Developmental changes in the germinability, desiccation tolerance, hardseededness, and longevity of individual seeds of Trifolium ambiguum

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ORIGINAL ARTICLE

Title: Developmental changes in the germinability, desiccation tolerance, hard-seededness and longevity of individual seeds of *Trifolium ambiguum*.

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Running title: Developmental changes in individual seeds of *Trifolium ambiguum*

ABSTRACT

Background and aims Using two parental clones of out-crossing *Trifolium ambiguum* as a potential model system, we examined how during seed development the maternal parent, number of seeds per pod, seed position within the pod, and pod position within the inflorescence influenced individual seed fresh weight, dry weight, water content, germinability, desiccation tolerance, hardseededness, and subsequent longevity of individual seeds.

Methods Near simultaneous, manual reciprocal crosses were carried out between clonal lines for two experiments. Infructescences were harvested at intervals during seed development. Each individual seed was weighed and then used to determine dry-weight or one of the physiological behaviour traits.

Key results Whilst population mass maturity was reached at 33-36 DAP, seed-to-seed variation in maximum seed dry weight, when it was achieved, and when maturation drying commenced, was considerable. Individual seeds acquired germinability between 14 and 44 DAP, desiccation tolerance between 30 and 40 DAP, and the capability to become hardseeded between 30 and 47 DAP. The time for viability to fall to 50 % (p_{50}) at 60 % RH, 45 °C increased between 36 and 56 DAP, when the seed coats of most individuals had become dark orange, but declined thereafter. Individual seed FW at harvest did not correlate with air-dry storage survival period. Analysing survival data for cohorts of seeds reduced the standard deviation of the normal distribution of seed deaths in time, but no sub-population showed complete uniformity of survival period.

Conclusions Variation in individual seed behaviours within a developing population is inherent and inevitable. In this out-breeder, there is significant variation in seed longevity

which appears dependent on embryo genotype with little effect of maternal genotype or architectural factors.

KEYWORDS

Seed development, seed-to-seed variation, seed longevity, seed coat colour, cohort and population measurements, model system, *Trifolium ambiguum*.

INTRODUCTION

Individual, apparently mature seeds, harvested at the same time from a single inter-breeding population of mother plants vary considerably in a wide range of traits including e.g. mass (McGinley *et al*., 1990; Castellanos *et al*., 2008; Jaradat and Rinke, 2008), level of dormancy / timing of germination (Biere, 1991; Hoyle *et al*., 2008), and enzyme activity (Still and Bradford, 1997; Mo and Bewley, 2003). Seed-to-seed variation is also evident during seed development. This is surmised from seed development studies where the population mean for traits such as seed mass are usually determined using replicate samples (typically 3-5) of multiple seeds (e.g. Rasyad *et al*., 1990; Hay and Probert, 1995; Mai-Hong *et al*., 2003). The standard error of the mean of these measures indicates that seed-to-seed variation occurs even though measurements are not made on individual seeds. This variation reflects differences in the growing environment of the mother plants, the timing of fertilisation, and the external and internal environments of the developing seeds. In crop seed-lots the mother plants will have shared a relatively uniform seed production environment resulting in substantially uniform flowering and fertilisation. In non-domesticated species this will be less so. In both, there may be significant effects of seed position within the fruit, fruit position within the inflorescence, and position of the inflorescence within the plant – as well as relative timing of fertilisation – on seed traits. These effects suggest significant competition for maternal resources between individual seeds.

The breeding system of the maternal plants will also exert significant effects. Seeds are genetic chimeras: the embryo is the diploid product of meiotic segregation and recombination; the endosperm is a triploid tissue resulting from the fusion of two copies of the maternal and one copy of the paternal gamete genomes; the seed coat is diploid maternal tissue. Repeated high levels of in-breeding, as in the production of crop varieties, will result in seed collections that are essentially genetically homogeneous between and within

individuals. Out-breeding, the usual situation in most wild species, will result in the opposite, with considerable levels of genetic heterogeneity between and within each individual in the collection.

In the case of seeds destined for long-term storage, the most significant trait showing seed-to-seed variation, in both crop and wild plant species, is longevity (survival period). The primary tenet of the viability equations (Ellis and Roberts, 1980), widely used to quantify seed longevity, is that the period to seed death is normally distributed within a population of seeds. Probit analysis determines the parameters of this distribution in accordance with the viability equation

$$v = K_i - p/\sigma \qquad 1$$

where v is the viability (NED) of a population of seeds with initial viability K_i (NED) after p days in air-dry storage and σ is the standard deviation of the distribution of seed deaths in time (days) (Ellis and Roberts, 1980). A further tenet of the viability equation is that σ is constant for all seed-lots within a species when placed in an identical storage environment. If seeds are stored in a favourable environment (low temperature, low moisture), the distribution is broadened; σ increases. In a less favourable storage environment, σ decreases. If we could eliminate inter-seed variation, σ should become zero; all the seeds would die at the same time during storage, regardless of the storage environment. The length of the lag before this catastrophic viability loss would be the parameter that changed depending on the storage environment. Furthermore, a seed-lot would ideally be harvested when all the seeds have attained the same maximum potential longevity.

Seed development studies can either attempt to limit sources of inter-seed variation, or to study their effects explicitly. Most studies of seed developmental physiology have employed crop cultivars as models. In consequence, studies have tended to be skewed towards seeds

where the seed coat and the embryo genotypes are identical and near homozygous. To better understand the interplay between maternal and zygotic genotypes which can be expected in the vast majority of non-domesticated species, which mainly out-breed, we have undertaken seed development and seed quality studies at the level of single seeds on *Trifolium ambiguum* (Caucasian or Kura clover; Bryant, 1974; Taylor and Smith, 1998; Abberton *et al*., 1998), a highly out-breeding species, which we developed as a model system. To achieve this, we have grown and near-simultaneously reciprocally-crossed two cloned lines under conditions which allowed other potential sources of variation to be controlled or quantified.

We chose *T. ambiguum* as our study species since:

i) Within the Fabaceae the genus *Trifolium* lies in the same sub-clade as *Pisum*, *Medicago* and *Vicia* while *Phaseolus*, *Glycine* and *Vigna* lie only two clades away. Hence behaviours similar to cultivated grain legumes can be expected.

ii) It is self-infertile and so, if bees are excluded, hand pollination will ensure that variation in pollination time is minimised. For pollinations carried out at the same time, the environmental conditions during subsequent seed development will be identical.

iii) Large numbers of genetically identical parent plants can be clonally propagated.

iv) Each parent plant produces many inflorescences each with many flowers: sufficient flowers are available for experimental purposes within the restricted pollination period.

v) The ovary of each flower has only one or two ovules: any variation due to sequential fertilisation can be easily observed.

vi) The seeds are non-endospermic with the endosperm substantially absorbed early in development and remaining only as an aleurone layer in the mature seed.

vii) The dormancy and germination characteristics of seeds of Fabaceae species are well understood.

We report this model system and the variation in seed characters during seed development. The parameters measured reflect the behaviours of the different genomes within the seed: seed coat colour and hardseededness to reflect the maternal genome; seed weight to reflect the interaction between the maternal and filial genomes; germination, desiccation tolerance and survival to reflect the filial genome. We also studied the factors controlling variation in individual seed lifespans (in subsequent experimental air-dry storage) during seed development. Our overall intention is to better understand how to obtain seed-lots of greater uniformity and optimum quality, when collecting seeds for *ex situ* conservation, particularly of wild out-breeding plant species. As seed coat colour is often used as an indicator of seed maturity. Understanding how coat colour changes in relation to quality measures may help to guide collectors in the field and this was also studied.

MATERIALS AND METHODS

Plant propagation and selection of clones

Approximately 100 scarified seeds of *Trifolium ambiguum* provided by the Institute of Grassland and Environmental Research (IGER), Aberystwyth (population Ah1471, a diploid variety from Australia) were sown in trays of Medium Grade Compost (William Sinclair Ltd.) at a depth of approximately 50 mm on 11 Dec. 2003. The seed trays were placed in a glasshouse at Reading University at 20 ºC with continuous supplementary lighting to promote

germination. After 35 d, seedlings were transferred to conditions of approximately 15/7 °C (12 h light/12 h dark). Once seedlings had produced two or three trifoliate leaves, individual seedlings were potted up into separate 70 mm diameter pots of Medium Grade Compost (William Sinclair Ltd.). A solution of *Rhizobium* mixture obtained from IGER was watered onto seedlings 42 and 59 d after sowing. Root nodules were evident once plant roots had become established. On 31 Mar. 2004, 30 vigorous plants were selected and transferred to a glasshouse at Wakehurst Place where they were grown under conditions of approximately 20/10°C (12 h light/12 h dark) with supplementary lighting. Plants were watered as necessary and given weekly liquid feeds of Vitafeed 111 (Vitax Ltd.) a balanced fertiliser plus trace elements (diluted 1:100 in water).

A few of the plants showing the most vigorous growth were selected for clonal multiplication, either by detaching plantlets or by taking cuttings. Rooting powder Rhizopon B 0.2% (Fargo Ltd., UK) was applied to the excised end of cuttings. Plantlets and cuttings were potted up into 70 mm diameter pots in a 2:1 mixture of Sylvamix potting compost (Melcourt Industries Ltd.) and perlite granules (Scotts UK Ltd.). Once plants were sufficiently large they were either multiplied again or potted on into 125 mm diameter (1 l) and 140 mm diameter (2 l) pots in a 1:23.33 mixture Osmocot slow release fertiliser (Scotts UK Ltd.) and nursery stock compost (Melcourt Industries Ltd). Early in this multiplication process, a couple of plants for each clone were allowed to establish and produce flowers. Reciprocal crosses were then carried out by hand (see below) between clones. Two of the clones that were found to be compatible and produce viable seeds from these reciprocal crosses were selected for further multiplication. Once sufficient plants of each of these two clones were in the largest pots and sufficiently large, flowering was induced by increasing day length to 16 h. Plants were given weekly liquid feeds of Peters Excel (Scotts UK Ltd.),

diluted 1:100 in water. In total 8 of the propagated plants of clone 1 (referred to as M1) and 10 plants of clone 2 (M2) were used in these experiments.

For Experiment 2, plants from earlier investigations were cut back and allowed to 'rest' at approx. 20/10°C (12 h light/12 h dark) for at least two months. Plants were then re-potted and flowering induced by again increasing day length to 16 h.

Hand pollination

To prevent pollination by bees, or other insects, and enable control of pollination (timing and source of pollen) plants were placed within a cage constructed from stainless steel and 4 × 4 mm mesh netting. The mesh size was sufficient to exclude all insects large enough to pollinate a papillionaceous flower. Manual cross-pollination of the two clones M1 and M2 was carried out on 8 Dec. 2004 (Experiment 1) or 11 Jan. 2006 (Experiment 2) following IGER protocols (Michaelson-Yeates, *pers. comm.*). First, the keel petal of each floret on the recipient (maternal) inflorescence was pushed down with a pair of forceps. The tip of a folded triangular piece of card was then inserted between the standard and keel petals of a few florets on a donor inflorescence in order to pick up pollen which was then deposited on the stigmas of recipient florets. Apart from old florets at the base of the recipient inflorescence and immature florets at the top which were removed using forceps, all the florets on an inflorescence were pollinated. A maximum of five inflorescences were pollinated on each plant. Flowers not pollinated were removed, as were all inflorescences appearing subsequently, thereby reducing competition for assimilate.

Seed harvest

Experiment 1. One inflorescence was taken from one of the M1 plants at each of 14, 22, 28, 30, 33, 36, 40, 44, 47, 50, 54, 58, 61, and 64 days after pollination (DAP). In addition one inflorescence was taken from one of the M2 plants at each of 22, 28, 36, 40, 47, 50, 58, and 61 DAP as fewer M2 inflorescences were available at pollination. For harvests when one inflorescence from both clones were sampled, all the seeds from one inflorescence were processed before harvesting the second. The stems were placed in a beaker of water whilst pods were removed and the seeds dissected out under a light microscope. For each seed, pod position within the inflorescence (top, middle or bottom), number of seeds within the pod, position within the pod (apical or basal; for single seeded pods this was not always possible to discern and so was not recorded), and fresh weight (FW; to nearest μg on a seven-place balance, Mettler Toledo) were recorded. After weighing, individual seeds were randomly assigned for testing moisture content, germination (of fresh seed), or desiccation tolerance (germination after desiccation).

Experiment 2. Seed harvests were made at regular intervals from 20 up to 70 days after pollination (DAP). For each harvest, a total of 5-7 inflorescences were removed from the two maternal types (seeds processed as above). Each seed was treated separately, recording the position of the pod in the inflorescence (top, middle or bottom), number of seeds in the pod (1 or 2), and its position [apical or basal; only recorded for double-seeded pods], before measuring the seed FW to the nearest μg using a seven-place balance (Mettler Toledo). The colour of the testa for each seed was assessed by eye and recorded as green, yellow, dark yellow, orange, or dark orange. After weighing, seeds were randomly assigned for testing moisture content (and hence DW) and, for seeds harvested on or after 36 DAP, germination (of fresh seed), desiccation tolerance, or germination after a variable period of experimental

storage. Relatively few dark yellow seeds were harvested overall. Hence, this colour category was not represented in the samples used for moisture content determination.

Dry weight / moisture content determination

Seeds destined for moisture content determination were dried in an oven at 103 °C for 17 h according to the low-constant-temperature-oven method (ISTA, 2005). After drying, individual seed DW was measured on the 7-place balance as above, and moisture content calculated as a proportion of FW.

Germination and desiccation tolerance

Individual seeds were tested for ability to germinate on 1% dH_2O agar each held within a single 10 mm diameter well of a Multidish culture plate (125 × 85 × 20 mm, H × W × D; Nunc AIS, Denmark) at 15 °C with light provided for 8 h d^{-1}. Seeds failing to imbibe within 28 d were recorded as hard-seeded, then scarified by removing a small portion of the seed coat using a scalpel and placed on fresh agar for a further 28 d.

To test for desiccation tolerance, individual seeds were dried for 28 d in separate empty Multidish wells without lids at 20 °C over silica gel in a 170 × 115 × 60 mm (H × W × D) polyethylene sandwich box sealed with Nescofilm (Bando Chemical Ind. Ltd., Japan). They were then tested for germination (and hardseededness) as above.

Seed longevity

Individual seeds that had been dried as above in Experiment 2 were scarified and returned to their allotted Multidish well. The Multidish plates were placed, without their lids, at 20 ºC over a non-saturated LiCl solution at 47 % RH (Hay *et al*., 2008) held within a sealed 300 × 300 × 130 mm Electrical Enclosure Box (Ensto UK Ltd., Southampton). After 20 d equilibration of the seeds, the Multidish plates were placed, again uncovered, at 45 ºC over a different non-saturated LiCl solution at 60 % RH held in an Electrical Enclosure Box as above. The experimental storage period was deemed to commence on transfer to 45 ºC, 60 % RH, the initial treatment at 20 ºC, 47 % RH having adjusted seed moisture content to that in equilibrium with 45 ºC, 60 % RH (Hay *et al*., 2008). Random samples of approximately 20 seeds were removed at 3 – 7 d intervals and tested for germination as above.

Data analysis

All analyses were carried out using GenStat for Windows 11th Edition (VSN International Ltd., UK). For experiment 1, a two sample Poisson test (normal approximation) was used to compare the numbers of seeds from M1 or M2 inflorescences. X^2-tests were used to compare proportions, *e.g.* of pods bearing 1 or 2 seeds. For both experiments, correlation coefficients were determined by plotting individual seed DW against their FW within each harvest. The Shapiro-Wilk test was used to test for normality of the distribution of FW, DW, weight of water, and moisture content within each harvest. The amount of skewness and kurtosis when fitting the normal distribution was also assessed. Where appropriate, *i.e.* a normal distribution could be fitted, T-tests were used to compare mean seed FW or DW for sub-sets of data and ANOVA (unbalanced design) with harvest age (DAP), maternal parent (M1 or M2), number of seeds per pod, or position in the inflorescence (top, middle, or bottom) was used to identify sources of variation in mean seed FW or DW. X^2 tests were used to compare proportions of,

for example, seeds germinating / not germinating within sub-sets of the data. Experimental storage data (experiment 2) were subjected to probit analysis in order to fit equation (1) and estimate p_{50} [period of experimental storage (in days) for population viability to be reduced to 50%, the product of K_i and σ]; analysis of deviance with an approximate F-test was used to assess whether or not survival curves could be constrained to common slope (σ^{-1}).

RESULTS

Trifolium ambiguum *as a model system*

Trifolium ambiguum was well suited to clonal propagation and hand (and so controlled) pollination. The mean number of seeds produced by M1 inflorescences (i.e. M1 = maternal parent, pollinated by M2) was 41 in experiment 1 and 44 in experiment 2 [**Supplementary Information**]. In experiment 1, M2 inflorescences (i.e. M2 = maternal parent, pollinated by M1) produced significantly fewer seeds (mean of 26; Poisson test; $P<0.001$), but this difference was not detected in experiment 2 (mean of 48). Two seeds developed in a greater proportion of pods than had been anticipated: in experiment 1, 49 and 70 % for M1 and M2 inflorescences, respectively. This maternal parent effect was significant ($X^2=16.68$, 1 d.f., $P<0.001$) in both experiments. Twice as many seeds were harvested from the middle of the inflorescences than from either above or below [**Supplementary Information**]. This region had twice the number of florets of each of the other regions available for pollination on the selected date, the florets developing sequentially from bottom to top.

Seed development

The pattern of increase in seed FW and DW over time was very similar for the two experiments (Fig. 1). Harvest FW and DW of individual seeds increased up to 33-36 DAP

(also shown in Fig. 2A,F for experiment 1). Up to this point, there was good correlation between DW and FW within each harvest (Fig. 1), however the relationship changed over time, as seed DW and the seed-to-seed variation in DW increased. For example, in experiment 1, at 14 DAP individual seed DW ranged from 0.07 to 0.33 mg; at 28 DAP the range was 0.63 to 1.24 mg (Fig. 1A).

Although absolute seed water content rose, relative seed moisture content measured at the population level declined steadily over this seed filling period (Figs. 2K and 3). Absolute seed water content then declined resulting in a more rapid drop in relative seed moisture content until equilibrium (approx. 11 % f.w.b.) with ambient conditions was reached at 47 DAP (experiment 1) or 49 DAP (experiment 2). During this phase of water loss, the correlation between FW and DW within each harvest was compromised. In experiment 1, the lowest R value was 0.21 at 40 DAP (Fig. 1A) and the standard error for the mean moisture content was greater at 40 DAP than any other harvest date (Fig. 2K). After 47 or 49 DAP (experiments 1 and 2, respectively), there was once again good correlation between individual seed DW and FW ($R>0.99$ within each harvest; Fig. 1). There were no further significant changes in either, or therefore, in moisture content, between these and the respective last harvests (Figs. 2A,F,K and 3).

Individual seed FW and DW within each harvest could generally be fitted using normal distributions (Table 1). However, in the phase after population mass maturity when mean seed moisture content was falling rapidly (40-47 or 49 DAP, for experiments 1 or 2, respectively), there were high levels of skewness and/or kurtosis for individual seed water content (actual mass and/or as a proportion of FW). Harvest time accounted for most of the variation in FW (86 and 68 % in experiments 1 and 2, respectively), but there were also significant main effects of maternal parent (M1 or M2; $P<0.05$; experiment 1 only), number

of seeds per pod (P<0.01), and position in the inflorescence (P<0.001; Table 2). For example, seeds from the top of the inflorescence tended to have lower FW than those from the middle or bottom (Fig. 2E). There were also significant interactions between harvest time and maternal parent (P<0.05; Table 2) or position in the inflorescence (P<0.001; experiment 1 only). Harvest time was also the largest source of variation in mean seed DW (37 %) between 33 and 64 DAP in experiment 1 (Table 2) appearing to fluctuate between sequential harvests (Fig. 2F), but not in experiment 2 (as expected, since by 36 DAP, mass maturity had been reached). Maternal parent and number of seeds per pod were significant factors (P<0.001) in both experiments (Table 2). Differences in mean seed moisture contents within each harvest depending on maternal parent, number of seeds per pod, and position within the pod were small or nil (shown for experiment 1 only; Fig. 2L-N). However, the rate of decline in moisture content after 36 DAP appeared to vary depending on the position within the inflorescence; desiccation occurred progressively more rapidly from the top to the bottom of the inflorescence (Fig. 2O).

Seed coat colour (experiment 2 only)

The relative proportions of seeds with different coat colours through the harvesting period reflected the sequence of colour change (green → yellow → dark yellow → orange → dark orange; Figs 2 and 3). At 36 DAP most of the seeds were green and a small proportion yellow; at 44 DAP, half the seeds were yellow or dark yellow, and the remainder were orange or dark orange. By 56 DAP most of the seeds were dark orange, however, a uniform population of dark orange seeds was never achieved, even at the last harvest (70 DAP). Categorising seeds according to colour meant that they were also, largely, sorted into groups that reflected their moisture content (Table 3; also apparent in Fig. 1B where individual seeds that were more advanced in the colour series tended to have the lower FW within their

harvest cohort). Green seeds harvested at between 36 and 42 DAP consistently had the highest moisture content (mean within each harvest > 60 %; Table 3). The moisture content of yellow seeds tended to be lower than that of green seeds; this difference was significant at 36 DAF (T-test, $P<0.05$; the difference was not significant or could not be tested for other harvest dates). Similarly, the moisture content of orange seeds was generally less than that of yellow seeds at each harvest (significantly different at 44 DAP; T-test, $P<0.05$). By 44 DAP there was no difference in the moisture contents of orange and dark orange seeds at each harvest.

The moisture content declined within each colour category as seeds were harvested progressively later. For example, at 36 DAP yellow seeds had a mean moisture content of 55.6 %, whereas at 44 DAP it was 47.6 % (Table 3). Sub-sets of seeds which showed more rapid decline in moisture content, also progressed more quickly through the sequence of colour change. For example, at 42 DAF, the ratio of green : yellow : dark-yellow : orange : dark-orange seeds from M1 plants (mean moisture content 50.0 %) was 0.12 : 0.54 : 0.13 : 0.12 : 0.09 (*i.e.* most seeds were yellow; Fig. 3D) compared with 0.01 : 0.18 : 0.08 : 0.61 : 0.12 (most seeds orange) for seeds from M2 plants (mean moisture content 29.2 %). These differences in colour progression and moisture content between seeds from M1 and M2 plants was also apparent at 38 and 44 DAF; for subsequent harvests there were no differences in mean seed moisture content between seeds from M1 and M2 plants and if anything, seeds from M2 plants appeared less mature, based on their colour (Fig. 3).

The colour ratios also suggested that seeds from 2-seeded pods matured more quickly than those from single-seeded pods between 40 and 49 DAF (Fig. 3D-F). There was not a consistent trend in the proportions of seeds in each colour category depending on whether they came from the apical or basal position of the pod (seeds from 2-seeded pods only; Fig.

3). Seeds from the top of the inflorescence appeared to mature more quickly than seeds from the bottom or middle of the inflorescence throughout the developmental period studied. This was also reflected in lower moisture contents for seeds from the top of the inflorescence.

Germinability, desiccation tolerance, and hardseededness

In experiment 1, ability to germinate was first detected at 22 DAP, most seeds were able to germinate by 28 DAP, but the ability of all seeds to germinate was not observed until 42 DAP (Figs 4A and 5A). The ability to tolerate desiccation was first seen in some individuals at 33 DAP, with all individuals desiccation tolerant 7 d later (Figs 4F and 5B). Thus some seeds appear to acquire desiccation tolerance 11 d after germinability whilst later maturing seeds acquire germinability and desiccation tolerance almost simultaneously or possibly even in a different order (i.e. desiccation tolerance first). No consistent differences were detected in these patterns between seeds from M1 and M2 inflorescences (Fig. 4B,G). Comparisons amongst individual seeds within harvests between 22 and 30 DAP suggested that it might be the heavier seeds within populations that were capable of germinating (only shown for seeds with maternal line M1 and thus only for 28 and 30 DAP; Fig. 5A). However, at 22 DAP, when ability to germinate was 31 %, the difference in mean FW between germinating and non-germinating seeds was not significant (T-test; $P > 0.05$). At 28 and 30 DAP, the mean FW of seeds able to germinate was greater ($P < 0.05$) than that of those which did not, but in both these cases the numbers of non-germinators was small (Fig. 5A). Similarly, visual examination of the results for individuals within a harvest over the period when populations began to acquire desiccation tolerance (at 33 and 36 DAP for seeds from maternal line M1; Fig. 5B) suggested that this might be acquired earlier in seeds that had begun to dry sooner, but this was not significant ($P > 0.05$).

Seeds that developed within double-seeded pods appeared to acquire the ability to germinate slightly later than those from one-seeded pods (Fig. 4C), whereas if anything the former may have tolerated desiccation slightly earlier than those from one-seeded pods (Fig. 4H). The effect of seed position within double-seeded pods had a similarly negligible but also potentially contradictory effect. Ability to germinate may have been acquired slightly earlier across the population by seeds from the base of pods (Fig. 4D), whereas desiccation tolerance may have been acquired slightly sooner in apical seeds (Fig. 4I). Position in the inflorescence gave the greatest effect on the development of ability to germinate: seeds that developed in the top of the inflorescence acquired this some 2 d earlier than those in the middle, whilst those at the bottom were some 2-5d later than those in the middle (Fig. 4E). In contrast, the development of desiccation tolerance was unaffected by inflorescence position (Fig. 4J). Number of seeds per pod, position within the pod, and position within the inflorescence had no effect on the incidence of hardseededness (Fig. 3M-O).

In experiment 2, harvesting seeds for germination and experimental storage did not commence until 36 DAP, when 83 % of fresh seeds had acquired germinability and 67 % were already desiccation tolerant [**Supplementary Information**]. All seeds achieved germinability and desiccation tolerance at 40 and 42 DAP, respectively. In the case of seeds harvested at 36 DAP, there was no difference in the mean seed FW of seeds that did germinate compared with those that failed to germinate, either before (mean 3.7 ± s.e. 0.11 mg for seeds that germinated compared with 3.6 ± 0.24 mg for those that did not) or after drying (mean FW of seeds that were desiccation tolerant 3.6 ± 0.08 mg compared with 3.8 ± 0.09 mg for seeds that were not) [**Supplementary Information**]. Seeds that did not germinate fresh at 36 DAP had green testas [**Supplementary Information**]. Similarly, the seeds that had not acquired desiccation tolerance at 36 and 38 DAP were green, even though

by 38 DAP there were some seeds in all categories of coat colour with the largest proportion being yellow.

On-plant hardseededness developed during late development and maturation (only recorded for experiment 1; Figs 4K and 5A), as individual seed moisture content approached equilibrium with ambient. Comparison of the hardseedness results for *in planta* desiccation with variation in mean moisture content suggests that at moisture contents close to 12 %, hardseededness may be lost or acquired depending on subtle variation in ambient humidity. Whilst the trait was associated with desiccation *in planta*, it was expressed earlier in development and in a much greater proportion of the population when dried *ex planta* (28 d at 20 °C over silica gel; Figs 4K and 5B). The developmental trend for hardseededness when dried *ex planta* generally followed the development of the ability to tolerate desiccation; in some individuals both traits may be acquired almost simultaneously whilst in others hardseededness is delayed by up to 7 d (Fig. 4K). The development of hardseededness upon drying did not differ between seeds from M1 or M2 maternal parents (Fig. 4L). The apparent difference in hardseededness of fresh seeds harvested at 50 DAP from different maternal lines may not be reliable due to low sample size (all three M2 seeds were hardseeded whilst none of the 17 M1 seeds was hardseeded).

Seed longevity (experiment 2 only)

Seed longevity in experimental storage (60 % RH, 45 °C) increased progressively between 36 (p_{50} = 4.3 d) and 56 DAP (p_{50} = 40.1 d), declining progressively thereafter until 70 DAP (p_{50} = 32.5 d) (Fig. 6). The harvest FW of seeds that germinated at each sample time during experimental ageing tended to be higher than the FW of those that failed to germinate at 36 and 38 DAP (Fig. 6A,B). However these differences were not significant (T-test, P>0.05) and were not consistently apparent for later harvests (Fig. 6C-I). The large increase in p_{50} between

36 and 44 DAP was due to an increase in K_i (eqn. 1), from 0.31 to 3.7 NED (Fig. 7A). It subsequently fell to an apparent plateau of *c.* 2.2 NED between 49 and 70 DAP. The estimates for σ fluctuated between 8 and 16 d. However, it was possible to constrain the survival curves for all the harvest times to a common estimate for σ without a significant increase in the residual deviance [$P > 0.05$; $\sigma^{-1} = 0.0733$ (s.e. 0.00332)], such that all the variation in survival among harvest dates was accounted for by differences in the estimates of K_i.

For seeds harvested at 36 DAP, a greater proportion of yellow seeds were able to germinate upon removal from experimental storage at each sampling time compared with those that were green [**Supplementary Information**]. Similarly for 38 DAP seeds, those that were green were less likely to germinate when removed from experimental storage. However, there was little consistent difference in the survival of yellow, dark yellow, orange, or dark orange seeds, within each subsequent harvest. The estimate of σ for colour-sorted cohorts of the population fluctuated in a similar way to that when the data was not sorted, but was generally lower, particularly for harvests between 36 and 44 DAP (Fig. 7). This apparently smaller amount of seed-to-seed variation in longevity within colour-sorted cohorts may be due to lower seed numbers within each cohort. Low seed numbers similarly confounds the possibility of assessing the extent to which factors such as maternal clone or plant, number of seeds per pod, *etcetera* influence seed longevity. For many of the analyses of the experimental storage data where seeds were allocated to two or more sub-populations, there was not a significant increase in residual deviance if the sub-populations were modelled using a common slope or even a single line through all the data.

DISCUSSION

Seed development

Few studies of physiological seed development have looked at the level of individual seeds. Those that have been reported, have been carried out on crop species (e.g. Munier-Jolain and Ney, 1998; Ishimaru *et al*., 2003). We chose to study *Trifolium ambiguum* as a model for other out-breeding, genetically heterozygous species, with the aim of increasing our understanding of the genotypic and architectural variables that affect the seed-to-seed variability in physiological traits and hence, we surmise, influence seed development. This understanding may help us optimise the quality of seed collections.

Working at either a population or individual seed level, it is not possible to monitor the same set of individuals through their development. Seed development studies inevitably require that seeds are removed from the maternal plant and the measurement of many of the parameters is destructive (including germination). Thus, such studies assume that all seeds follow the same development sequence and time is used as the independent variable. Recognising that the development of individual seeds may vary, we have considered the use of individual seed FW at harvest, a non-destructive measurement, as an alternative internal (*i.e.* characteristic of the seed) independent variable to chart seed development. Additionally we have monitored changes in seed coat colour to reflect changes in the state of the maternal tissue and its relationship to the behaviour of the enclosed embryo.

Using population measurements of developmental parameters, by calculating the means or overall percentages of the measurements made on individual seeds, seed development over time in *T. ambiguum* is similar to other orthodox species (Figs 2A,F,K and 4A,F). A key point in population studies of seed development is the cessation of seed filling and achievement of mass maturity, defined as the attainment of maximum DW (Ellis and Pieta Filho, 1992). A single value (± s.e.) is usually given as the maximum DW for the population

based on samples taken at regular intervals during seed development. Adopting this whole population approach, our results show mass maturity occurred between 33 and 36 DAP when seeds reached a mean DW of 1.47 mg (shown for experiment 1 only; Fig. 2F). Achievement of mass maturity was followed by a rapid decline in seed moisture content (Fig. 2K), 'maturation drying' (Kermode and Bewley, 1985). However, it is clear these population parameters can be misleading at the individual seed level. Comparing the range in individual seed DW for seeds that were either in the process of drying or had dried to equilibrium with ambient, some seeds can be deduced to have reached their 'individual' maximum DW as early as 28 DAP in experiment 1 (Fig. 1A) or 27 DAP in experiment 2 (Fig. 1B). Identifying the latest individuals to achieve their maximum dry weight is problematic. Despite having a near common time of pollination, seeds that were still increasing in DW at *e.g.* 30 DAP and those that have already achieved their relatively low maximum DW and have started to dry, could not be distinguished with certainty. It is perhaps more likely that those individuals that had the fastest rate of seed filling were also those that were going to reach a higher maximum DW and would therefore be the heaviest within any harvest.

In our study we used only two maternal lines. Analysis of variance showed that at the population level, harvest date, maternal parent, number of seeds per pod, position within the inflorescence, and the interactions of these factors, accounted for large proportions of the variation in individual seed harvest FW over the developmental periods studied (94 and 86 % for experiments 1 and 2, respectively) and DW (77 and 62 %; Table 2). More rigorous partitioning of the phenotypic variance would require more lines and all combinations of reciprocal crossing. Furthermore, the efforts to eliminate any confounding effects of microclimate by randomising and/or rotating the positioning of maternal parents within the glasshouse would need to be greater than here. Nonetheless our results are consistent with the results of Castellanos *et al*. (2008), who found strong maternal effects, accounting for 59 %

of the phenotypic variation, in dispersal mass (analogous to our DW measurements) of seeds of *Aquilegia pyrenaica* subsp. *cazorlensis*, but did not rule out zygotic genotype effects. The residual variation we observed suggests that the embryo genotype is to some degree determining final seed mass in *T. ambiguum*. Looking at individual seeds also shows that, despite the small variation in pollination times, the variation in DW increases during seed development (Fig. 1). This is consistent with variation in the rate of filling between individual seeds. The similarity in seed DW between one- and two-seeded pods (Fig. 2H), between apical and basal seeds (of two-seeded pods; Fig. 2I), and between seeds taken from different parts of the inflorescence (Fig. 2J) implies little competition for assimilates. Nonetheless, ANOVA indicated that these factors were significant sources of variation. Overall the rate of seed filling appears to vary between individuals and depends not only on maternal genotype or environment and architectural effects, but also on the embryo genotype. Furthermore, the wide variation in DW of individual seeds within each maternal clone suggests an interaction between embryo and maternal genotypes. This is inconsistent with the maternal genotype influencing seed size by controlling embryo cell numbers as reported for genetically-homogenous pea cultivars (Lemontey *et al*., 2000).

The accumulation of seed DW ceases when the abscission layer forms within the maternal tissue and cuts off the supply of water and nutrients to the developing seed. From population studies, all seeds are thought to start losing water at this point and eventually equilibrate with ambient (Le Deunff and Rachidian, 1988). Our data (Fig. 1) suggests that the two processes are independent to a degree. Thus, as with variation between individual seeds in the timing of individual seed mass maturity, there is variation in the timing of the onset of maturation drying, despite the maternal genotype being identical. The non-normality of the water content of individual seeds during the period of rapid drying (Table 1) reflects the interactions between the similar maternal tissue and the genetically varied embryos. The rate at which

seeds dry may also vary, and again this may be determined by the embryo genotype or due to architectural / micro-environmental effects. Whilst there was little effect of seed number per pod (Fig. 2M) or position within a pod (Fig. 2N), seeds from the top of the inflorescence appeared to dry more quickly than seeds lower down the inflorescence (Fig. 2O). The radiation incident upon inflorescences may have declined progressively from top to bottom. If so, the temperature and relative humidity of the air surrounding pods would have declined and increased, respectively, from the top to bottom of inflorescences resulting in the variation in rate of maturation drying observed.

The maturation drying period was also the phase when seed coat colour changes were occurring, but again at different times for different individuals (Fig. 1B) and to some extent depending on maternal parent and position within the inflorescence from which the seed was taken (Fig. 3A-F). Although seed coat colour was not a precise indicator of seed moisture content across harvest times, within each harvest time, mean seed moisture content sequentially differed for each colour category, being highest for green seeds and lowest for dark orange seeds (Table 3). Seed coat colour change has been associated with changes in physiological activity and the onset of drying in other legumes. For example, in *Glycine max* respiration rate reduced as the seed coat changed from yellow-green to yellow (TeKrony *et al*., 1979). Hyde *et al*. (1959) reported that seed coat colour in *T. pratense* and *T. repens* started to change when the mean seed DW was 80 % of the DW at population mass maturity. In *T. ambiguum*, we might now interpret this variation in seed coat colour to reflect some individuals having reached their individual mass maturity earlier together with an earlier onset of drying. This again suggests an interaction between the embryo and the maternal seed coat which contrasts with the controlling role of the seed coat reported for seed filling in genetically homogeneous cultivars of legumes evolutionarily close to *Trifolium* (Lemontey *et al*., 2000). The acquisition of desiccation tolerance largely occurred just prior to the seed coat

turning from green to yellow (Fig. 1), although this was not a definitive marker in all individuals; some green seeds were desiccation tolerant at 36 and 38 DAP.

Germinability was first observed at 22 DAP (experiment 1; Figs 4A and 5A), before any individual seeds reached their maximum DW, however it was not necessarily the seeds with greatest harvest FW that were more physiologically advanced (Fig. 5B). Rather, it could have been those seeds that were closer to reaching their individual mass maturity that acquired germinability sooner. The onset of germinability occurred at essentially the same time for seeds from M1 and M2 maternal inflorescences suggesting that maternal genotype is not significant. Differences in architectural factors such as number of seeds per pods or position of the seed within the pod (Fig. 4B-D) also had little effect. However, seeds from the bottom of the inflorescence appeared to acquire germinability 3-5 d later than seeds from the middle or top of the inflorescence (Fig. 4E).

Desiccation tolerance was first observed at 33 DAP (experiment 1; Figs 4F and 5B), 11 days after germinability was first observed. Again, those seeds that acquired desiccation tolerance the soonest, may have been the individuals that reached their maximum DW earlier and started maturation drying earlier; the FW of individual 33 and 36 DAP seeds that germinated after rapid enforced drying tended to be towards the lower part of the FW range (Fig. 5B). This would again suggest some embryo genotype control. Looking at the population level, Sinniah *et al*. (1998) found that ending irrigation progressively earlier to plants of *Brassica campestris* resulted in maturation drying, onset of ability to germinate, and onset of desiccation tolerance all occurring sooner. However, whilst *T. ambiguum* seeds at the top of the inflorescence dried more rapidly (Fig. 2O), there is no evidence that they acquired desiccation tolerance sooner (Fig. 4J).

Hardseededness *in planta* was aquired late during development with 55 % of the seeds harvested at 54 DAP failing to imbibe when placed, fresh, on agar (Fig. 4K). However, all seeds harvested at 58 DAP did imbibe; this mirrors the reversible hardseededness reported in *Lupinus digitatus* (Gladstones, 1958). This loss of hardseededness coincided with an increase in seed moisture content (Fig. 2K), presumably a consequence of high ambient humidity at that time. At 54 DAP, mean seed moisture content reached 12.2 %, similar to the onset moisture content for hardseededness reported in other legumes; for example, 15 % in *Peltophorum pterocarpum* (Mai-Hong *et al.*, 2003); 12 % in *Pisum sativum* (Ellis and Roberts, 1982), *Lupinus arboreus*, *Trifolium pratense*, and *T. repens* (Hyde, 1954), and 11 % in *Lupinus digitatus* (Gladstones, 1958). With enforced desiccation post-harvest, however, hardseededness was induced considerably earlier (from 33 DAP) in some individuals and almost coincident with their acquisition of desiccation tolerance. That is, the ability of embryos to survive considerable enforced, rapid desiccation and of testas to become impermeable in response to extreme desiccation developed at similar points during seed development and maturation. This is compatible with seed development studies in *Ornithopus compressus* (Revell *et al.*, 1999), *Lathyrus maritimus*, and *Lathyrus sativus* (Chinnasamy and Bal, 2003). Because of the way hardseededness can be overcome in legumes it is usually considered a characteristic of the testa and hence maternal genotype. Since in our experiments there are only two maternal genotypes, we might expect there to be two sub-populations within each of which hardseededness affects all individuals identically. As this is not the case (Fig. 4L), this suggests the origins of hardseededness might be due to other factors we were unable to consider (such as location of the inflorescence on the plant) and/or under embryonic control, this capability developing between 33 and 47 d (Fig. 5B). Callose has been proposed as the cause of hardseededness in other *Trifolium* species (Bhalla and Slattery, 1984). Our results suggest that embryos of different genotypes exude callose at

different times during these early stages of maturation and the callose is then 'cured' by drying to produce the impermeable testa. *In planta* the variation in the frequency of expression of hardseededness and its reversibility suggests that the curing of the callose in the testa may be dependant on the extent of desiccation, its duration or some combination of them.

Whilst limited as an internal non-destructive parameter of development, FW when combined with more traditional measures such as period since pollination and if applied at the single seed level can provide insights into the developmental physiology of seeds (of out-breeding plant species). As Figure 1A shows, a seed with FW 2.5 mg could have been harvested anytime between 22 and 40 DAP (experiment 1), however only those harvested at 40 DAP might have been desiccation tolerant. When the other non-destructive measure of the condition of the maternal tissue, seed coat colour, is also taken into consideration, the diagnosis becomes more certain.

Seed longevity

The longevity of a population of developing seeds increases over time. Using probit analysis to fit survival curves to all the experimental storage data within each harvest, the estimate of time for viability of the seed population to fall to 50 % (p_{50}), increased from 4.3 d at 36 DAP to 40.1 d at 56 DAP (Fig. 3). The improvement in longevity (in subsequent experimental storage) for 20 or so days after population mass maturity in *T. ambiguum* seeds is also similar to other species (*e.g.* Pieta Filho and Ellis, 1991; Demir and Ellis, 1992*a*,*b*; Hay and Probert, 1995). Thus, the optimum time to harvest seeds of *Trifolium ambiguum* in order to have maximum longevity coincided with the seeds reaching equilibrium with ambient conditions and was after all the seed coats had become orange or dark orange (Figs 1B and 3). In most

wild plant species, reaching moisture equilibrium with ambient is also when the seeds will reach the point of natural dispersal (Hay and Smith, 2003). However the *T. ambiguum* pods did not dehisce and there were on-plant declines in longevity between 56 and 70 DAP; p_{50} dropped to 33 d (Fig. 6). On-plant decline in longevity is characteristic of species, mainly crops, which desiccate considerably but have been selected to not release their seeds. For example, on-plant declines in seed longevity were reported in millet (*Pennisetum glaucum*; Kameswara Rao *et al*., 1991) and barley (*Hordeum vulgare*; Pieta Filho and Ellis, 1991). Between 36 and 44 DAP, increases in p_{50} were largely due to increases in K_i (eqn. 1), presumably as all the developing seeds finally acquired desiccation tolerance (Fig. 7A). K_i can be a more sensitive measure of the desiccation tolerance of a population of seeds than a germination test that uses a relatively small number of dried seeds, provided that the most advanced seeds have not started to lose viability.

There is not yet any means to identify the exact moment when individual seeds lose the ability to germinate. Further, in the most immature *T. ambiguum* seeds tested for longevity (36 DAP), it is not possible to distinguish between those seeds that died early in experimental storage and those that had not acquired desiccation tolerance (Fig. 6A). For seeds harvested at 40 DAP, there is evidence that some seeds had lost the ability to germinate by the time that the 7 d sample was withdrawn from experimental storage (Fig. 6C). However, some individuals were still able to germinate after 28 d in storage. Similarly, for seeds harvested at 63 DAP, some seeds lost the ability to germinate after between 14 and 21 d in experimental storage, but one seed was still able to germinate after 63 d (Fig. 6H). Given their common albeit heterozygous parents and their near synchronous fertilisation, this 3- to 4-fold variation in individual seed longevity indicates the large effects that genotype and maternal environment can have on this seed behaviour.

This variation in the time to seed death can be assessed by σ (eqn. 1). For seeds from 195 geographically and taxonomically diverse species, estimates for σ under the same storage conditions as used here ranged between 0.9 and 397 d (actual σ values not reported, but see Probert *et al*., 2009). For eight legume species, the predicted value of σ under these experimental storage conditions ranged between 11 (*Arachis hypogaea*) and 72 (*Vigna radiata*) days. The estimate for another *Trifolium* species, *T. subterraneum* was 15 d [estimated using the 'predict days to lose 1 probit' module of the Seed Information Database (Liu *et al.*, 2008)]. Here, in *Trifolium ambiguum*, the population estimates for σ ranged between 8.3 and 16.6 d (Fig. 7A). For all seed-lots (i.e. populations of mature seeds) within a species, the value σ is expected to be constant if they are stored under precisely the same storage conditions (Ellis and Roberts, 1980). This was also the case here during seed development: the *T. ambiguum* survival curves for the different harvest times (not shown) could be constrained to a common estimate of σ (13.6 d) without a significant increase in residual deviance (P>0.05). Comparing the common estimate of σ for *Trifolium ambiguum* with that for *T. subterraneum*, we have perhaps slightly reduced seed-to-seed variation in the time to seed death – by synchronising pollination to an 8 hour period and only using two parental lines – but it has certainly not been eliminated. We have not determined σ for *T. ambiguum* seed lots produced following normal collecting (and growing) practice.

The FW distributions for seeds that did germinate did not differ from those that did not, for each period of experimental storage and within each harvest (Fig. 6). For instance, for seeds harvested at 36 DAP, when some seeds may not have reached their individual mass maturity, the stored seeds that did not germinate and were perhaps not desiccation tolerant did not have the highest (*i.e.* had not started to dry) harvest FW (Fig. 6A). Individual seed FW is not a reliable indicator of individual seed storability. Furthermore, for *Trifolium ambiguum* seeds that have dried to equilibrium with ambient by the time they are harvested (49 – 70 DAP),

greater DW does not infer greater lifespan (Fig. 6). Thus a similar statement can be inferred for FW since after 49 DAP, FW and DW are correlated (Fig. 1B).

Even for highly-bred crop species, which are largely genetically homogeneous, a seed-lot where all the seeds lose the ability to germinate at the same time (*i.e.* a square survival 'curve' with a long lag before very rapid decline in germination) has not been reported. However in out-breeding species, it would seem that, rather than variation in lifespan being simply a reflection of physiological maturity, not all seeds within a developing population have the potential to acquire the same level of resistance to ageing in dry storage. Individual seed lifespan may be another trait that, like final seed mass, is determined by the unique genotype of the embryo. Furthermore, longevity is likely to be under multi-gene control. In rice, three quantitative trait loci for seed longevity, mapped to different chromosomes, have been identified (Miura *et al*., 2002; Xue *et al*., 2008). The lack of reduced seed-to-seed variation in lifespan of seeds of crop species suggests that although genetically homogeneous, the variation of seed maturation environment significantly effects individual seed longevity.

We did attempt to identify whether any of the factors that were a source of variation in individual seed FW or DW also influenced seed-to-seed variation in lifespan, by looking at the survival data for cohorts of seeds within each harvest, however consistent trends across the harvest dates could not be detected (not shown; Butler, 2007). The estimates for σ were almost always lower for sub-populations compared with the estimates for the whole population at each harvest, suggesting that there was greater synchronisation in the time to seed death. However, the estimates could usually be constrained to common values for the different cohorts (P>0.05) and the lower estimates for σ are more likely to be due to having less data rather than a real reduction in variation in individual seed lifespans.

Trifolium ambiguum *as a model system*

Overall controlled crossing of clonally-propagated self-incompatible *Trifolium ambiguum* provided an appropriate model system for studying individual seed development. This model species provides a valuable contrast to the restricted view gained from the genetically homogeneous seeds of frequently studied crop species. Already some improvements to our experimental design have become clear. Each harvest involved sampling entire inflorescences and processing each seed individually; the number of seeds included in the experiments was limited by the time required to process individual seeds rather than by the number of seeds available for observation. However the number of seeds and pods varied between harvests, as did the number of seeds falling into each cohort and some combinations of maternal parent, number of seeds per pod, position within the inflorescence, and position within the pod were not represented at each harvest in experiment 1. A better sampling procedure may have been to sample pods from across the population, ensuring good representation of all the factor combinations, although this may have had an impact on the development of those which remained to be sampled later. Rather more two-seeded pods were produced than expected. This may have been the result of selecting the most vigorous clones and then selecting the pair of clones with the greatest seed set. Nonetheless, the effect of number of seeds per pod on the developmental parameters studied was comparatively small. Perhaps surprisingly (given the potential for competition for assimilates within a pod), this was as true for seed DW as it was for, say, the development of desiccation tolerance.

By growing the plants in a controlled environment and synchronising (to within 8 hours) pollination, we aimed to investigate the extent to which seed-to-seed variability could be reduced in the developing population of *T. ambiguum* seeds. However, significant variation in physical and physiological development remained. Despite using only two parental lines, since *T. ambiguum* is an out-breeder, the heterozygosity within these parental genotypes

created populations of genetically-heterogeneous embryos whose genotypes exerted significantly varied effects on seed development. The effects of number of seeds per pod, seed position within the pod, or position of the pod on the inflorescence, or their interactions with harvest time were relatively small, albeit in some cases significant, possibly because later flowers were removed, so ensuring that resources to the developing seeds were not limited. The extent to which the picture presented here would change in a more natural, open-pollinated situation, where more competition and greater main or interaction effects of these architectural factors could be anticipated, remains unclear.

Conclusions

Whilst not without some difficulties of interpretation, we have shown that a tightly defined seed production system combined with both destructive and non-destructive measurement of seed behaviour, allows the physiology of seed development to be studied at the level of individual seeds. We have shown

- The behaviour of individual seeds varies greatly compared with that which might be expected by extrapolation from the population means.
- The different physiological stages of seed development can be significantly varied temporally between individuals.
- Development processes appear to be under the control of both genetic and maternal environment factors. There is little evidence that these maternal factors are due to limited supplies of resource, rather they appear more associated with maternal plant architecture.

- Interaction between the maternal and filial genomes during development can be deduced to account for the variation in the completion of grain filling and the commencement of maturation drying between individual seeds.
- From our results we deduce that the variation found in individual seed behaviours within a population of out-breeding species could be expected in the variation between seed populations of varieties of in-breeding crop species provided they have not been unintentionally lost during the processes of domestication and breeding.
- As we concluded elsewhere (Butler *et al*., 2009) and has been demonstrated by others (Still and Bradford, 1997; Mo and Bewley, 2003), caution should be taken when drawing conclusions from studies using samples of multiple seeds and calculating mean values or proportions.
- Individuals within a population of developing seeds differ with respect to the potential lifespan that they can acquire and that variation is likely to be influenced by the embryo genotype.
- The value of σ for *T. ambiguum* seeds aged at 60 % RH and 45 °C did not appear to be reduced by controlling the time of pollination and using only two maternal lines, Since it may not be possible to ever make a collection of seeds with uniform lifespan, those involved in the storage of seed can continue to exploit the normally-distributed variation and use the time when viability of an accession falls to 85 % germination to indicate the point when the percentage viability of a seed lot starts to decline rapidly and thus a prompt for regeneration or recollection of that accession.

Our results also show the value of understanding the genetic background of the different seed tissues when interpreting physiological behaviour. Both in-breeding homogeneous crops and highly out-breeding undomesticated species produce seed development plots of similar shape which require different interpretation. In our highly controlled experiments with

simultaneously pollinated *T. ambiguum* flowers, explanations of the variation in behaviour between individual seeds can properly include variation in embryo genotype as well as factors of plant architecture and internal maternal environment. At the other end of the spectrum, in self-pollinating crops, similar seed-to-seed variations will need to be exclusively interpreted in terms of the timing of fertilisation and the external and internal maternal environments. The possibility that these variations can also vary between seasons and locations makes the likelihood of repeatedly creating seed lots of similar population structure small. This frustrates attempts to understand the molecular behaviour of seeds through the homogenisation of many individuals from a population, in a similar way that the identification of mass maturity in the population obscures the variation in individual maturities.

SUPPLEMENTARY INFORMATION

SI 1. 'Passport information' including harvest FW, DW, and moisture content of individual *Trifolium ambiguum* seeds harvested between 14 and 64 DAP (experiment 1, pollinated 8 Dec. 2004).

SI 2. Harvest FW, DW, and moisture content of individual *Trifolium ambiguum* seeds harvested between 20 and 70 DAP (experiment 2, pollinated 11 Jan. 2006).

SI 3. 'Passport data' recorded for each individual *Trifolium ambiguum* seed harvested between 36 and 70 DAP (experiment 2, pollinated 11 Jan. 2006) and tested for fresh or dry

germination or germination after the period of dry storage indicated [‘time aged’ (at 60 % RH, 45 ºC)].

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FIG. 1. The relationship between individual seed DW and FW at harvest during seed development in *Trifolium ambiguum.* (A) Experiment 1, hand-pollinated 8 Dec. 2004, harvests from M1 maternal line only; a similar pattern was apparent for seeds with maternal line M2, however not all harvest dates were represented and hence data is not shown. (B) Experiment 2, hand-pollinated 11 Jan. 2006. Correlation coefficients (R values) are within each harvest except for seeds harvested at 44 DAP (experiment 1), where a split-line model was used and for seeds harvested at or after 47 DAP (experiment 1) or 49 DAP (experiment 2), where the data was combined for the different harvests. In (B), symbol labels for 36 to 44 DAP indicate seed coat colour as assessed by eye: G = green; Y = yellow; O = orange; DO = dark orange. All seeds at 49 to 70 DAP were orange or dark orange.

FIG. 2. Changes in (A-E) harvest FW (mean ± s.e.), (F-J) DW (mean ± s.e.), (K-O) moisture content (mean ± s.e.) during seed development in *Trifolium ambiguum* (experiment 1; hand-pollinated 8 Dec. 2004). Means calculated for all seeds (A,F,K) or for cohorts sorted by maternal parent (B,G,L), number of seeds per pod (C,H,M), position within the pod (D,I,N), position within the inflorescence (E,J,O).

FIG. 3. The proportions of *Trifolium ambiguum* seeds from experiment 2 (pollinated 11 Jan. 2006) falling into five colour categories (bars, left hand axis) and the mean seed moisture content (± standard error; solid squares, right hand axis). Proportions shown for all seeds (1st column) and for cohorts sorted depending on whether they were harvested from M1 or M2 maternal parent; from a single-seeded pod or position within the pod if 2-seeded; from the bottom, middle, or top of the inflorescence. Each moisture content shown was determined with approximately 20 seeds; no seeds taken from the top of the inflorescence at 63 DAF were used for moisture content determination.

FIG. 4. Changes in (A-E) fresh seed germination, (F-J) desiccation tolerance (seeds dried over silica gel at 20 ºC for 28 d), (K-O) hardseededness of fresh (solid symbols) or dried (hollow symbols) seeds during seed development in *Trifolium ambiguum* (experiment 1, pollinated 8 Dec. 2004). Proportions shown for all seeds (A,F,K) or for cohorts sorted by maternal parent (B,G,L), number of seeds per pod (C,H,M), position within the pod (D,I,N), or position within the inflorescence (E,J,O).

FIG. 5. The FW of individual seeds of *Trifolium ambiguum* (experiment 1, pollinated 8 Dec. 2004) harvested between 14 and 64 DAP from M1 maternal line that did or did not germinate and were or were not hardseeded when placed to germinate (A) immediately after harvest or (B) after drying over silica gel (for 28 d at 20 °C).

FIG. 6. The FW of individual seeds of *Trifolium ambiguum* (experiment 2, pollinated 11 Jan. 2006) harvested between 36 and 70 DAP that did (open triangles) or did not (closed circles) germinate after different experimental storage periods (x-axis) at 60 % RH and 45 ºC. The vertical dotted lines indicate the time when viability had fallen to 50 % (p_{50}), estimated by probit analysis.

FIG. 7. Changes in longevity parameters during seed development in *Trifolium ambiguum* (experiment 2, pollinated 11 Jan. 2006). (A) Estimates of K_i (open circles) and σ (closed squares) when all the data within each harvest was included in the probit analysis. (B) estimates of σ for cohorts of the population sorted according to seed coat colour. The common value of σ was 13.6 d (see text).