles of 15/50°C). These seed samples were not assessed for moisture content and this co-variant may have improved discrimination.

The distribution of hybrids for hard-seed residual after exposure to two days of 60°C followed by eight cycles of temperature fluctuation may be the most reliable measure of diversity and possible heritability of hard-seed breakdown. Based on this response, the soft-seeded Genotype D1 did not
strongly influence hard-seed breakdown when hybridised with the dominant hard-seed genotype A1.1. However, the D1 x B1.2 F<sub>2</sub> hybrids generally had greater levels of residual hard-seed than genotype B1.2. This could be formed into two groups centering around 30% and 60% hard-seed. This suggests that although genotype D1 produces soft-seed, it can influence hard-seed breakdown behavior in hard-seeded hybrid progeny. All B1.2 x A1.1 F<sub>2</sub> hybrids showed some level of hard-seed residue after the combined temperature treatment, and may have shown segregation to two groups; a broad distribution of 75 plants around 67% and 20 plants around 27%.

The hypothesis that the relative response to the various treatments used in this study could elicit clear segregation among hard-seed producing hybrids was not confirmed by the results obtained. However, the procedures could be refined to better discriminate the difference between requirement for dehydration to establish irreversible seed coat impermeability, and the accrual of pre-conditioning heat units required to initiate the response to fluctuating temperature.
Chapter 8. Modelling changes in seed germination of *Ornithopus sativus* Brot. populations over time: Effect of hard-seed inheritance, cross pollination and hard-seed breakdown.

8.1 Introduction

The production of dormant seed is a survival strategy observed in many plant species and the dynamics of this dormancy has implications on the ecology of species in both natural and managed settings. As a survival strategy it has two main functions. The release from dormancy may synchronise the timing of seed germination with environmental or edaphic conditions most favourable for plant establishment and eventual reproduction (Bewley and Black, 1982; Bradbeer, 1988; Baskin and Baskin, 2001). Alternatively, longer lasting seed dormancy can disperse seed germination over a number of seasons for plant establishment and eventual reproduction.

The longer-term role of seed dormancy is particularly important to the survival of short lived species where reproductive success is uncertain in every seasonal cycle (Taylor, 1972; Norman *et al.*, 1998; Norman *et al.*, 2002; Taylor, 2005). Failure to achieve reproduction may be due to biological pressures, such as animal, insect or disease predation; climatic variability in
both the timing and amount of seasonal rainfall; or external intervention such as human cultivation or fire (Jansen, 1969). Seed dormancy may also function as a strategy to reduce parent-offspring competition in longer lived species with multiple opportunities for reproduction across a life cycle (Ellner, 1986; Kobayashi and Yamamura, 2000).

The production of dormant seeds by a species can have both positive and negative effects in agricultural systems. Seed dormancy can be a problem when the species is undesirable in a particular situation (“One year of seeds, ten years of weeds”). However, the capacity of a species to self regenerate can be useful in other settings. Species that spontaneously establish from a persistent pool of seed in the soil can provide animal fodder as well as ameliorate and stabilize the soil when the land is not sown to a crop. This principle is applied in southern Australian farming systems using T. subterraneum and several other annual legume species.

The production of fodder and grain crops generally involves repeated harvesting and sowing of seed. With this management, seed dormancy can be a hindrance to successful cultivation. Reliable and timely plant establishment is often required to optimize yield and for the sown species to be competitive with weeds. Under cultivation there may also be selective pressure for the development of genotypes with non-dormant seeds. Only the seeds which germinate can form adult plants that in turn produce seed to be harvested and sown for the next generation of crop.
As a result of the selective pressures of cultivation, and through breeding programs, many domesticated plant species that are propagated by seed produce little or no dormant seed (Lester, 1989). This includes legumes (Leguminosae subfamily Papilionoideae) where the principal form of seed dormancy is imposed by an impermeable seed coat (testa). As the seed coat is derived from maternal tissue (cells of the ovule integument), maternal phenotype controls seed coat characteristics. Legume species and genotypes that produce seeds with an impermeable seed coat are referred to as hard-seeded and those where seed coat impermeability does not develop are called soft-seeded.

*Ornithopus sativus* Brot. is a legume species that is cultivated for fodder and as a soil ameliorant. The cultivated forms of *O. sativus* do not normally produce hard-seed. However, hard-seed producing genotypes can be isolated from predominantly soft-seeded populations by mass selection (Chapter 3). Hybridisation studies using this species have shown that the capacity to produce hard-seed may be inherited under the influence of a single gene. However, in the *O. sativus* populations studied there appeared to be both recessive and dominant inheritance of hard-seededness relative to soft-seededness (Chapter 5). Simple “Mendalian” inheritance for hard-seed and soft-seed production has also been found in *Lupinus angustifolius*, *L. luteus*, *Lens culinaris*, *Glycine max*, *Vicia faba* and *V. sativa x V. angustifolia* hybrids (Lebedeff, 1947; Forbes and Wells, 1968; Gladstones, 1970; Donnelly, 1971; Donnelly et al., 1972; Kilen and Hartwig, 1978; Ladizinski, 1985).
The aim of this chapter is to model the changes in gene frequency that could occur in populations when genotypes that produce either dormant or non-dormant seed are both present. The model assumes simple inheritance of a single gene (or multiple but linked genes) and examines several factors whose influences and interactions are difficult to understand without mapping population change over several generations. These factors include maternal phenotype control on seed coat characteristics, dominance, cross-pollination, accrued reserves of dormant seed (and subsequent release from dormancy) and the incidence of failed reproduction. This will provide insights into how hard-seed production of a mixed population may change depending on management decisions, and predict the efficiency of different selection procedures to change the level of seed dormancy in a population.

8.2 Methods.

Two scenarios are considered in a stepwise generational model to examine the dynamics of hard-seed dormancy in a legume population (Figure 8.1). Both involve an initial establishment using scarified seed (non-dormant). The first scenario involves seasonal harvesting of seed and re-seeding (without scarification) on “new” land, where dormant reserves of seed in the soil do not contribute to the reproducing population. In the second scenario, the population is considered in situ, and through release from seed dormancy, soil seed reserves contribute to annual reproducing populations. A potential yield of 1 or 0 is applied to each cycle to simulate successful or failed reproduction. These are two common scenarios in agriculture.
Figure 8.1. Flow chart representing the major influences on genotypic frequencies relating to heritable seed dormancy (hard-seed production) in a population harvested for re-sowing (A) or self regenerating *in situ* (B).

**Generation 1**
Population with known frequencies of genotypes established from scarified seed (Table 8.1, scarified)

- Self pollinated
- Cross pollinated

Seed at end of season 1

Seed harvested

**Generation 2**
Germination of seed according to maternal genotype (Table 8.1, unscarified)

- Self poll’n
- Cross poll’n

Seed at end of season 2

A

Seed in soil bank

Loss of viability over summer

Germination of soft seed and softened hard-seed according to maternal phenotype (Table 8.1, after dormancy release)

- Self poll’n
- Cross poll’n

Seed at end of season 2 (0 or 1)

B
In both cases the populations are described using the following assumptions;


2. No difference in reproductive potential between genotypes.

3. Potential population yield is not influenced by density.

4. Hard-seed dormancy is determined by the maternal phenotype and not influenced by progeny genotype.

5. No difference between cross pollinated and self pollinationed plants in yield or fitness and there is no self incompatibility for fertilisation.

The model is constructed as spreadsheet workbook, where the reproducing genotypes present in each successive generation relates to their respective maternal genotype for possible mating combinations and maternal phenotype for seed germination (Figure 8.2). The model uses three alternative alleles of the same locus that control seed coat permeability (SCP$_{1,2,3}$) (Chapter 5). The effect of the relative dominance of these alleles on seed germination and hard-seed breakdown is shown in Table 8.1.

With three alleles, segregation and cross pollination produces 24 different combinations of maternal and progeny genotypes. With only self pollination this is reduced to 12 combinations. An example of the equations used for calculating mating and the frequency of genotypes within a seasonal generation is shown in Figure 8.2. Within the model any range in cross pollination can be applied, however for this study 0% and 25% are examined (25% allogamic cross pollination was estimated in O. sativus, Chapter 4).
The initial introduction (sowing) into the model is determined as a mixture of homozygous and/or heterozygous genotypes, but it is specified that in the first generation the seed is scarified and not subject to the maternal phenotype (Table 8.1). In subsequent generations, the germination of un-scarified seed is applied to harvested and re-sown seed, while breakdown relates to the proportion of seed released each season from an accrued pool of dormant seed. The values in Table 1 are based on data relevant to *O. sativus* presented in previous chapters of this thesis (Chapters 5, 6, and 7) although the model is designed to accommodate to interact with any specified values. An additional factor considered in the *in situ* model is the loss of viability in seed produced by soft-seeded maternal phenotypes (expressed as seed present after loss, Table 8.1). This variable could also be used to simulate selection for a particular seed type.

The expressions associated with the three proposed seed coat permeability (SCP) alleles are as follows;

SCP<sub>1</sub> – Dominant, producing high levels of hard seed at harvest and moderate amounts of seed released from dormancy in an annual cycle *in situ*.

SCP<sub>2</sub> - No hard seed and recessive to SCP<sub>1</sub>.

SCP<sub>3</sub> - Moderate levels of hard seed at harvest, and high levels of seed released from dormancy in an annual cycle *in situ*. The lowest level of hard-seed recorded from this type of *O. sativus* was 10% (at 8% moisture content and after 60 days of moist conditions) while the highest was 85% (after
drying to below 4% moisture content) (Chapter 7). Recessive to SCP_1 and SCP_2.

Table 8.1. Scarified and un-scarified seed germination (1 = 100%) of harvested seed and the rate of release from a dormant pool and loss of seed in situ, according to maternal seed coat permeability genotype and phenotype. SCP_{1,1} denotes a homozygous genotype for SCP_1 hard-seed and SCP_{1,2} is a heterozygous genotype of SCP_1 and SCP_2 alleles. Alternative variables used in some scenarios are sown in brackets.

<table>
<thead>
<tr>
<th>Germination of seed</th>
<th>Germination of seed for maternal genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCP_{1,1}</td>
</tr>
<tr>
<td>Scarified</td>
<td>1</td>
</tr>
<tr>
<td>Un-scarified</td>
<td>0.1</td>
</tr>
<tr>
<td>After dormancy release</td>
<td>0.4</td>
</tr>
<tr>
<td>Seed after loss</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 8.2. Equations between related spreadsheets to model frequencies of genotype in a population with three alternative alleles controlling seed germination and dormancy in seasonal generation N. (R:C denote row and column for navigation in spreadsheet).

A Population present at the end of a growing season.

<table>
<thead>
<tr>
<th>R:C</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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</thead>
<tbody>
<tr>
<td>a</td>
<td>Maternal Genotype</td>
<td>SCP,1,1</td>
<td>SCP,1,2</td>
<td>SCP,1,3</td>
<td>SCP,2,2</td>
<td>SCP,2,3</td>
<td>SCP,3,1</td>
<td>SCP,3,2</td>
<td>SCP,3,3</td>
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<tr>
<td>b</td>
<td>Progeny Genotype</td>
<td>SCP,1,1</td>
<td>SCP,1,2</td>
<td>SCP,1,3</td>
<td>SCP,1,2</td>
<td>SCP,2,2</td>
<td>SCP,1,3</td>
<td>SCP,2,3</td>
<td>Etc.</td>
</tr>
<tr>
<td>c</td>
<td>N-1</td>
<td>Frequency in N-1 sheet G Season production (harvest and re-sow) or sheet G Season production + sheet H Dormant seed pool</td>
<td></td>
<td></td>
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</tbody>
</table>

B Potentially germinating population at start of growing season

<table>
<thead>
<tr>
<th>R:C</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Maternal Genotype</td>
<td>SCP,1,1</td>
<td>SCP,1,2</td>
<td>SCP,1,3</td>
<td>SCP,2,2</td>
<td>SCP,2,3</td>
<td>SCP,3,1</td>
<td>SCP,3,2</td>
<td>SCP,3,3</td>
</tr>
<tr>
<td>b</td>
<td>Progeny Genotype</td>
<td>SCP,1,1</td>
<td>SCP,1,2</td>
<td>SCP,1,3</td>
<td>SCP,1,2</td>
<td>SCP,2,2</td>
<td>SCP,1,3</td>
<td>SCP,2,3</td>
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</tr>
<tr>
<td>d</td>
<td>N</td>
<td>Frequency in N-1 from sheet B Population present x Germination associated with maternal phenotype and seed condition (Table 8.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</table>

C Established population after seed loss

<table>
<thead>
<tr>
<th>R:C</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Maternal Genotype</td>
<td>SCP,1,1</td>
<td>SCP,1,2</td>
<td>SCP,1,3</td>
<td>SCP,2,2</td>
<td>SCP,2,3</td>
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<tr>
<td>b</td>
<td>Progeny Genotype</td>
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<td>SCP,1,2</td>
<td>SCP,1,3</td>
<td>SCP,1,2</td>
<td>SCP,2,2</td>
<td>SCP,1,3</td>
<td>SCP,2,3</td>
<td>Etc.</td>
</tr>
<tr>
<td>d</td>
<td>N</td>
<td>Frequency in N from sheet C Established population x Seed after loss associated with maternal phenotype and seed condition (Table 8.1)</td>
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</table>
**Figure 8.2. Cont.**

### D Mating of genotypes

<table>
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<th>4</th>
<th>5</th>
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<th>8</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>Genotype</td>
<td>SCP₁₁</td>
<td>SCP₁₂</td>
<td>SCP₁₃</td>
<td>SCP₂₂</td>
<td>SCP₂₃</td>
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<td></td>
</tr>
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</table>

| d   | N | Sum of SCP₁₁ in sheet C (3 poss.) | Sum of SCP₁₂ in sheet C (6 poss.) | Sum of SCP₁₂ in sheet C (6 poss.) | Sum of SCP₁₂ in sheet C (6 poss.) | Sum of SCP₁₂ in sheet C (6 poss.) | Sum of all frequencies |

### E Mating of genotypes (standardized to equal 1)

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<tr>
<th>R:C</th>
<th>2</th>
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<tr>
<td>a</td>
<td>Genotype</td>
<td>SCP₁₁</td>
<td>SCP₁₂</td>
<td>SCP₁₃</td>
<td>SCP₂₂</td>
<td>SCP₂₃</td>
<td>SCP₃₃</td>
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| d   | N | Relevant cell in sheet D divided by the sum of all frequencies (sheet D d9) |

### F Allele frequencies in mating population

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<tr>
<td>a</td>
<td>Allele</td>
<td>SCP₁</td>
<td>SCP₂</td>
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| d   | N | ((SCP₁₁*2)+SCP₁₂+SCP₁₃)/2 from sheet E | ((SCP₂₂*2)+SCP₁₂+SCP₂₃)/2 from sheet E | ((SCP₃₃*2)+SCP₁₃+SCP₂₃)/2 from sheet E |   |
### G Season production

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<tr>
<td>a</td>
<td>Maternal Genotype</td>
<td>SCP₁₁</td>
<td>SCP₁₂</td>
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<tr>
<td>b</td>
<td>Progeny Genotype</td>
<td>SCP₁₁</td>
<td>SCP₁₂</td>
<td>SCP₁₃</td>
<td>SCP₁₁</td>
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<tr>
<td>d</td>
<td>N</td>
<td><a href="#">Equations for calculating frequency of self and cross pollinated progeny</a></td>
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For each relevant cell calculate frequency of self and cross pollinated progeny for each maternal genotype. There are 4 basic equations used, for example –

- **SCP₁₁ to SCP₁₁**: \((\text{sheet } E d₃ \times (1 - \text{cross pollination})) + (\text{sheet } E d₃ \times \text{sheet } F d₃ \times \text{cross pollination})) \times \text{Seasonal yield (1 or 0)}\)
- **SCP₁₁ to SCP₁₂**: \((\text{sheet } E d₃ \times \text{sheet } F d₃ \times \text{cross pollination})) \times \text{Seasonal yield (1 or 0)}\)
- **SCP₁₂ to SCP₁₁**: \((\text{sheet } E d₄ \times 0.25 \times (1 - \text{cross pollination})) + (\text{sheet } E d₄ \times \text{sheet } F d₃ \times 0.5 \times \text{cross pollination})) \times \text{Seasonal yield (1 or 0)}\)
- **SCP₁₂ to SCP₁₂**: \((\text{sheet } E d₄ \times 0.5 \times (1 - \text{cross pollination} )) + (\text{sheet } E d₄ \times 0.5 \times \text{sheet } F d₃ \times \text{cross pollination}) + (\text{sheet } E d₄ \times 0.5 \times \text{sheet } F c₃ \times \text{cross pollination})) \times \text{Seasonal yield (1 or 0)}\)

### H Dormant seed pool

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<tr>
<td>a</td>
<td>Maternal Genotype</td>
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<tr>
<td>b</td>
<td>Progeny Genotype</td>
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<tr>
<td>d</td>
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Frequency in N-1 from sheet B Population present – (sheet B Population present x Germination associated with maternal phenotype and seed condition) (Table 8.1)
8.3 Results

8.3.1 Harvesting and re-sowing mixed populations of hard and soft-seeded genotypes.

In the simplest scenario, where there is no cross pollination and no contribution of seedlings from a soil seed reserve, the selective pressure is strongly against the presence of seed dormancy in a population (Figure 8.3). The intensity of selection is dependent on the relative germination between the hard-seeded and soft-seeded phenotypes. For instance, if the hard and soft-seeded phenotypes have 10% and 100% germination, the presence of the hard-seed allele is reduced by a factor of 0.1 with each reproductive cycle in the absence of cross pollination. The recessive SCP3 is predicted to be more persistent in the population, due to the lower amount of hard-seed (at high moisture content) believed to be associated with this type of genotype in *O. sativus* (Figure 8.3). If seed of genotype SCP3,3 was sufficiently dried to express high levels of hard-seed, this advantage would be lost and the SCP3 allele would be removed from the population almost as rapidly as SCP1.

Cross pollination reduces the rate of removal of both the dominant and recessive expression of the hard-seed trait in the harvest and re-sow model (Figure 8.3). At 25% cross pollination the effect is only slight for the dominant SCP1 allele and more pronounced for the recessive allele SCP3, due to the soft-seeded phenotype of the SCP2,3 genotype.
Figure 8.3. Changes in the frequency of three alleles (SCP₁ ■, SCP₂ ▲, and SCP₃ ○) associated with seed coat permeability under repeated harvesting of seed and re-sowing with un-scarified seed. Solid line denotes no cross pollination, dashed line 25% allogamic cross pollination. Initial populations established as scarified seed with equal frequencies of homozygous genotypes (i.e. 1/3 SCP₁₁, 1/3 SCP₂₂, and 1/3 SCP₃₃). The germination of the various genotypes is shown in Table 8.1.

8.3.2 *In situ changes in mixed populations of hard and soft-seeded genotypes with reliable seasonal production of seed.*

In a self sustaining population the frequency of alleles associated with hard and soft-seededness will reach a balance in situ once the input and output of seed from the dormant seed pool reaches equilibrium (Figure 8.4). Until equilibrium is achieved the soft-seeded allele SCP₂ and the greater rate of dormancy breakdown with SCP₃ are favoured with respect to SCP₁. With reproductive success in each production cycle there is little effect of inheritance or cross pollination (Figure 8.4a).
The fitness of the soft-seed associated allele SCP₂ is reduced, however, if the loss of seed viability with seed remaining on the soil is taken into consideration. If the soft-seeded genotypes SCP₂,₂ and SCP₂,₃ lose 50% viability, the hard-seeded alleles are favoured (Figure 8.4b) and SCP₂ is removed from the population. With the successful production of seed in each production cycle, SCP₃ dominates the population due to the greater rate of dormancy breakdown.

Cross pollination provides a buffer to the selection pressure against SCP₂, due to the protection of the SCP₂ allele in the hard-seeded SCP₁,₂ phenotype. However, it also slightly favours the hard-seed allele SCP₁ compared to SCP₃ due to the loss of seed viability of the soft-seeded phenotype of SCP₂,₃ (Figure 8.4).
Figure 8.4. Change in the frequency of three alleles (SCP₁ ■, SCP₂ ▲, and SCP₃ ○) associated with seed coat permeability in a self sustaining population in situ with reproductive successs in each production cycle and no seed loss (A) or 50% loss of seed in the soft-seeded phenotypes SCP₂,2 and SCP₂,3 in the summer/autumn before the growing season (B). Solid line denotes no cross pollination, dashed line 25% allogamic cross pollination. Initial populations established as equal frequencies of homozygous genotypes and scarified seed (i.e. 1/3 SCP₁,1, 1/3 SCP₂,2, and 1/3 SCP₃,3). The germination of the various genotypes is shown in Table 8.1.
8.3.3 *In situ changes in mixed populations of hard and soft-seeded genotypes with failure to achieve seasonal production of seed in every cycle.*

The advantage of dormant seed production is seen in the event of reproductive failure (Figure 8.5). The selective pressure towards hard-seed is strongest when there is no cross pollination as one reproductive failure removes all soft-seed associated alleles from the population. Likewise, the accelerated breakdown of dormancy associated with the hard-seeded allele SCP₃ produces reduced fitness relative to SCP₁. The degree of selection favouring SCP₁ increases with the frequency of failure to achieve reproduction (Figure 8.5).

Cross pollination has a strong influence on reducing the selective pressure under frequent failure to reproduce, with both the soft-seeded SCP₂ allele and the rapid dormancy breakdown associated with the SCP₃ allele (Figure 8.5). This increased persistence is driven by protection in the hard-seeded phenotype of SCP₁,₂ and SCP₁,₃ genotypes.
Figure 8.5. Changes in the frequency of three alleles (SCP\textsubscript{1} ■, SCP\textsubscript{2} ▲, and SCP\textsubscript{3} ○) associated with seed coat permeability in a self sustaining population \textit{in situ} with reproductive failure every second (A) and every fourth seasons (B). Solid line denotes no cross pollination, dashed line 25% allogamic cross pollination. Initial populations established as equal frequencies of homozygous genotypes and scarified seed (i.e. 1/3 SCP\textsubscript{1,1}, 1/3 SCP\textsubscript{2,2}, and 1/3 SCP\textsubscript{3,3}). The germination of the various genotypes is shown in Table 8.1.
8.4 Discussion.

The described model for hard/soft seed production demonstrates how there will be no or a low incidence of seed dormancy in a population that is harvested then cultivated each year from seed. It also demonstrates the advantages of seed dormancy in natural settings where reproductive failure occurs. Although the possibility of co-existence of the two traits is influenced by inheritance and cross-pollination, the hard-seed trait is unlikely to persist within a cultivated population without intervention. This intervention would require crop management that ensures a high level of germination of hard-seeded genotypes, through dehulling and scarification.

When a mixture of heritable hard and soft-seed characters is present within a population the model predicts the hard-seed trait can be rapidly removed from a population in a scenario of sowing, harvesting and re-sowing on new land. The selective pressure is largely determined by the dominant maternal phenotype. When hard-seed has a dominant inheritance, it is rapidly removed from the population due to the low germination of seed produced by both homozygous and heterozygous hard-seeded maternal genotypes. Removal of hard-seed dormancy by treating seed prior to sowing is essential to maintain this character in a population (of mixed seed types) with this inheritance and management (i.e. by scarification).

A hard-seed character is more persistent in a harvested and re-sown population, if it is inherited recessively because the hard-seed allele is carried in the population in non-dormant seeds produced by heterozygote
(soft-seed producing) maternal plants (Figure 8.3). This effect is pronounced with the occurrence of cross pollination. In this model the germination of the recessive hard-seed associated allele was relatively high compared to the dominant allele (60% vs 10%). This was based on data presented in previous chapters which showed high levels of germination are possible in the recessive expression of hard-seed (Genotype B1.2) when the seed is at high moisture content. Intervention to remove dormancy prior to sowing may not be as necessary with this type of hard-seed if high moisture content could be maintained after harvest and during storage.

Hard and soft-seed alleles can remain in balance in a self-regenerating situation once the hard-seed pool in the soil reaches equilibrium (for fresh seed input and release from dormancy). The selective pressure would shift to favor hard-seed production if there was any loss of soft-seeds through untimely germination, loss of viability or particularly if reproduction occasionally fails (Figure 8.4, 8.5). The selective pressure towards hard-seed in these situations is virtually the opposite of the harvest and re-sowing scenario. A soft-seed allele can be carried in a population when it is recessively inherited, due to the production of hard-seed by a heterozygous maternal plant. In contrast, a dominant soft-seed allele can be rapidly removed from a population with reproductive failure unless substantial cross-pollination occurs.

The two hard-seed alleles as described in this model, in concept, produce some interesting interaction, particularly in the presence of cross-pollination.
In longer pasture cereal rotations (equivalent to infrequent reproductive failure, Figure 8.6b), mixtures of these genotypes could provide very dynamic pastures that would become dense quickly in the pasture years after cropping. The accelerated breakdown which described SCP₃ was applied because induced softening of hard-seed of Genotype B1.2 (recessively inherited) was twice the rate of Genotype A1.1 (dominantly inherited). Further evaluation of hard-seed breakdown in the field would be needed to further investigate this hypothesis.
Chapter 9. General Discussion.

*Ornithopus sativus* has been described as a self-pollinating, annual legume with little or no seed dormancy (hard-seed). This study has found that the description of *O. sativus* should be expanded to include a capacity for both facultative cross pollination and high levels of seed dormancy. The development of hard-seeds was found to have two expressions that differed in their pattern of inheritance. The observation of two different hard-seed behaviours in *O. sativus* may also have relevance when studying hard-seed in other legumes.

The initiation of flowering in *O. sativus* is promoted by an accumulation of growing days, modified by delaying effect of short day length. A genotype that is relatively insensitive to photoperiod was identified which rapidly develops to anthesis regardless of when it is sown during the year. The availability of genotypes with both early flowering, when sown in late autumn/winter, and production of dormant seeds greatly expands the potential of *O. sativus* for agricultural purposes.

9.1 Cross pollination in *O. sativus* and implications for genotype selection and purity.

There is no apparent barrier to self fertilisation in *O. sativus* and anthers dehisce before the flower opens fully. These observations, combined with a paucity of definitive morphological characters to distinguish between
genotypes, have promoted the conclusion that autogamous self pollination is the predominant breeding system of this species (and other species of *Ornithopus*) (Wojciechowski, 1971; 1972a; 1972b; Fu *et al.*, 1994).

Segregation away from the flower colour of maternal plants has been cited as evidence of cross pollination in *O. sativus* (Klinkowski, 1942; Pfeffer, 1963). However, the extent of cross pollination has not been reported. Using segregation away from a recessive white flower colour, this study estimated 25% of seed produced in an open field in which honey bees were active were the result of cross pollination (Chapter 4). This estimate was based on the first flowers formed by the maternal plants, with the assumption that the pollen carried by vectors would most likely have been from different, non-maternal, plants. During full flowering, there will be higher levels of fertilisation from pollen of a different flower on the same individual.

Insects are the most likely vectors for cross pollination in *O. sativus*, therefore the actual level of cross pollination is likely to be site and weather dependant. Even so, the possibility of significant cross pollination in *O. sativus* has important implications for the cultivation, cultivar improvement programs and genetic conservation of the species.

Given the high level of self compatibility evident in *O. sativus*, the incidence of cross pollination prompts the question of whether pollinator activity results in greater ovule fertilization. Alternatively, fertilisation by out-crossed pollen may be at some advantage through more rapid pollen tube growth than
selfing pollen (Richards, 1997). This could result in pollen substitution as well as improved fertilisation.

Although not examined directly, observation during this study suggests that pollinator activity increases fertilisation and seed production. Pods of plants grown in the field generally have more segments/seeds than those from plants grown in isolation. If improved fertilization accompanies pollinator activity, seed set could be increased by bee foraging. This hypothesis merits testing, as the gain in seed yield could be of sufficient economic value to encourage the placing of bee hives near *O. sativus* seed crops.

The certification of plant cultivars provides a quality control system that aims to ensure that seed is free of weed seed and disease and has a minimum germination requirement. It also provides a guarantee that cultivars will reliably express a set of described characters. The important characters vary with particular species and the way they are utilized. To ensure cultivar integrity, certification rules usually specify spatial separation and restriction of the numbers of generations from a basic seed generation.

For *O. sativus*, cultivar certification is restricted to two generations away from a basic seed source and separation by at least 200m from other *O. sativus* (OECD). The incidence of cross pollination reported in Chapter 2 and the changes in population structure that were predicted in Chapter 8 would support these requirements in the absence of more precise information. The relationship between distance of separation and pollen travel has not been
measured for *O. sativus*. The genotypes with pink and white flower colours that were used to quantify cross pollination in Chapter 4 could be a useful tool for this purpose.

Further work is required to test the value of certification requirements for *O. sativus* cultivars, however, crop management should also be considered when soft or hard-seededness is a key character of a cultivar. The relative importance of restricted generations and management can be demonstrated by the model described in Chapter 8. Given little contamination in basic seed stocks, there would be little change in the presence of a targeted soft-seeded cultivar if it is harvested and re-sown in a new paddock each generation. Under this management regime, more than two generations could be considered as meeting certification requirements provided there was no change in other important characteristics, such as maturity. There is a greater potential for change if a soft-seeded cultivar is harvested from a self-regenerating crop, particularly if loss of soft-seed occurs through exposure on the soil surface or due to unseasonal rainfall, and the two generation restriction for certification is prudent in this case.

To ensure the integrity of a hard-seeded cultivar, it is essential to dehull and scarify the seed to be sown for the further harvesting of seed. Otherwise there is potential for a rapid shift to soft-seededness even with a low contamination with the soft-seeded allele. There is less risk of population change if seed is harvested from self-regenerating swards. However, purity of a hard-seeded cultivar can be assured by harvesting seed only from
regenerating swards that follow a rotational cereal or oil seed crop. Under this management regime more than two generations could be considered for certification of cultivar status provided the regulatory authorities could manage this additional requirement.

The incidence of cross pollination has important implications for genetic resource conservation and breeding activities with *O. sativus*. When large numbers of different genotypes or accessions held in collections are grown for seed production, isolation from pollinators or maintaining sufficient isolation distances may be a logistical problem. Managing this could be improved by testing the effectiveness of planting intervening flowering species as a diversion to bee foraging and as a barrier/filter to cross pollination. Genetic conservation should also consider growing reduced numbers of genotypes in a nursery and producing sufficient seed to minimize the need for further seed multiplication (and generations away from the initial seed source). For the purposes of maintaining diversity in germplasm collections, seed of *O. sativus* should have any hard-seeds present dehulled and scarified when undertaking seed increase, in order to maintain the presence of this character in populations.

The observation of cross pollination in *O. sativus* leads to the conclusion that accessions may contain a significant level of heterogeneity. In contrast, breeding activities and genetic studies are improved with the generation of homozygous genotypes. In this study, progeny testing was applied to identify homozygous hard and soft-seeded genotypes and early flowering maturity.
An alternative approach would have been line breeding (ensuring self-pollination) from single seed descent for a number of generations. Utilising the flowering response in *O. sativus* reported in Chapter 3, accelerated generational turnover, at more that two generations per year, could be achieved under artificial lighting with long day length.

The selection targets in this study were early maturity and hard-seed dormancy. Heritable forms of both of these characters were successfully isolated from *O. sativus* populations. In addition, the presence or absence of fine hairs on the surface of the pods was observed to be highly heritable (Chapter 5). The existence of heterogeneity in populations for these characters and segregation of progeny away for the maternal expression can now be explained by the incidence of cross pollination.

### 9.2 Hard-seed dormancy in *O. sativus*.

*O. sativus* plants examined in this study showed three types of seed behavior. Along with soft-seededness (non-dormant) there were two different types of hard-seed dormancy. They are distinguishable by inheritance (one dominant (SCP₁) and the other recessive (SCP₃) to soft-seed production (SCP₂)), interaction with atmospheric moisture and hard-seed breakdown behaviour (Chapters 3 and 5). No evidence of recombination was observed in hybrid progenies between the three expressions although the number of hybrids, particularly with Genotype B1.2, were two few to possibly indentify this type of segregation. For simplicity, seed coat behaviour is therefore
described here as the result of three alternate alleles at a single locus although the possibility of more complex genetic makeup is not denied.

*O. sativus* is normally a diploid species although tetraploid types have been induced artificially. The genotypes in this study were assumed to be diploid due to their appearance and fecundity. Given the assumption of diploid genetics, the phenotype expressions as they relate to the genotypes and order of dominance are;

High hard-seed Phenotype SCP\(_1\) = Genotypes SCP\(_{1,1}\), SCP\(_{1,2}\), and SCP\(_{1,3}\)

All soft-seed Phenotype SCP\(_2\) = Genotypes SCP\(_{2,2}\) and SCP\(_{2,3}\)

Variable hard-seed Phenotype SCP\(_3\) = Genotype SCP\(_{3,3}\)

Furthermore, the phenotype of the maternal plant determines the behaviour of the seed coat surrounding the seeds that it produces (as the coat is derived from maternal tissue). Therefore, the embryo contained within a seed can be a genotype that, when grown to maturity, expresses the opposite character. That is, a hard-seed may produce a soft-seed producing phenotype and *visa versa* (Chapters 3 and 5).

Soft-seeded phenotypes of *O. sativus* (SCP\(_{2,2}\) and SCP\(_{2,3}\)) produce seeds that do not form an impermeable coat upon drying. This is likely to be due to the persistence of channels between the cells of palisade layer of the seed coat, which in hard-seed become sealed at low seed moisture content. Similar channels were observed in the palisade layer of seeds produced by
soft-seeded genotypes of *Lupinus angustifolius* (Serrato-Valenti *et al*., 1989). Through these channels the whole seed rapidly dehydrates and rehydrates according to the ambient relative humidity and available moisture. The stress associated with diurnal cycles in relative humidity, and exposure to short periods of free moisture (i.e. morning dew), creates cracks over the seed coat in general.

The initial colour of soft-seeds is light beige and similar to hard-seeds. However, through the action of the pores or cracks that develop in the seed coat of the soft-seeds produced by SCP₂ phenotype plants, they rapidly turn brown. The stresses associated with fluctuations in seed moisture in the soft seeded phenotypes are also likely to generate seedlings with detached radicals or cotyledons when they germinate. Freshly harvested seed of cv. Cadiz produced 77 and 79% viable seedlings from the Mingenew and Muresk sites of production respectively. However, when seed of this cultivar were exposed on the soil surface for three months, the number of viable seedlings was reduced to 52% with a corresponding increase in abnormal seedlings.

Without the soft-seed expression with which to create hybrid progeny, the two hard-seed alleles would be hard to differentiate. The amount of hard-seed produced by SCP₁ type phenotypes (SCP₁₁, SCP₁₂, and SCP₁₃) is high after plant senescence and the drying of seeds to between 7 and 8% moisture content (according to seed dry weight). Below this moisture content, the seeds do not readily absorb moisture until a breach is formed in the lens.
structure of the seed coat. This behaviour is typical of many small seeded, annual Mediterranean-type legumes (Quinlivan, 1971; Ballard, 1973; Taylor, 2004).

The alternate, recessively inherited SCP₃ hard-seed expression, is more variable in the amount of hard-seed measured at any particular time. This appears to be driven by an imperfect impermeability to the seed coat compared to the SCP₁ expression. High amounts of hard-seed can be induced if the seed is tested for germination soon after drying to very low seed moisture content. However, dry seeds of the SCP₃ phenotype appear to slowly absorb moisture either in high relative humidity or exposed free moisture. At a moisture content of 8%, seed will show a protracted germination pattern when exposed to moisture. This creates potential for stress on the seed coats of seeds that do not germinate immediately rendering them permeable when next exposed to free moisture. This could account for the interaction between maturity and initial hard-seed levels produced by hard-seed genotypes from the Emena populations (type SCP₃) in Chapter 3.

Although a single locus with three alternate alleles has been implied from the results of this study, the hypothesis that hard-seed dormancy is determined by the interaction of more than one locus with high levels of linkage can not be discredited. If the occurrence of the recessive form of hard-seed had been predicted at the outset of this study, then a greater emphasis could have been placed on hybridisation with the hard-seeded genotypes of Emena
origin to test this hypothesis. Even so, the trends predicted by the three allele model for change of hard-seed expression in a population would also relate to more complex inheritance.

Soft-seededness in *L. angustifolius* is inherited recessively as a single gene associated with the *mollis* locus. The absolute expression of hard and soft-seededness in *L. angustifolius* is similar to that observed with the SCP$_1$ hard-seed and SCP$_2$ soft-seed in *O. sativus*. In *Vicia faba*, the production of hard-seed has been associated with two genes, one with dominant expression and the other recessive (Ramsay 1997). A similar result was obtained with inter-specific crossing in *Vicia* and *Lens* (Donnelly *et al.*, 1972; Ladizinski, 1985). The possibility could not be excluded that an interaction between two tightly linked loci occurs in *O. sativus*. Molecular marker comparison between the various types of seed character, such as conducted by Ramsay (1997) for *V. faba* and Nelson *et al.* (2006) for *L. angustifolius*, may assist in discriminating between two loci, or several alleles of a single gene in *O. sativus*. The hybrid genotypes developed in Chapter 5 could be a useful resource for this type of study.

The development, structure and chemistry of legume seed coats are likely to be highly complex and involve the interaction of many genes. The results of this study and those reported for other legume species, suggest that the one or two genes associated with the hard/soft-seed relationship have a regulatory function (Ramsay, 1997). Therefore, some potential aspects of seed coat behaviour may not be evident under the expression of soft-
seededness. Due to this epistatic effect, some useful aspects of hard-seed behaviour could be inherited from a soft-seeded parent.

9.3 *O. sativus* cultivation in agriculture.

The application of *O. sativus* as a cultivated fodder has been restricted to farming systems where it is frequently re-sown. The existence of heritable hard-seed dormancy in *O. sativus* will expand its role in systems that require long term persistence after an initial establishment. In combination with the other desirable attributes of *O. sativus*, particularly ease of seed propagation, hard-seeded forms may be utilised in various ways for pasture improvement.

The SCP₂ phenotype is the traditional representation of the species, where it produces soft-seed and there is no requirement for dehulling and scarification to improve germination. Improvement of this *O. sativus* phenotype for a particular situation could be achieved through selection for the most appropriate timing of flowering to the region and type of utilisation. Fu *et al.* (1994) considered this to be a balance between maintaining the more nutritious vegetative stage as long as possible and a concentrated period of flowering for efficient seed harvesting. Even so, early maturity is required for reliable seed production in low rainfall, short growing season environments. Also, *O. sativus* is a useful green manure crop and for this purpose maturity is not a critical character. For these reasons the early flowering, soft-seeded genotype identified in this study should be evaluated
and possibly released for cultivation, particularly in low and variable rainfall environments.

The timing of flowering in the *O. sativus* germplasm in this study was controlled by a qualitative influence of photoperiod. The initiation of flowering was either delayed under short days or induced by long days. The timing of sowing and latitude would determine the type of pressure applied by selection for a particular maturity. Selection for early or late flowering plants when sown in late spring will provide pressure to change the position of the first flowering node along the stems (growing days). However, selection for early or late flowering plants when sown in late autumn can be the result of changing not only the requirement for a minimum amount of accumulated growing days, but also qualitative photoperiod response.

In systems with rotational cropping, the SCP\(_1\) phenotype of hard-seed would be valuable because of its more reliable and longer lasting dormancy. It would dominate a mixed population in the longer term with increasing selection pressure favouring the SCP\(_1,1\) genotype under single years of pasture between rotational crops. The seed characteristics of the recessive SCP\(_3\) phenotype could also have useful application for certain situations. Seeds produced by homozygous SCP\(_{3,3}\) maternal plants have a greater level of reversible hard-seededness. Under dry conditions the seed coat is likely to be impermeable, or slowly permeable. Additionally, dry SCP\(_3\) type seeds appear to have a higher degree of softening when subjected to alternating
temperatures. This combination of characters could have several applications.

Disadvantages of the soft-seeded SCP$_2$ phenotype are the potential loss of seed through germination on non-seasonal rainfall and the loss of viability when pods are exposed on the soil surface. The seed coat behaviour of the SCP$_3$ phenotype may provide the opportunity to overcome these problems. Harvested pod of the SCP$_3$ phenotype can have high levels of germination at moderate seed moisture contents. In this study SCP$_3$ seed at a moisture content of 8.7% showed germinations of 66% and 95% after 15 and 60 days of moist conditions respectively (Table 7.4). If seed moisture around this level can be captured at harvesting and maintained under storage, this level of germination would be adequate for establishing a pasture without the need for seed extraction and scarification. Furthermore, pods of the SCP$_3$ genotype could be dry sown either in summer or autumn where dry conditions would enhance the expression of hard-seed providing insurance against short periods of moist conditions. As previously mentioned, pedigree seed crops of this genotype (as opposed to pasture improvement) should be sown with scarified seed to minimize selective pressure toward soft-seededness.

**9.4 Implications for protocols to be followed when analysing hard-seededness in legumes.**

Inheritance of hard-seed production in *O. sativus* and the three associated seed behaviours, SCP$_1$, SCP$_2$, and SCP$_3$, observed in this study has
parallels in other species. The importance of methodology for obtaining meaningful results when assessing hard-seededness has also been highlighted by this study. The relationship between the moisture content of legume seeds and the establishment of irreversible hard-seededness has been understood for some time (Quinlivan, 1971). The dynamics of seed moisture in a particular seed sample may also influence the progression of hard-seed breakdown and pattern of germination (Taylor, 2004). However, as highlighted by Taylor (2005), the importance of this relationship is often ignored and results presented on hard-seed behaviour have reduced value if they are not accompanied by some measure of seed moisture. The results of this study support this view and also suggest the possible mechanisms underlying this relationship.

The seed moisture content of hard-seeds was found to increase and decrease at different times of the year when exposed in the field (Chapter 6). Hard-seeds of both the SCP₁ and SCP₃ phenotypes were also found to interact with atmospheric moisture (Chapter 7). Relative humidity is intrinsically linked with temperature and, as biological tissue expands and contracts to a greater extent in response to hydration level than temperature per se, fluctuating diurnal temperatures may drive a hydration/dehydration cycle in hard-seeds.

Slight changes in seed moisture may act primarily on the elongated hypodermal cells underlying the lens structure, creating stress associated with expansion and contraction of these cells. This could explain the
enhanced hard-seed breakdown observed when seed is slightly covered with soil compared to exposure on the soil surface (Revell et al., 1997; Chapter 6).

The moisture content of buried seed was consistently higher than seed exposed on the soil surface, presumably due to the soil modifying the diurnal dehydration and hydration cycle. Facilities that control the fluctuation of both relative humidity and temperature are required to elicit the interaction between these two interlinked environmental conditions on hard-seed breakdown.

Given the possibility of interaction with atmospheric humidity, measurement of germination and hard-seed must be conducted as quickly as possible after sampling. Also, consistent post harvest treatment and storage needs to be applied to all seed. This can be problematic when comparing seed produced by genotypes of different maturity and where large numbers of samples are involved. This was experienced in the experiments reported in Chapter 3. There was a positive interaction between maturity and hard-seed content of pod produced by selections derived from the Emena populations (Figure 3.3 and 3.4). This may have been due to moist conditions and/or irrigation resulting in the imbibition of moisture in early formed seeds resulting in damage to the seed coat. The amount of hard-seed in the selections was also variable between selections and within and between their offspring. Some of this variability may have been due to the conditions and length of storage prior to the germination test.
The direct measurement of seed moisture and dry weight is a destructive process and requires a sufficient seed quantity to detect small relative changes in weight. A possible non-destructive solution for estimating moisture content of seed lots is to use indicator samples during post harvest treatment and storage. Samples of seeds known to be hard or soft (or scarified hard-seed), and for which the dry weight has been measured, could be stored in sealed containers with the experimental samples. After equilibration, the indicator hard-seed sample will show the lowest moisture content possible, while the soft-seed sample will display the greater moisture content under the particular storage treatment. The experimental seed lots would be at an ideal moisture content for comparison when the two indicator samples were estimating a similar moisture content.

The simple inheritance of hard and soft-seededness in *O. sativus* and the variability in the amount of hard-seed with the S₃ phenotype also presents a challenge when interpreting results based on a group of plants rather than individuals. Low to moderate amounts of hard-seed in freshly harvested seed from a “population”, could be the result of a mixed hard and soft-seed population. This would be a particularly important consideration when the species has a high proportion of cross pollination. For example, *Hedysarum coronarium* appears to have a high requirement for cross pollination (Satta *et al.*, 2000). The variation in germination between accessions of *H. coronarium* reported by Bell *et al.* (2003) could therefore be explained by the presence of both hard and soft-seeded genotypes in some of these accessions. The
variation reported could equally be explained by the equivalent of the S₃ type hard-seededness of *O. sativus* being present in *H. coronarium*. In this case, differences in seed moisture content are responsible for the variation. At least some testing of hard-seed level on an individual parent plant basis and an estimate of the moisture status of the seed is needed to either confirm or discredit these possibilities.

9.5 Concluding remarks.

This study has elucidated important factors for effective harnessing of the variability in flowering time and hard-seededness in *O. sativus*, and the maintenance of certified lines of the species. *O. sativus* is a valuable forage and nitrogen source for agriculture. The knowledge gained in this study will enhance the exploitation of this species, particularly with the trend towards an appreciation of the increasing financial and environmental burden of artificial nitrogen in agricultural systems. In addition, the new insights gained into genetic control and expression of hard-seededness will allow better experimental design for studying this trait in other legumes.
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