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IMPROVEMENT OF SPROUTING CONTROL IN WHEAT; ROLES OF EMBRYO CAVITY WAXES AND GRAIN COLOUR GENES

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IMPROVEMENT OF SPROUTING CONTROL IN WHEAT; ROLES OF EMBRYO CAVITY WAXES AND GRAIN COLOUR GENES

by

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ABSTRACT

Pre-harvest sprouting presents the greatest single obstacle to increased incorporation of home-grown wheat into high value, such as bread flour. It is generally agreed that the solution to the problems of sprouting must be solved by breeding greater control of dormancy into new varieties but establishment of the dormancy status of new varieties is difficult and may take several years. Improvement thus comes slowly and inclusion of sprout resistance characters from one generation to the next cannot be guaranteed. In the absence of adequately reliable tests for resistance to sprouting the identification of markers which distinguish dormant from non-dormant types was an important objective of this project. Markers of two types have been examined: an established marker, grain colour, has been scrutinised in order to make it more readily and reliably usable by breeders, and the significance of a novel embryo cavity wax, indicated to be associated with sprout resistance in an earlier study, has been critically evaluated.

In relation to genes controlling grain colour, the aims of this project were to measure the effects of individual *R* genes and to identify molecular markers for the *R* genes and to identify the *R* genes present in U.K. breeding stock.

The dormancy-enhancing effects of genes for red grain colour were demonstrated in near-isogenic lines carrying five different *R* genes in a white spring wheat background. These lines provided compelling evidence that the *R* genes are directly responsible for the greater sprout-resistance of red-grained (as opposed to white) varieties and are not simply acting as genetic markers for other dormancy factors. Additional observations suggested that this effect depends on the presence of a continuous layer of red/brown pigment in the seed coat. Different *R* genes each have a similar effect on grain dormancy, delaying germination by a factor which diminishes with time after harvest ripeness.

Genetic markers for each of the *R* genes are described for the first time, with genetic maps showing the gene order and recombination frequencies between diagnostic DNA sequences and the *R2* gene on the long arm of chromosome 3A, *R3* on 3B, and *R1* on 3D. These genetic maps support growing evidence that the chromosomes of different cereal crops share a common ancestral structure, with similar genes in similar arrangements.

The number, and in most cases the identities of *R* genes carried by 109 wheat varieties and breeding lines were analyzed with respect to their frequencies in the U.K. gene pool. Although U.K. varieties were virtually all red-grained, they showed no evidence of effective selection against any of the three *r* (white-grained) alleles, probably because these are masked by the presence of dominant *R* alleles. This survey included the first description of varieties carrying *R3* in the absence of other genes for grain colour, allowing genetic mapping of *R3* without confounding effects from duplicate *R* genes on other chromosomes.

Sprout-resistance scores were shown to vary between different U.K. varieties in confirmation of the acknowledged resistance of red- as opposed to white-grained types, however variation within the red-grained group is independent of *R* dosage and is not associated with any particular *R* gene more than the others. The presence and practical importance of grain dormancy genes independent of the red pigment effect is inferred, and

evidence for simple additive gene effects on grain dormancy in crosses between *R1 R2 R3* varieties is presented.

It is concluded that adequate sprout-resistance depends upon a combination of traits. Red grain colour is one of the necessary factors, but very little is known about the other components. In order to understand the mechanisms of resistance, and to discriminate conveniently between resistant and susceptible wheats, marker traits associated with different aspects of resistance to pre-harvest deterioration in Hagberg Falling Number were investigated. Potentially useful marker traits including embryo cavity waxes, the rate of water penetration into different grain tissues during imbibition, and the production of germinative-type *alpha*-amylase enzymes during ripening were evaluated.

Variation for *alpha*-amylase content in ripe, ungerminated grains is described among recombinant lines from a low-amylase x high-amylase cross, in which the high-amylase character appears to be controlled by two or more recessive genes. Evidence is described supporting the involvement of a gene on the long arm of chromosome 3D, either the *r1* gene or a different, linked gene.

In relation to embryo cavity waxes, the objective of the project was to investigate their composition and their relation to grain colour/dormancy differences, and to investigate mechanisms underlying variation for resistance to sprouting. Success in relation to this objective has been severely limited by technical difficulties associated with handling extremely small quantities of very complex materials. While broad generalisations concerning the composition of embryo cavity waxes can now be made, it has not been possible to distinguish components or relationships among components that are indicative of a particular dormancy condition. The same problems of scale and complexity also proved too great for conclusions to be reached about the physical properties of the waxes. At an early stage in the investigation, the surface activity of embryo cavity extracts promised to provide the basis of a rapid test by which sprout resistance might be recognised. However, excessive variation obscured any systematic differences that might be present.

When the pursuit of waxes as markers was abandoned, attention was focused on another possible marker for *alpha*-amylase activity. Unlike colour and the hypothesised role of embryo cavity waxes, grain size has more significance in relation to the enzymes that are produced in endosperm in the late stages of grain maturation, than to those produced during germination, although there is some evidence for a connection here also. Evidence from several sources suggests that, in varieties with large grains, higher levels of activity than those found in small grained varieties are likely. The observation that this was the case came originally from coordination of data recorded elsewhere but it has been substantiated in this project. Also in this project, the unusual (and unlikely) enzyme source has been identified as the aleurone tissue in the crease region of the grain. The relationships between grain size and both late maturity and germination *alpha*-amylases are considered worthy of further investigation.

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GENERAL INTRODUCTION

Background to the project

The investigation reported here arose from the recognition of potential synergy to be derived from collaboration between two groups working under independent project funding from the same sources, on different aspects of an extremely complex and economically important problem. As a preliminary to full integration of endeavours, a joint report to HGCA was prepared by the two groups involved, *viz* Flour Milling and Baking Research Association (FMBRA), now part of Campden and Chorleywood Food Research Association (CCFRA), and the IPSR Cambridge Laboratory at Norwich (now part of the John Innes Centre). The report was published as **Project Report No 77 Genetic and Physiological Studies on Sprouting in Wheat by HGCA** (Flintham, Evers and Kratochvil 1993).

That report reviewed the economic importance of the problem of excessive *alpha*-amylase in cereals and showed that contributions to solving the problem were likely to emerge from further investigation of two relevant factors: grain colour and the occurrence of a waxy substance identified in the embryo cavity of some wheat varieties. Grain colour had already been shown to exert a major influence on susceptibility to premature sprouting and this had led to the strategy of growers sowing only red varieties since the 1960's. This policy has contributed more than any other factor, to the production of unsprouted harvests in all years except those with the most adverse weather conditions.

Grain colour is determined by three major genes, designated *R*, located separately within the A, B and D genomes present in wheat. The work on grain colour undertaken in the project has been:

a) continued examination of the detailed relationship between resistance to sprouting and the *R* genes, both separately and in all possible permutations;

b) establishing the *R* gene constitution of varieties; and

c) precisely locating the genes on their respective chromosomes.

Dormancy

The significance of premature germination, or sprouting, lies in the production of enzymes, and particularly *alpha*-amylase, that induces sprouting. Within a grain sample, the amount of enzyme produced is related to the length of time for which germination continues, but among samples, enzyme levels can vary for other reasons also. Variations may result, for example, from inherent differences in ability to respond to hormones that initiate enzyme synthesis in the aleurone cells that constitute the outermost layer (or layers in some species) of the endosperm tissue.

Dormancy indicates the inability of a viable seed to germinate when subjected to conditions that might be expected to give rise to germination. It is a characteristic capable

of conferring selective advantage on seeds of plants growing in changing environments, since it can prevent germination under conditions that are favourable for an unduly limited period. Variable dormancy among seeds of the same plant can also provide a stock of potential plants, capable of being generated over an extended period, thus increasing the chances of some of the plants growing to maturity under favourable conditions (Simpson, 1990).

In cultivated plants, such as cereals, the concept of selective advantage has little significance since the grower attempts to minimise the selective pressures that apply to his crops. In relation to dormancy, the time of sowing is chosen to ensure the establishment of plants during a season when conditions are favourable. The value of dormancy, as a device for guarding against germination during conditions that misleadingly appear favourable, is thus reduced. The presence of variable degrees of dormancy among seeds produced at the same time in a crop plant is clearly disadvantageous as the establishment of a uniform stand of plants at a similar stage of development is required. As cultivation methods have improved the need for dormancy has declined and selective breeding has progressively produced varieties in which dormancy is reduced.

In some species, total absence of dormancy leads to a condition known as vivipary, in which it is the norm for grains to germinate on the ear as soon as they attain physiological maturity, irrespective of ambient conditions. In wheats, such extreme absence of dormancy is unknown and even white wheats germinate only when ambient conditions are sufficiently moist, following attainment of ripeness.

Dormancy is a complex factor, its several causes residing in several grain tissues. The viviparous condition is simple to interpret as it is indicative of an absence of all dormancy-inducing mechanisms. Where any dormancy exists however the cause may be one or more of any of the known or even unrecognised mechanisms and it is conceivable that the same degree of dormancy may be induced by several mechanisms acting singly or in concert. A further complication arises as degree of dormancy can be influenced by environmental conditions during growth and maturation of reproductive and possibly vegetative parts of a plant.

Markers

In a breeding context, grain colour constitutes a "marker" for dormancy or resistance to sprouting. The requirements of a marker are that it should be an easily perceived feature, the presence of which may not be an important breeding criterion in itself, but which signals a more important, but less easily recognised characteristic. The degree of success of markers varies and although redness is a high grade marker, as demonstrated by the influence that it has exerted on breeding and agricultural practices, it has two frustrating shortcomings, the first of which is intellectually challenging and the second is experienced in a more practical fashion. The intellectual frustration arises from an inability to understand the association between colour and dormancy. Many useful observations have been made in this context but, as yet, no satisfactory physical, hormonal or physiological connection has been made between the presence of pigmentation in the seed coat and the

dormancy that is associated with it. The question of whether redness is a mere "marker", or a causal factor in maintaining dormancy is addressed in this study.

The second frustration associated with redness is that it is not totally reliable, for although red grains are almost always more dormant than white, their degree of dormancy is variable. Further, although an additive relationship exists between degree of dormancy and *R* gene dosage, the relationship is non-linear and even inconsistent. In consequence, additional markers remain highly desirable in the quest for optimum control of germinability and this urgent requirement justified interest in the second factor described in Project Report No 77 (Flintham *et al.*, 1993). Unique tufts resembling surface waxes encountered on leaves of many plants had been observed in apparently crystalline form, in embryo cavities of red wheat types that had been stored for some 20 years, but not in similarly stored white types. In fresh grains, evidence had been presented for systematic variations among red and white wheats, in contents of waxes of similar composition to the crystalline types. In this project we further investigated the association, through extension of the sample set on which microscopical observations were made, and through chemical analysis of extracts from tissues surrounding the embryo.

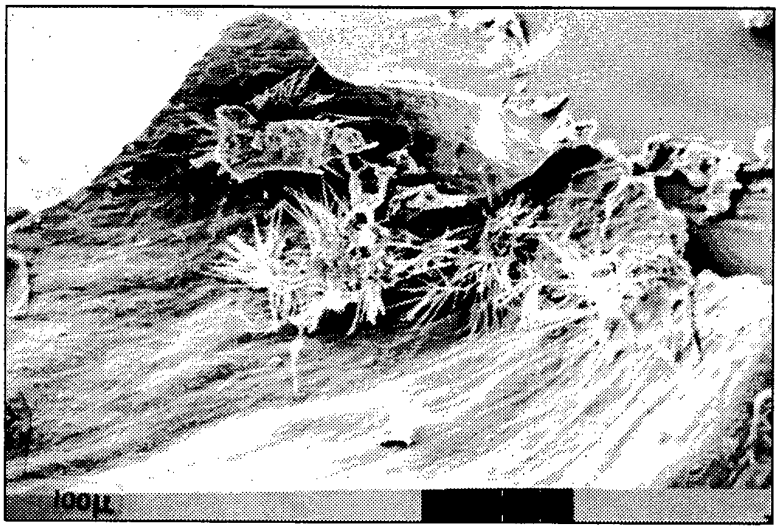


Fig 1.1 Tufts of wax found in the embryo cavity of long-stored red wheat grains. SEM micrograph.

Types of *alpha*-amylase

Alpha-amylase catalyses the digestion of starch stored in the starchy endosperm, thus reducing the mass of solids present in that tissue and contributing to a reduction in crop yield. Of greater importance however is the effect that the enzyme has on processing. During the early stages of breadmaking, for example, conditions favouring enzyme activity

are created, and the combination of these with an increase in the susceptibility of the substrate, starch, brought about by the onset of gelatinisation, leads to rapid deterioration in the condition of the dough. As well as adversely affecting the handling characteristics of the dough the deleterious changes are carried on into the baked bread itself as products of the enzymic hydrolysis are dextrins which cause stickiness in the crumb and excessive darkening of the crust (Buchanan and Nicholas 1980).

Both redness and the presence of the waxy substance in embryo cavities have been pursued primarily or exclusively because of their perceived relationship with excessive enzyme production resulting from germination. This is not however the only cause of excessive production of enzymes and other conditions have been recognised in which higher than normal *alpha*-amylase activities are present. These are now well documented (Kruger 1980) and the conditions have been identified with names that go some way towards defining them. Also, the iso-enzymes typically detected in each by iso-electric focusing have been established (Marchylo *et al* 1980), and this provides some help in distinguishing them, although there are few bands that are unique to any one type. In chronological order of their appearances during maturation they are known as: "pericarp", "late maturity", and the "germination" *alpha*-amylases, the last being the only one considered so far in this report. The nomenclature provides a clear distinction between the last two types but it does not provide adequate distinction between the first and second as, in some cases the pericarp enzyme can persist into late maturity. During this project we have established that the source of late maturity enzyme is the endosperm and hence we adopt the precise, if somewhat cumbersome, description: "late-maturation endosperm *alpha*-amylase" throughout.

Physiological functions of hydrolytic enzymes

The presence of germination enzymes is capable of a logical explanation since the energy requirements of the growing embryonic axis, and of the plant that it becomes, can be met by the soluble sugars produced by starch digestion, to which *alpha*-amylase contributes. A further endorsement of this functional relationship is the polarisation of its distribution, initially in the scutellum, and later in the aleurone layer of the endosperm, with activity being evident firstly at the embryo end and progressively towards the brush end (Gibbons 1980). This distribution is compatible with an assumption that the stimulus for enzyme production originates in the embryo itself, and, although the full details remain to be clarified, there is much supporting experimental evidence for such an origin. Starch digestion, this time in the pericarp of developing grains, also provides a logical explanation of the presence of the more recently discovered "green" *alpha*-amylase, found in that tissue, where starch granules form an ephemeral reserve in the early stages of grain development.

Unlike the other two types, late maturation endosperm enzyme defies rational physiological explanation. It arises in grains in which no germination is detectable and indeed, in some experimental instances, in grains that have been protected against germination-inducing conditions. Neither can any alternative need for starch hydrolysis be proposed. It is possible that the presence of the enzyme signifies an aberrant condition.

Unlike germination related enzymes, its distribution is inconsistent (Seymour 1984),

suggesting that its production is independent of initiation by any non-endosperm grain component. While germination enzymes, produced in scutellum, aleurone or both, are found in other cereals, non-germination *alpha*-amylases have been reported, to our knowledge, only in wheat and its interspecific hybrid with rye: triticale.

Some authors have sought to use information on pericarp enzyme levels as a means of predicting levels of equivalent enzymes in mature grain. While this is possible in established stable varieties, it should be remembered that in segregating populations the genetic constitution of pericarp, being a maternal tissue, is different from that of the filial embryo and endosperm (the endosperm is even different from the embryo because, although of the same generation, endosperm nuclei have twice the maternal contribution present in embryo nuclei, as endosperm results uniquely from a fusion of one paternal and two maternal nuclei). No predictive relationship has been proposed between levels of late-maturity endosperm *alpha*-amylase and germination enzyme production or dormancy. In spite of this, the relationship between the two types is significant to processing when they occur together, as they combine to exceed the threshold of acceptability determined by users.

To processors of wheat flours, pericarp enzymes have little significance because they usually decline to negligible levels before harvest and because they arise in a tissue that is removed, at least from white flour, during milling. When germination and late maturity endosperm enzymes are present in large enough amounts however, they are deleterious to the quality of a sample or a product. To researchers seeking to minimise the problem through maximising control over enzyme production, knowledge of which enzyme, or combination of enzymes, is causing problems in a given situation, is vital.

Red grain colour genes in near-isogenic lines

Although it has been known for many years that red-grained wheats are much more resistant to sprouting than white-grained wheats, several questions regarding the role of grain pigmentation as a marker for grain dormancy remained unresolved prior to this project. In particular, as has already been indicated, it was not clear whether red grain colour itself is directly involved in the control of germinability, or whether the genes for redness simply act as markers for other dormancy genes which are co-inherited due to close association on the same chromosome. This question is addressed by measuring the germinability of grains from closely related "isolines" carrying different *R* (red) or *r* (white) genes in a common genetic background from a white-grained wheat. Because these lines were produced by repeated backcrossing to a white-grained parent, virtually all of their genes are identical to that parent except for the particular *R* gene which was selected after each round of crossing.

Differences between these lines and the white parent are thus attributed to the *R* genes rather than to chance association with other dormancy factors. Comparisons between the red-grained isolines provide a test of the equality of different *R* gene effects, and a sequential series of germination tests reveal the duration of *R*-mediated resistance to sprouting.

Because there are three different *R* genes, and a single copy of any one of them is sufficient to produce red pigment, one cannot tell which genes are carried simply by looking at the grain. Two different methods for revealing the exact *R* gene status of any wheat have been investigated, one relying on a traditional test-cross breeding technique, and a rapid alternative based on new molecular methods.

The test-cross method has been employed to assign *R* genotypes to a large sample of wheat varieties which have been widely grown in the U.K. in recent years. This was done in order to see whether there might be scope for improving the sprout resistance of future varieties by increasing the number of *R* genes carried or by selecting particular combinations of genes. This genetic survey complements a survey of resistance to sprouting in the same varieties, enabling comparisons to be made between the propensity of a particular wheat to sprout in the ear and the number or identity of *R* genes which it carries.

The test-cross method involves a minimum of three cross-pollinations and three generations of plants for every wheat tested, and is certainly not a viable technique for screening large numbers of new breeders' lines. Advances in molecular biology allow breeders to use DNA as a marker system. It involves cutting the DNA of the plant at specific sites to generate DNA fragments of many different sizes. Genetic differences between plants can be related to differences in the sizes of their DNA fragments. The degree of association between a target trait such as grain redness and specific DNA fragments is expressed as a genetic map, in which the chromosome is represented as a straight line with the DNA and other markers located at intervals along it. The distance between any two markers on the map denotes the frequency with which they will both be inherited together. DNA markers located close to the *R* genes can be used to identify the *R* genes carried by a plant from just a small leaf sample, providing a much more rapid alternative to the cumbersome and time-consuming test-cross technique.

Other genes for sprout-resistance

The existence of other genes for resistance to sprouting had been suspected from previous work, and is confirmed by the finding that different varieties carrying the same *R* genes can nonetheless vary quite widely for grain dormancy. There are two complementary approaches to investigating such "non-colour" effects and examples of both are described in this report.

The breeding behaviour of genes controlling resistance is determined amongst progeny from crosses between resistant and susceptible wheats. Differences between the parents, F_1 hybrids, and new recombinant lines arising from such crosses are analyzed to estimate how many resistance genes are involved, and how they interact with one another. Such breeding experiments can also be used for molecular tagging of dormancy genes in an extension of the genetic mapping technique described above.

As an alternative to this type of breeding experiment, physiological marker traits which are considered to have a potentially direct association with resistance to sprouting are also investigated. Investigations into embryo cavity waxes, rate of water uptake by dormant

and non-dormant grains, production of *alpha*-amylase during ripening, and effects associated with grain size differences are examples of this approach.

The value of archives

It is well established that the subject of seed germination is a complex one. As a result of this and its importance in the context of seed establishment, both of crop and weed plants, a wealth of literature and data has accumulated. In some cases it has been possible to save samples also. This study has benefitted from conservation, by the participants and others, of all these classes of material.

The value of collecting and conserving crop data on an annual basis is not always apparent, and perhaps insufficient attention is paid to archives by many undertaking original research. It is particularly valuable in the case of agricultural or other experiments where conditions such as weather cannot be controlled. The resulting variation often renders single experiments of little predictive value but a well planned archive allows deductions to be made on the sound basis of many years experience. By reviewing archives, much repetition may be saved and discovery of new relationships, unsuspected at the time of data collection may be made. In this work examination of data from NIAB and ADAS was instrumental in the uncovering of the apparent relationship between grain morphology and enzyme status, a relationship that has been further examined as part of this project.

2) METHODS

2.1) Selection and dormancy testing of varieties

2.1.1) Selection

Wheat varieties were selected for study from NIAB Lists of Varieties in Trials from 1983 on, with bias towards entries which achieved Classified List status (NIAB 1983-1992a,b). These wheats were taken as representative of the contemporary U.K. gene pool, which will make a major contribution to new varieties in the future. Additional wheats were included for study, either as genetic standards, or because they are parents of special genetic stocks such as chromosome substitution lines, or for their known extreme (high or low) dormancy. All wheats were sampled from the AFRC Cereals Collection at JIC and a single typical plant of each accession was used to multiply seedstocks by enforced self-pollination.

Near-isogenic lines carrying different *R* genes in a Russian white spring wheat background also became available during the course of this project. The white variety Novosibirskaya 67 and the red lines ANK-1A, ANK-1B, ANK-1C, ANK-1D and ANK-1E, bred by Dr. S.F. Koval at the Institute of Cytology and Genetics, Novosibirsk, were donated by Dr. A. Vershinin of the Russian Academy of Science.

2.1.2) Dormancy test

Resistance of varieties and F_1 hybrids to sprouting in the ear was assessed by misting ripe ears in a "rain chamber". Duplicate samples of ten ripe ears from each entry were after-ripened for four weeks following harvest, then misted for three days, allowed to dry in a glasshouse, hand-threshed and scored for percentage visibly sprouted grains. Varietal sprout-resistance data accumulated from a previous project (Flintham *et al* 1993) were cross-referenced to the *R* genotypes of the same varieties in order to test for resistance effects attributable to particular *R* genes or dosages.

Grain dormancy in individual ear samples was investigated by gently teasing out ten grains from basal florets of central spikelets on one side of each ear, the remainder being stored for eitherembryo cavity wax analysis or for subsequent dormancy tests. For each wheat sampled ten ripe grains from each of ten ears were placed embryo uppermost on two 9mm diameter Whatman filter papers with 5ml deionised water in a petri dish in the dark at 25°C. Germinated grains were counted and discarded from each dish daily and the dishes compensated for evaporative water loss. Sequential tests of aged matching grains from the same ears were carried out at intervals, comparisons between cumulative germination frequency curves from sequential test series revealing the relative rate of dormancy loss in different samples. Ageing of samples was by storage in paper packets at 25°C except for samples in which dormancy was preserved for extended periods by drying to less than 15% moisture content and storing at -20°C (Mares 1989). Viability of any dormant grains which failed to germinate during the course of an experiment was proved by chilling at 4°C for twenty four hours followed by a return to 25°C to complete germination.

2.2) Test-crosses

The number and identity of *R* genes carried by 54 different varieties were determined from red : white segregation ratios in F_2 populations derived from F_1 hybrids between tested wheats and standard white (*r1 r2 r3*), red (*R1 r2 r3*), and red (*r1 R2 r3*) tester lines. Various white tester lines were used, the *R1* standard was Chinese Spring and the *R2* standard was Red Bobs. No *r1 r2 R3* tester line had been described prior to this project (McIntosh 1988) and genotypes at the *R3/r3* locus were therefore inferred from the three test-crosses described above.

For each cross, grains from up to six self-pollinated F_1 hybrid plants were sown to give a stand of up to 400 F_2 plants which were then scored for grain colour using the sodium hydroxide test as described by Flintham (1993). Colour scores were confirmed under a binocular microscope. In each case the aim was to discriminate between the possible frequencies of white F_2's of 1/4 or 1/16 or 1/64 or zero. For each test-cross the probabilities attached to all expected models for the observed ratio of whites to reds were calculated by the χ^2 procedure. Models with probabilities < 0.05 were considered to be excluded and in all cases this resulted in unequivocal identification of the *R* genotype of the tested wheat.

A diallele analysis of genetic variation for grain dormancy amongst *R1 R2 R3* wheats was carried out using the low-dormancy varieties Boxer and Galahad, the dormant varieties Sentry and Soleil, and all twelve reciprocal F_1 hybrids between these four parents. A double row of 2 x 11 hand-sown plants of each genotype was grown in each of four randomised blocks in the field at Morley Research Centre over the 1993/94 season. Four weeks after harvest ripeness, ten grains from each of ten ears per replicate were subjected to a dormancy test and a further two duplicates of ten ears each were tested for resistance to simulated rainfall. Reciprocal crosses for each F_1 hybrid revealed no maternal effects and variance between reciprocals was therefore pooled with replicates error. Germination curves for grains from each of the F_1 hybrids were then compared to those of the parents, to establish whether the observed genotypic variation for dormancy was due to predominantly additive gene effects, or to non-additive interaction.

In order to generate near-isogenic lines more relevant to U.K. conditions, for assessment of multiple accessions of each *R* gene and their interactions with different genetic backgrounds, a breeding programme was carried through during this project for the production of near-isogenic lines of four U.K.-adapted white-grained winter wheats. Crosses between the white-grained varieties Holdfast, Wilma, Abele and JBW (a PBI breeding line) as recurrent parents, and Chinese Spring (*R1*), Red Bobs (*R2*), Cappelle Desprez (*R2, R3*) and RL4137 (*R1, R2, R3*) as *R* gene donors, were carried through a series of six backcrosses with selection for red grain colour in each generation to eliminate donor genes except for the segment of chromosome carrying an *R* gene. After six backcrosses the material was self-pollinated for the extraction of duplicate lines carrying the *R1, R2* and *R3* genes from each of two or three different donors in each of the white wheat backgrounds.

2.3) Embryo cavity waxes

2.3.1) Samples

Original samples of grain in which tufts of wax were discovered and subsequently sought were from an archive at CCFRA at Chorleywood. Further samples were kindly provided from an archive at National Institute of Agricultural Botany, Cambridge.

Recently harvested samples were provided from stocks, or were grown especially for the project by the John Innes Institute.

A standard sample set on which most work was carried out comprised samples with extreme *R* gene constitution and a narrow (necessarily, as a result of the association of colour with resistance) range of sprouting resistance within both red and white sets. They are listed in Table 2.I

Table 2.I. Sample set used for investigation of relationships between embryo cavity waxes and dormancy.

Cultivar	Harvest year	Sprout resistance (1, little to 5, extreme)	*R* gene constitution
RL 4137	1991	5	R1 + R2 + R3
RL 4137	1992	5	" " "
Soleil	1991	4	" " "
Soleil	1992	4	" " "
Sentry	1991	4	" " "
Chinese Spring (CS)	1991	4	R1 + r2 + r3
Boxer	1991	3	R1 + R2 + R3
Galahad	1991	3	" " "
Galahad	1992	3	" " "
Kenya 321	1992	2	r1 + r2 + r3
CS (Cappelle 3D)	1991	1	" " "
Timgalen	1992	1	" " "
Abele	1991	1	" " "
JBW	1991	1	" " "

2.3.2) Dissection and extraction

Variation in sprouting resistance can occur even among grains on the same ear, along the length of both the spike and the spikelet (Evers and Ferguson, 1980, Gale *et al*, 1987). Greatest uniformity occurs among the basal grains within the 3 central spikelets on either side of the rachis (Bremner and Rawson, 1978), and it was from these positions that grains were selected when whole ears were available. Where samples were available only as threshed grains, the largest grains were selected in an attempt to ensure uniformity and consistency with on-ear samples.

To provide an authentic reference embryo cavity wax, for comparison with extracts from more recently harvested grains, the covering layers were removed from 1,000 grains of the red variety Yeoman. Grains were held fast on an adhesive surface with dorsal side uppermost so that the loose outer pericarp (beeswing) could be removed from the embryo end of the grain. The remaining inner pericarp and the seed coats were removed with greater precision by cutting with a sharp needle around the periphery of the cavity. The flap of tissue, thus loosened, could be removed and stuck, inner-side uppermost on an adhesive surface. The crystals thus exposed could be carefully removed and accumulated in chloroform. From 1,000 grains approximately 1mg of wax was collected.

In manipulations of all grains from which solvent extracts were made, care was taken to avoid contact with hands or other potential sources of contamination with oils or fats. To this end, rubber gloves were worn and instruments were cleaned with solvent before use and between samples. Separation of tissues was achieved by a fairly consistent practice, although, since the method was subject to improvement, not all details applied in all cases.

Solvent extracts were prepared, with minimum dissection, from grains of long-stored samples known to contain solid waxes and also from more recently harvested grains. Initially, after removal of beeswing, an incision was made only half-way round the embryo so that access might be gained by solvent, to its contents, when the embryo end of the grain was dipped five times, for one second each time, in chloroform.

As lack of consistency in the analysis and surface pressures was experienced in early work, contamination was suspected and increased care was later taken to ensure that no waxes from the embryo itself, or from other grain tissues, were present. Techniques progressed through stages including complete removal of embryo covers and extraction by immersing these in chromic acid-cleaned, screw-capped, narrow-ended tubes, which were subjected to 1 min vortex mixing, to the final resort of using elaborately cleaned dissecting equipment and swabbing only the inner surface of the embryo covers with brushes of pre-extracted cotton thread.

Extracts were prepared from between 20 and 100 grains or parts. In general the number of grains used increased as the lack of reproducibility emphasised the need for a more representative extract.

All glassware and quartz wool was pre-cleaned in fresh strong chromic acid before use, following detection of peaks corresponding to previous samples when thorough but less stringent cleaning regimes (including previously used chromic acid) had been used. Good lipid laboratory practice was used to prevent contamination with plastics and plasticisers following detection of dioctyl phthalate in early extracts.

Extracts were filtered through acid cleaned quartz wool into narrow-ended 2ml tubes graduated to 650µl. The solution was reduced to that volume by blowing N gas over the surface.

2.4) NMR Imaging

Dormant and non-dormant grains from the same ears of the highly dormant *R1 R2 R3* variety Soleil were obtained by drying freshly harvested grains from plants grown in a heated glasshouse over the winter of 1992/93, separating them at random into equal portions, and ageing the "non-dormant" sample at ambient laboratory temperature while dormancy was preserved in the second sample by storage at -20°C.

Nuclear Magnetic Imaging was kindly carried out by Dr Jeremy Wright at the Herschel Smith Laboratory for Medicinal Chemistry, in the University of Cambridge School of Clinical Medicine.

At the time of examination the grains had been stored for six months. Immediately following the examination, germination tests on the two sets of grain were carried out to confirm that the differences in dormancy resulting from the different storage conditions had materialised.

2.5) Genetic mapping

2.5.1) Breeding

A breeding programme was initiated to provide lines for mapping as yet unidentified genes causing wide variation in dormancy between different *R1 R2 R3* winter wheats. Doubled-haploid recombinant lines were produced from F_1 hybrids between the parents Soleil (highly dormant) and Boxer (very low dormancy). Ears on F_1 hybrid plants were emasculated prior to anthesis, then fertilized with maize pollen. Two days after pollination the upper internode and all florets of the tiller were filled with 2,4-D solution ($20mg.l^{-1}$) to promote the development of haploid recombinant wheat embryos (Laurie and Reymondie 1991). Haploid embryos were dissected out two to three weeks after pollination, germinated *in vitro* (on Gamborg's B5 medium with vitamins, $20g.l^{-1}$ sucrose and $9g.l^{-1}$ Difco bacto agar), grown on to the three/four tiller stage, treated with $0.5g.l^{-1}$ colchicine for 5½ hours to double up chromosome numbers to the normal hexaploid complement, and self-pollinated to generate pure-breeding homozygous lines.

In addition to the breeding programmes described above, three populations of recombinant lines were produced for genetic mapping of the grain colour genes. Each population was derived from an initial cross between a standard single-*R*-gene red wheat and a white wheat, to generate homozygous red, heterozygous, and homozygous white F_2 recombinant plants. *R1* was mapped from 130 lines out of the cross Spica (*r1 r2 r3*) x Chinese Spring (*R1 r2 r3*); *R2* from 140 lines out of Red Bobs (*r1 R2 r3*) x Kenya 321 (*r1 r2 r3*); *R3* from 160 lines out of Dollar (*r1 r2 R3*) x Kenya 321. A random sample of 23 F_3 grains was grown on from each F_2 to provide a line-bulk DNA sample containing balanced proportions of the genes present in the original F_2, and to score the F_2 as either homozygous red (*R/R*), heterozygous red (*R/r*), or homozygous white (*r/r*).

2.5.2) Sampling for *alpha*-amylase determination

Alpha-amylase activities in bulk F_4 grain samples from F_3 recombinant families out of the cross Spica x Chinese Spring were assayed quantitatively using the Phadebas test (see 2.7.1). A single row of 23 plants from each F_3 family (representing a random bulk progeny from a single F_2 plant) was grown under glass from a sowing in March 1992. Replication errors were estimated from triplicate rows of the parent varieties.

Parents of mapping populations (Chinese Spring, Spica, Red Bobs and Kenya 321) were screened for variation at the *Est-5* loci on chromosome group 3 long arms by isoelectric focusing of aqueous grain extracts. No differences in isoelectric points were detected

The recombinant lines from Chinese Spring x Spica, used for RFLP mapping of the *R1* gene, have also been scored for *alpha*-amylase production in ripening grains in the absence of sprouting. The parent varieties, an F_2 population, and the 130 F_3 mapping families were grown at spaced-plant rows under glass from a sowing in the spring of 1993. No visible sprouting occurred and clean, sound samples from each entry were assayed for *alpha*-amylase activity following harvest ripeness.

2.5.3) DNA markers

DNA clones used for genetic mapping were prepared either as bacterial plasmid inserts, or by amplification in the Polymerase Chain Reaction (PCR - Devos and Gale 1992). Clones were selected for study on the bases of low genomic copy number and of genetic linkage to the long arms of group 3 chromosomes. The clones were screened against genomic DNA from the mapping parents Chinese Spring (the laboratory standard genotype), Spica, Red Bobs and Kenya 321, digested with a set of six restriction enzymes, to identify probe/enzyme combinations which revealed useful polymorphisms (in order to be of use for mapping the polymorphism must be on the relevant chromosome). **Table 3.VII** lists the restriction enzymes used, the clones screened to date, the laboratories from which they were obtained, and the overall frequency of polymorphism detected with each. Clones from barley chromosome 3H were investigated in addition to those from wheat, since 3H is known to carry a number of similar genes, in similar linkage arrangements, as 3A, 3B and 3D and hence these barley clones can be expected to map to homoeologous loci in wheat (Devos and Gale 1993).

Wheat homoeologues to the barley marker genes *XBcd131* and *XGlb33* map extremely close to the expected positions of *R* loci, however the frequencies of polymorphism detected with these clones were too low to allow their general use as *R* gene markers. Since these two markers could prove very useful for manipulating *R* genes, methods for revealing hitherto undetected polymorphisms at or near these loci were investigated. Nucleotide sequences of the two barley genes were used to design primers for PCR amplification of homoeologous wheat DNA in order to target potentially polymorphic sequences. Pharmarcia LKB Gene Assembler Plus was used to raise PCR primer sets for the two clones Glb33 and BCD131. The sequence for Glb33 and surrounding region came from Wang, Peilin and Fincher (1992). The BCD131 clone was obtained from Cornell University (Heun *et al.* 1991) and approximately 300 base pairs were sequenced from each

end using an AutoCycle sequencing kit with an Automated Laser Fluorescent DNA Sequencer (sequences courtesy of Mr. B. J. Smith, JIC). Four sets of primers were raised from Glb33 and three from BCD131. The length of the primers varied from 18 to 22 base pairs. Reaction conditions used were: template 50 ng, 50 µl PCR buffer, dNTPs 100 mM, Taq polymerase 0.8 U, primers 50 ng. Amplification was performed using a Perkin-Elmer Cetus DNA Thermal Cycler, with 45 cycles of 1 min. 94°C / 1 min. 53-57°C / 2 mins. at 72°C. Annealing temperature varied with the melting temperature (Tm) of the primer.

2.5.4) RFLP mapping

Recombination frequencies between the *R/r* loci and DNA markers known to lie on the same chromosome were analyzed to produce genetic maps showing the positions of the *R1, R2* and *R3* genes on the long arms of chromosomes 3D, 3A and 3B respectively. DNA markers in the area of interest were identified by reference to nullisomic/tetrasomic aneuploid lines of Chinese Spring and to mapping data from other wheat and barley crosses (Devos *et al.* 1992, Devos and Gale 1993, Kleinhofs *et al.* 1993). Genetic mapping was carried out by analysing the frequency of recombination between different parental genes on the same chromosome amongst progenies from the crosses Spica x Chinese Spring, Red Bobs x Kenya 321, and Dollar x Kenya 321. For each gene examined, parental lines of each cross were first screened for differences (polymorphisms). Recombination between non-polymorphic genes cannot be detected (for *Est-5* grain esterases all parents shared identical patterns when their isozymes were separated by iso-electric focusing and this marker system was therefore abandoned). Other marker systems yielded polymorphisms including variation for grain colour at the *R1, R2* and *R3* loci, and DNA restriction enzyme fragment length polymorphisms (RFLP's).

RFLP's were detected by extracting high-molecular-weight genomic DNA from leaves of F_2 plants or F_3 families, cutting this at specific sites with restriction enzymes, separating the fragments by size using gel electrophoresis, transferring the fragments to nitrocellulose filters, and hybridising with ^{32}P radiolabelled DNA probes. Genomic/probe hybrids were detected following exposure of X-ray films against radioactive filters, differences in genomic restriction fragment length being revealed by differences in signal position on developed films. Restriction enzyme digestion was enhanced by the addition of casein (Dreyer and Schulter-Holthausen, 1991). Further details of molecular protocols may be found in Devos *et al.* (1992) and references cited therein. Analyses of recombination data were performed using Mapmaker 3.0 computer software (E.S. Landers, Whitehead Institute for Biomedical Research, Cambridge U.S.A.).

Random Amplified Polymorphic DNA (RAPD) analysis of bulked segregants (Devos and Gale 1992; Michelmore *et al.* 1991) was investigated as an alternative to map-based approaches as a strategy for identifying markers for the *R2* gene. Briefly stated, this technique involves screening a number of random DNA oligomers for their ability to direct the synthesis of different DNA polymers from genomic templates, differences being associated with presence or absence of the *R* gene in bulked recombinant lines used as template DNA source. Bulked homozygous *r1 R2 r3* and bulked homozygous *r1 r2 r3* lines from the cross Red Bobs x Kenya 321 were used to target the *R2* gene using this technique, however out of 120 random decamer primers screened none gave rise to

polymorphisms detectable by size separation in agarose gel electrophoresis.

2.6) Archival analysis

The possible association of late maturation endosperm *alpha*-amylase with larger grains was explored in records made available by two agricultural research institutes, NIAB and ADAS. The extremely useful archive of the former allows retrospective searching for relationships among varietal characteristics. Records of Falling Number (FN) scores accumulated between 1986 and 1993, and made available by Mr R Fenwick, were analysed for individual varieties. A relationship emerged between varietal grain size, as described in Anon (undated) and HFN ranking for the years in question. As the varieties present in the records changed considerably over the period, the criteria recorded were the frequency of appearance in the annual tables and the proportion of those years in which the variety fell among the four varieties with the lowest HFN for the year.

2.7) Analytical methods

2.7.1) *Alpha*-amylase determinations

Phadebas method

For grain bulks the Phadebas method, adapted from Mares (1989), was used. Each entry was subsampled, milled, and its enzyme activity was determined from an aqueous saline extract of the wholemeal flour.

Iso-electric focusing

Iso-electric focusing was performed on breeding stocks (2.5.1) by the method of Ainsworth *et al.* (1984).

Optical methods for detecting site of enzyme production

Grain cut faces and microtome sections, as well as aleurone layer surfaces were examined after application of fluorescein dibutyrate in acetone. In the presence of esterase this dye fluoresces and, using a suitable microscope the location of the enzyme can be detected. The technique has been proposed and used as an indirect means of quantifying *alpha*-amylase activity since both esterase and *alpha*-amylase are members of the suite of enzymes produced in germinating grains (Jensen and Heltved 1982).

Agarose gel semi-quantitative assay

While the fluorescein dibutyrate method provided a useful initial indication, its indirectness disqualified its use for a definitive indication of starch digesting enzymes. Attempts were made to develop a sensitive blotting technique, in which half- or part-grains were incubated on the surface of a gel containing *alpha*-amylase substrates. Powdered Phadebas tablets proved successful in that clear areas appeared near the source of amylase without further staining but, because the powder particles were large, the sensitivity was low. Soluble starch and *beta*-limit dextrin, flooded following incubation with iodine/potassium iodide both gave improved resolution and, although the starch gave the best contrast, the limit dextrin was adopted for routine application because of its greater specificity. The method adopted is based on that of Daussant and Renard (1983) using 1% agarose and 2% *beta*-limit dextrin, 2mM calcium chloride, 50mM sodium chloride, 50mM sodium malate, pH5.2. Samples were incubated on gels for 25 mins at 30°C.

Samples were carefully removed before flooding with 0.15% iodine/3% potassium iodide solution. The size of the clear area is proportional to the enzyme activity and the location of the source, within the grain part can be divined from its position in relation to the impression left.

Micro-titre plate assay

A quantitative colorimetric assay was developed for measuring *alpha*-amylase activity in microtitre plates, using a commercial reagent kit. This procedure, using benzylidine-blocked *p*-nitrophenyl maltoheptaoside as a substrate, together with two ancillary enzymes: glucoamylase and *alpha*-glucosidase (Randox) was adapted from those methods described by Sirou *et al*, (1990) and by McCleary and Sheehan (1987). Grains were extracted with 2mM calcium chloride, 50mM sodium chloride, 50mM sodium malate, pH5.2 for 30 mins. and then centrifuged. The supernatants were carefully sampled in duplicate and assayed in triplicate. In some cases, single grains were cut in half longitudinally for analysis by the agarose/*beta*-amylase limit dextrin gel, then immediately frozen for subsequent analysis by the microtitre plate assay. In preparation for experiments in which the same grain parts were assayed by gel blotting and (following storage in a freezer) microtitre plate, control experiments established that freezing did not affect the enzyme activity within the grain halves.

2.7.2 Chromatographic methods

Thin layer chromatography (TLC)

Separations were carried out using 0.2% ethanol in chloroform (Aldrich spectroscopic grade, stabilised by amylenes). This was the best solvent found for separating wax into constituents on 20cm silica gel 60 plates (Merck).

Gas Chromatography (GC)

A Perkin-Elmer 8500 gas chromatograph equipped with the PTV injection system and flame ionisation detector was used.

Trimethylsilylether derivatives of non-volatile constituents such as fatty acids and alcohols in wax extracts were prepared for analysis the same day to enable wax composition to be determined from a single chromatogram. Pyridene (2.5µl) and N-O-bis(trimethylsilyl) acetamide (7.5 µl) were added to dry sample and standard residues, and the reagents were whirlymixed and rotated to dissolve wax constituents. The vial was heated for 1h at 50°C, during which the volume usually decreased slightly.

Packed column chromatography - Neither resolution nor sensitivity were satisfactory in packed column separations using a 1m x 0.3mm ID stainless steel column of Dexsil 300 on Chromosorb W-HP, and work was transferred to capillary columns.

Capillary column chromatography - The first type of column used was a 12m x 0.32mm ID 0.1µm fused quartz, polyimide clad capillary column, bond coated with HT5 (max. operating temperature 370°C) supplied by SGE. Volumes injected were 0.1-0.2µl standard solutions and 0.2-0.3µl sample solution. A temperature programme from 100-370°C at 5°C min^{-1}, with a final hold of 10 min at 370°C separated triglycerides, fatty acids and alcohols and hydrocarbons, but failed to separate hydrocarbons from wax esters.

As the high temperature destroyed the polyimide coating, leading to column fracture, more durable, stainless steel coated 15m x 0.28mm ID 0.15μm MXT-1 columns (Restek Corpn) were substituted. The final PTV temperature was reduced to 390°C. Temperatures of 100°C, 5min; 100-200°C, 4.5°C min^{-1}; 200-370°C, 5.5°C min^{-1}; 370°C, 10 min, separated triglycerides, fatty acids and alcohols and hydrocarbons, but failed to separate alcohols from wax esters.

Heneicosane was the internal standard. Peaks were integrated using the SUMMIT system and estimated amounts relative to the internal standard were transferred by direct data link to the EXCEL data processing programme for further calculations. Response factors have not been used; they are reported to differ not only between constituent groups, but also with chain length relative to internal standard (Dr P Holloway, Long Ashton Research Station, private communication).

Routine identification was by comparing retention times with those of a mixed standard solution prepared from all commercially available wax constituents, as listed in Table 2.

Table 2.II. Standards used in capillary gas chromatography

hydrocarbons (HC):	C16:0 - C30:0, C32:0 - C34:0 odd and even; C36:0 - C40:0, C 50:0 even only
fatty alcohols (OH):	C 12:0 - 30:0 even.
fatty acids (AC):	C10:0 - C30:0 even
triglycerides (TG):	C12:0 - C22:0 even, (C18:0 - C22:0 not detected in 70 min.)
wax esters (E):	C24:0 - C44:0

The standard mixture contained approx 1mg of each of the standards in approx 2 ml chloroform. Sterol, sterol ester and dioctyl phthalate solutions were also used to identify individual peaks and a β-diketone fraction prepared from *Eucalyptus gunnii* was also examined.

Recoveries of three alcohol and three acid standards were determined as percentages using the derivatisation procedure and GC. The compounds selected spanned a range of retention times of 20-45min. Concentrations of alcohols were: C12:0, 6.48μg.ml^{-1}, C24:0, 4.65μg.ml^{-1} and C30:0, 3.26μg.ml^{-1}; and of acids: C14:0, 4.57μg.ml^{-1}, C24:0, 5.19μg.ml^{-1}, and C30:0, 2.31μg.ml^{-1}. Either 250μl ("1:1") or 50μl ("1:0.2") aliquots were mixed with 250μl of heneicosane 5 μg.ml^{-1} and amounts were determined relative to this internal standard.

Gas chromatography/Mass spectroscopy (GC-MS)

GC-MS was carried out by Dr P Holloway at Long Ashton Research Station, using the HT5 capillary column in the Carlo-Erba GC fitted with a Kratos MS 80RFA magnetic sector type mass spectrometer and comparing with a spectral library of 140,000 compounds. As retention times were affected both by the use of a fixed temperature rather than a PTV injector, and also by the vacuum applied to the end of the column by the MS, retention times of sample peaks were related to those of the mixed standard, run on the same equipment.

2.7.3) Surface pressure measurement

Surface films of wax extract spread on the surface of doubly distilled, deionised water at 25°C, contained in a Joyce-Loebl Langmuir surface balance, were compressed in order to obtain "surface pressure vs area" isotherms. Chloroform extracts were concentrated to approximately 200µl by blowing nitrogen gas over the samples. Transfer to the water surface was achieved by use of a microsyringe. Drops (2-4µl) at a time were injected on to the surface in random positions. The sides of the sample container were washed with a further 100µl of solvent and this was also applied to the water surface. After allowing 10 min for the chloroform to evaporate, the wax surface film was compressed by means of a moving belt at a speed of 1.1cm min^{-1}, and surface pressure vs area isotherms were obtained on a connected chart recorder.

Three replicate measurements were made from each extract. Before taking the first aliquot and between taking subsequent aliquots the bulk solution was placed in a freezer for a minimum of 30min.

3) RESULTS

3.1) Effects of individual *R* genes on grain dormancy in near-isogenic lines

The accessions ANK-1A, ANK-1B, ANK-1C, ANK-1D and ANK-1E constitute a set of near-isogenic lines of the white-grained (*r1 r2 r3*) spring wheat Novosibirskaya 67 (NS67).

Table 3.I Grain colour segregation ratios in test-cross F_2's from near-isogenic lines

Cross		Grain colour of F_2 plants Red	White	χ^2_{1df} for two-gene segregation (15 red : 1 white)	
C.CS	(ANK-1C x Chinese Spring)	269	0	17.93	P < 0.001
RB.D	(Red Bobs x ANK-1D)	210	0	14.00	P < 0.001
$.A	(Dollar x ANK-1A)	245	11	1.67	P > 0.1
C.D	(ANK-1C x ANK-1D)	133	17	6.62	P < 0.02
D.B	(ANK-1D x ANK-1B)	146	11	0.15	P > 0.5
C.A	(ANK-1C x ANK-1A)	150	0	10.00	P < 0.01
A.B	(ANK-1A x ANK-1B)	215	15	0.29	P > 0.5
A.E	(ANK-1A x ANK-1E)	242	20	0.56	P > 0.3
B.E	(ANK-1B x ANK-1E)	203	0	13.53	P < 0.001

Note : Cross C.D (ANK-1C x ANK-1D) gave a poor fit to the 15 : 1 model for two genes segregating; however, the deviation consisted of an excess of white-grained F_2 plants. The model is therefore accepted since alternatives would yield even lower expectations for the frequency of white grains. Crosses $.A, D.B, A.B and A.E segregated for two independent grain colour genes. No segregation was observed in the remaining crosses C.CS, RB.D, C.A, or B.E and in each of these crosses the two parents are deduced to share an *R* gene in common.

Each "isoline" carries a single *R* gene from a different red-grained donor variety and was selected through a series of nine backcrosses to NS67 (Koval, Metavosky and Sozinov, 1988). These lines are thus expected to be genetically identical to NS67 except for the segment of chromosome carrying the *R* gene. Differences between these lines are therefore ascribed to effects of the individual *R* genes, although the possibility of confounding effects due to other donor genes co-segregating with the *R* genes cannot be discounted in theory.

Test-crosses and intercrosses between the ANK lines and standard tester lines were carried out to confirm the identity of the *R* gene carried by each isoline, and to generate material for the extraction of isolines carrying combinations of two or three *R* genes. Chinese Spring and Red Bobs each carry a known, single *R* gene, and each of the ANK lines also carries a single *R* gene as shown by the two-gene segregation patterns in the following crosses listed in Table 3.1

ANK-1A: crosses $.A, A.B, A.E;
ANK-1B: crosses D.B, A.B;
ANK-1C: cross C.D;
ANK-1D: crosses C.D, D.B;
ANK-1E: cross A.E.

The *R1* gene is carried by lines ANK-1A and ANK-1C (from lack of segregation in crosses C.CS and C.A), and *R2* is carried by ANK-1D (lack of segregation in cross RB.D). There is no direct evidence regarding the identity of the gene carried by lines ANK-1B and ANK-1E, however both carry the same gene (cross B.E) and this gene is neither *R1* nor *R2* (crosses D.B, A.B and A.E). ANK-1B and ANK-1E are therefore deduced to carry the *R3* gene. Crosses between these two lines and the *R3* tester line Dollar have been made in order to confirm this deduction.

Two sets of germination experiments were carried out with these lines: a small-scale pilot study using grains from pot-grown plants and a subsequent, more detailed investigation of grains from a soil planting of an eleven-plant row of each line under glass; only data from the latter study are reported in detail. In both experiments marked and consistent differences were obvious between the white-grained parent NS67 and the other, red-grained lines. Smaller differences were also recorded between the different red-grained lines, however these were not consistent over experiments or replicates and data from the red lines were therefore pooled, i.e. the effects of different *R* genes from different donors were considered to be equivalent. For the main experiment ten anthesing ears of each line were tagged on 29th June 1994, to minimise variation for maturity between samples. Tagged ears were cut at harvest ripeness and stored dry at 25°C for one week prior to commencement of the first of three sequential germination tests on 19th August 1994. The data from this and two subsequent tests (after a further seven and fourteen days' dry storage of sibling grains in the same ears) are summarised in Figure 3.1.

Each of the germination curves in Figure 3.1 may be considered as comprising a lag period, during which no grains germinate, followed by a phase in which individual grains within a sample germinate at different times - some on the first day, some on the second, and so on. All but two of the curves attained 100% germination during the course of the experiment and all remaining grains germinated within twenty four hours of a cold treatment at 4°C. Each grain sample can thus be regarded as a population of individual grains each of which has latent propensity to germinate after a particular period of hydration; the shape of the sample germination curve results from the distribution of different latent periods between individual grains.

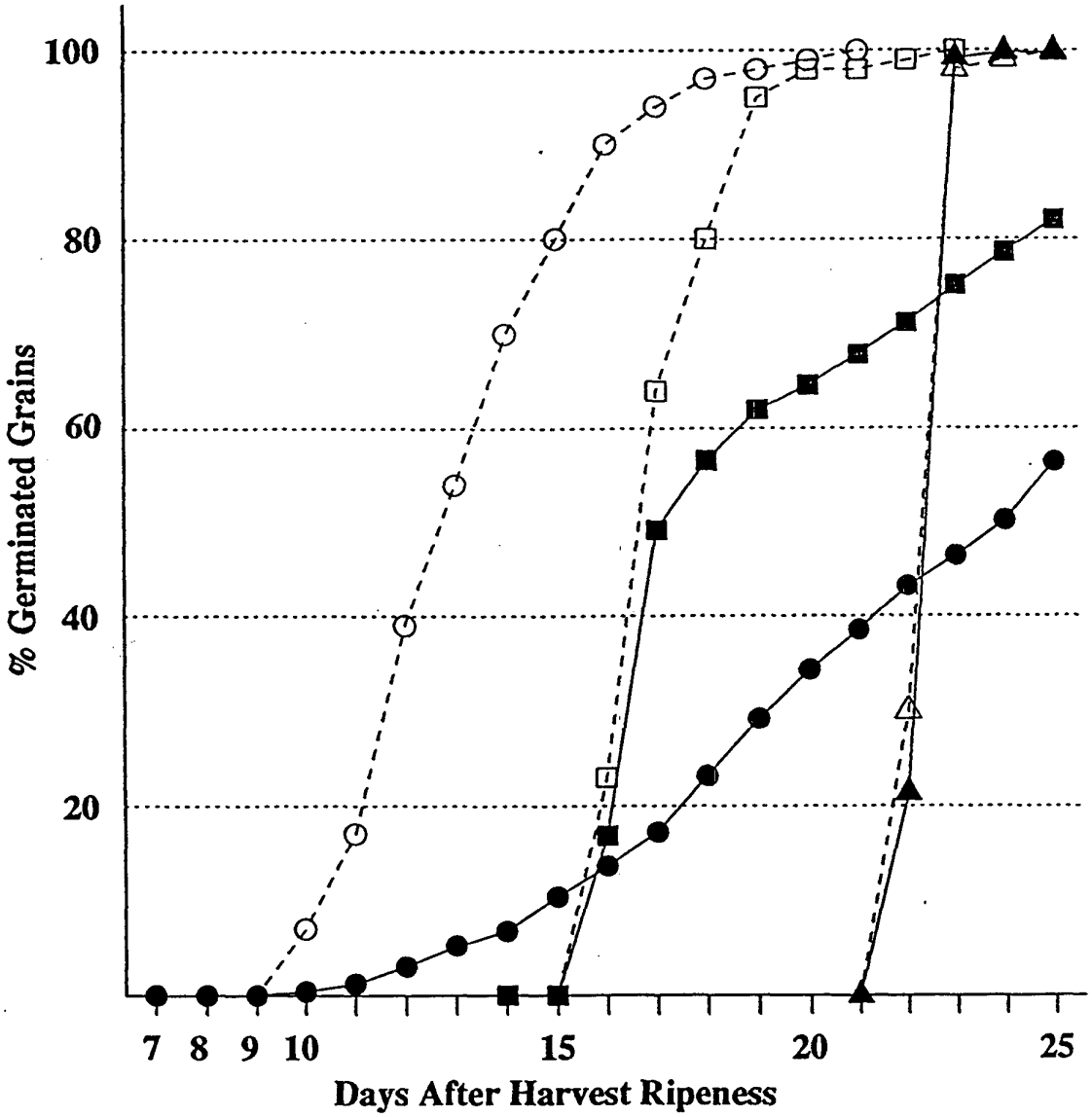


Figure 3.1 Cumulative germination curves for grains of Novosibirskaya 67 and near-isogenic red-grained lines.

Note: Separate curves are plotted for NS67 (open symbols) and for means over five red-grained ANK isolines (filled symbols) for three sequential germination tests started seven days (circles), fourteen days (squares) and twenty one days (triangles) after harvest ripeness.

Grain colour did not appear to affect the lag period of germination - onset of germination occurred in both red and white lines after two days in the first test, after one day in the second test, and within 24 hours in the third test. The lag period was thus dependent upon the post-harvest age of the grain rather than upon genotype and diminished progressively with storage at 25°C. In contrast, the "rate" of germination (i.e. the date at which individual grains became germinable) varied according to genotype, time of dry storage, and time in each germination test. In the first test more than 50% of grains from the white line germinated within four days of emerging from lag, while hydrated red grains emerged from dormancy at a much slower rate and only 5% germinated within four days of emerging from lag (sixth day hydrated). Grains from the red lines continued to become germinable at a slow and more or less constant rate and did not attain 50% germination until fifteen days after the lag period.

In the second test, started seven days later than the first, the white grains germinated more rapidly than at first and attained 50% germination within two days of emerging from lag. The red grains were slightly slower but also attained 50% by the second day after lag, however subsequent germination of red grains was much slower and occurred at a rate similar to that seen in the first test. It appears that the effect of seven days' ageing was to convert 50% of the red grains to a behaviour similar to that of white grains of a similar age, the remaining 50% of red grains retaining dormancy characteristic of the first sampling. It is evident from a comparison between the first and second red-grained samples in Figure 3.1 that dormancy loss under dry conditions is considerably quicker than its loss in the hydrated state, with the implication that in hydrated grains dormancy is actively maintained by a water-dependent process. In the third test red and white grains showed no significant differences, attaining 50% germination in about 36 hours. Three weeks of dry storage at 25°C (one week before the first test and two more after) were thus sufficient to completely eradicate the dormancy-enhancing effects of all five *R* genes studied. The prompt germination of previously dormant grains following cold treatment demonstrates the well-known temperature sensitivity of wheat dormancy.

These findings are consistent with a model in which a minimal but demonstrable degree of dormancy inherent in the white-grained line is enhanced to a similar degree by any one of five different *R* genes. Both the degree of inherent dormancy and the degree of *R*-enhancement decay with time in dry storage such that germination accelerates in all genotypes and eventually differences between them disappear.

A novel grain pigmentation pattern, consisting of variegation for white and red-spotted patches, was observed at low frequency in seed coats of genotypically *r1 R2 r3* recombinants from the cross Red Bobs x Kenya 321. Dormancy tests carried out with grains from the parents and from variegated F_7 single-seed-descent lines revealed that the variegated material was less dormant than either the red or the white-grained parent (data not shown). This result indicates that presence of the *R2* gene is not of itself sufficient to confer grain dormancy, and that the enhancement of dormancy described above may be dependent upon the presence of a contiguous layer of pigmented cells covering the entire grain.

3.2) *R* genes - genotypic and phenotypic characters

3.2.1) Identities of *R* genes carried by different wheats

Table 3.II lists 109 wheats according to their *R* genotypes as determined from crosses to white-grained testers (to determine the number of *R* genes present - Flintham, Evers and Kratochvil, 1993) and from crosses to the red-grained standard genotypes Chinese Spring (to test for presence of *R1*) and Red Bobs (to test for presence of *R2*). Presence or absence of *R3* was inferred from the number of *R* genes carried and presence of *R1* and/or *R2*. Excluding Atlas 66, Bezostaya 1, Carmen, Cheyenne, Favorits, Grana, Luna, Mara, Rannyaya 12, Sava, Supreme, Vuka, and the white-grained controls, the listed wheats may be taken as representative of the gene pool for breeding new varieties for U.K. agriculture.

Of the 93 wheats representative of the U.K., all are red-grained, 36 carry a single *R* gene, 39 carry two *R* genes, and 18 carry all three and are therefore classed as *R1 R2 R3*. This result confirms and extends previous findings (Flintham, Evers and Kratochvil, 1993) in that white wheats are rarely if ever submitted for release as varieties, and that amongst the different red wheats there is no evidence for selection towards increased dosage of *R* genes (the observed ratio of 36 *R* : 39 *RR* : 18 *RRR* is in agreement with the ratio of 39.9 *R* : 39.9 *RR* : 13.2 *RRR* expected from random assortment of genes with equal frequencies of *R* and *r* genes at each genetic locus and elimination of white-grained lines: χ^2_{2df}=2.15, 0.5>P>0.3).

The groups of wheats carrying either one or two *R* genes were further examined for evidence of preferential selection of individual, known *R* genes. Amongst the representative wheats with just one *R* gene, 13 carried *R1*, 9 carried *R2*, and 2 carried *R3*. These observations are significantly different from the expectation of 8 in each allelic class on the basis of equal gene frequencies (χ^2_{2df}=7.75, 0.05>P>0.02) and there is clearly a statistically significant deficiency in the frequency of the *R3* gene. In the group carrying two *R* genes, 4 carried *R1+R2*, 8 *R2+R3* and 7 *R1+R3* compared with the random expectation of 6.33 in each class. Thus among this second group there is no significant evidence for unequal representation of different genes (χ^2_{2df}=1.37, 0.7>P>0.5). The low frequency of *R3* amongst wheats with a single *R* gene is probably due to chance, given the high frequency of *R3* amongst wheats carrying more than one *R* gene.

On balance the wheats surveyed show no convincing evidence of selection for increased *R* dosage or for the presence or absence of particular *R* genes, and these findings are in agreement with the hypothesis of random distribution of equal frequencies of *R* and *r* genes at each locus previously advanced (Flintham, Evers and Kratochvil, 1993). One implication of this for wheat breeders is that one in eight true-breeding lines derived at random from this gene pool can be expected to be white-grained and will be discarded due to their susceptibility to sprouting. Apart from revealing the potential for increasing *R* dosage in new U.K. wheats this survey is also intended to be of use to breeders in predicting the *R* genotypes of lines bred out of this gene pool, for instance the cross Boxer x Soleil will only give rise to red (*R1 R2 R3*) lines, whereas lines from the cross Minaret x Dollar will be white-grained (*r1 r2 r3*), red (*R1 r2 r3*), red (*r1 r2 R3*), or red (*R1 r2 R3*) in equal proportions.

Table 3.II. *R/r* Genotypes and sprouting scores of wheat varieties and breeding lines

White-grained wheats with no *R* genes *(r1 r2 r3)* :
Abele (0.61), Holdfast (0.71), JBW (0.94), Wilma (0.56)

Red-grained wheats with a single *R* gene :

R1 r2 r3	*r1 R2 r3*	*r1 r2 R3*
Alexandria	Baron (0.53)	Canon
Apollo (0.58)	Hustler (0.10)	Dollar
Axona	Maris Widgeon (0.79)	Grana
Dwarf 'A' (0.24)	Mercia (0.39)	Supreme
Fortress (0.27)	Motto (0.38)	
Jerico	Sapphire	
Longbow (0.18)	Slejpner (0.02)	
Luna	Talent (0.73)	
Mardler (0.26)	Wembley	
Maris Huntsman (0.33)		
Minaret		
NFC 75/93/27A (0.13)		
Rapier (0.40)		
Voyage (0.62)		
Vuka		

Wheats with a single, unclassified *R* gene :
Avocet, Captor (0.68), Copain (0.68), Guardian (0.55), Hammer (0.08), King, Mara, Norton (0.55), Peacock (0.30), Polaris, Professeur Marchal (0.30), Sava, Sober, TJB 370/500.

Red-grained wheats with two *R* genes :

R1 R2 r3	*r1 R2 R3*	*R1 r2 R3*
Bezostaya 1	Avalon (0.49)	Fenman (0.47)
Brigand (0.15)	Bersée (0.43)	Norman (0.19)
Brock (0.49)	Cappelle Desprez (0.44)	Pastiche (0.10)
Broom (0.40)	Feuvert (0.24)	Riband (0.27)
Kronjuwel (0.47)	Mission (0.63)	Sperber
	Parade (0.07)	Squadron (0.10)
	Rendezvous (0.35)	Urban (0.53)
	Yuri	

Wheats with two, unclassified *R* genes :
Apostle (0.63), Argent, Atlas 66, Bounty (0.79), Champlein, Corin, Corinthian (0.30), Coxswain (0.15), Depot (0.30), Desprez 80 (0.51), Flanders (0.41), Kanzler (0.53), Kudu, Mantle (0.56), NFC 97/84B (0.33), Pageant (0.23), Poet (0.45), Prince (0.34), Proton, Renard (0.55), Stetson (0.60).

Red-grained wheats with all three *R* genes *(R1 R2 R3)* :
Ambassador (0.35), Boxer (0.57), Brimstone (0.18), Carmen, Cheyenne, Cub, Dauntless, Drum, Durin, Favorits, Fresco, Galahad (0.64), Haven (0.58), Hornet, Kador (0.62), NFC 70/84C (0.15), Probus, Rannyaya 12, Rektor (0.56), Sentry (0.26), Soleil (0.12), Tonic.

Note : Numbers in parentheses show mean sprouting scores, where available, for wheats considered to be representative of the UK winter wheat gene pool. Scores were derived from ripe, field-grown ears subjected to simulated rainfall, 0 = no sprouting, 1 = fully sprouted, averaged over two sequential tests in each of three years (Flintham, Evers and Kratochvil, 1993).

A further aim of the survey was to identify wheats of the genotype *r1 r2 R3*, to enable genetic mapping of the *R3* locus. The spring wheat Dollar was the first variety to be identified as carrying *R3* alone and the cross Dollar x Kenya 321 was made for the production of a population segregating for *R3/r3* in a (*r1+r2*) background (presence of additional *R* genes would prohibit genetic mapping by masking segregation of the target gene).

3.2.2) Variation for grain dormancy in relation to *R* genotype

Sprouting data for representative wheats in the *R* gene survey are summarised in Table 3.III. The scores are directly derived from the frequency of sprouting in ripe ears under simulated rainfall: a low score indicates resistance, and a high score susceptibility to sprouting. Figure 3.3 shows frequency distributions for susceptibility to sprouting amongst representatives of the U.K. winter wheat gene pool classified by *R* dosage (individual scores are shown in Table 3.II, non-representative and spring-sown varieties are excluded).

The marked susceptibility of white-grained wheats is evident, as is wide variation within each genotypic group. Amongst the red wheats there is no obvious relationship between sprouting and the number of *R* genes carried. This is in apparent contrast to the *R* dosage effects previously detected in experimental lines (Flintham, Evers and Kratochvil 1993) however those effects were small and equivalent variation within the present data set may well be masked by the effects of different varietal genetic backgrounds. The presence of background effects is demonstrated by the range of scores within each genotypic group; even among the white-grained wheats, some show a significant degree of dormancy compared to the most susceptible entries. Each of the red-grained groups includes several wheats at least as susceptible as the more resistant whites - there is an overlap between red and white-grained types which is not significantly affected by changing *R* genes or dosage. This overlapping variation between different genotypic groups implies the existence of other genes, independent of grain colour, which have important effects upon resistance/susceptibility to sprouting. It is quite probable that some of these are genes affecting flowering time, in view of the known interaction between maturity and grain dormancy, however not all of the observed variation can be attributed to maturity differences.

Evidence for non-colour effects of other genes on group 3 chromosomes comes from comparisons of chromosome substitution effects (Flintham, Evers and Kratochvil, 1993) with the *R/r* genotypes of the recipient and donor varieties used to develop the substitution lines (Table 3.II). A reduction in grain dormancy resulted from the substitution of chromosome 3B of Dwarf A by its homologue from Bezostaya 1. Since Dwarf A and Bezostaya 1 both carry the *r3* gene on 3B the reduction in dormancy cannot have been due to a change in *R* genotype and must be attributed to the loss of other dormancy-enhancing gene(s) on chromosome 3B of Dwarf A and their replacement by genes for low dormancy on the 3B chromosome introduced from Bezostaya 1. Similarly, chromosome 3B of Champlein led to increased dormancy when substituted into Bersée, while the reverse effect (reduced dormancy) was obtained from the 3D substitution; since Bersée has the genotype *r1 R2 R3* and Champlein carries two (unclassified) *R* genes the enhanced dormancy of the 3B substitution cannot be due to gain of *R3* and loss of dormancy in the

3D substitution cannot be due to loss of *R1*. These three examples all demonstrate genetic variation for dormancy at loci other than those controlling grain colour, on chromosomes 3B and 3D. Substitution effects have also been detected in other chromosome groups and it is unlikely that genes affecting grain dormancy are confined to group three chromosomes.

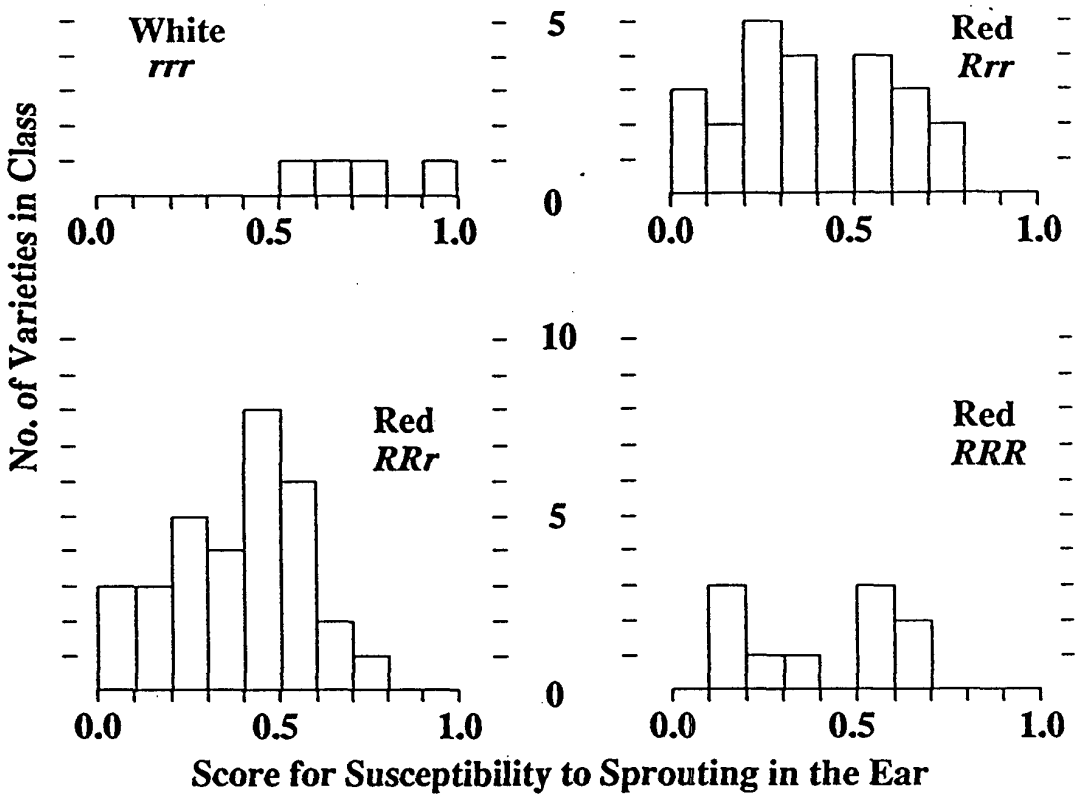


Figure 3.2 Frequency distributions for sprouting in the ear amongst white-grained controls and amongst red-grained varieties carrying one, two, or three *R* genes.

Note: Scores are proportional to the mean degree of sprouting in ears exposed to simulated rainfall in two sequential tests in each of three successive years (Flintham, Evers and Kratochvil, 1993). A score of zero represents complete absence of visible sprouting, a score of one indicates heavy sprouting in all ears tested. Only varieties considered to be representative of UK winter wheat breeding stock are included. Scores for individual varieties are given in Table 3.II

Table 3.III Summary of sprout-susceptibility statistics for wheats of different *R* genotypes

Genotypic Group	Sprouting Scores under Simulated Rainfall			
	n	Mean	Min	Max
White controls				
r1 r2 r3	4	0.71	0.56	0.94
Reds with one *R* gene				
Overall	23	0.40	0.02	0.79
R1 r2 r3 group	9	0.33	0.13	0.62
r1 R2 r3 group	7	0.42	0.02	0.79
r1 r2 R3 group	0			
Reds with two *R* genes				
Overall	32	0.39	0.07	0.79
R1 R2 r3 group	4	0.38	0.15	0.47
r1 R2 R3 group	7	0.38	0.07	0.63
R1 r2 R3 group	6	0.28	0.10	0.53
Reds with three *R* genes				
R1 R2 R3	10	0.40	0.12	0.64

Note: See Table 3.II for individual variety scores, Figure 3.2 for frequency distributions.

3.2.3) Variation for grain dormancy amongst adapted *R1 R2 R3* winter wheats

Two breeding experiments were initiated for studies of the variation for grain dormancy between wheats carrying a full complement of *R* genes. In order to eliminate effects of *R* dosage, to minimise "trivial" differences in maturity and adaptation to local conditions, and to maximise variation for grain dormancy, the wheats Boxer, Galahad, Sentry and Soleil were selected for further study. All four of these varieties were included in NIAB Classified Lists of winter wheats within the period 1985 to 1987, each carries all three *R* genes, and each gave consistently high (Boxer, Galahad) or consistently low (Sentry,

Soleil) sprouting scores over three years' tests (Table 3.II). Preliminary germination tests on glasshouse-grown samples confirmed that the variation for sprout-resistance was due to grain dormancy, that the differences between the low-dormancy and high-dormancy varieties were large and consistent, and that dormancy decreased with storage time in a similar manner to that observed in red and white isolines, albeit at a slower rate. The first breeding programme, for the rapid production of pure-breeding recombinants out of the cross Boxer x Soleil, was designed to furnish experimental lines for future investigations into the number of genes determining the wide difference in dormancy between the two parents, and the magnitudes (and hence the potential usefulness) of their individual effects. The second experiment consisted of an F_1 dialled analysis to determine the relative importance of additive versus dominant gene action in determining grain dormancy in the presence of *R1, R2,* and *R3.*

Boxer and Soleil were chosen as parents for the production of doubled-haploid recombinant lines (Methods) because of their close synchrony of flowering and because they were bred from genetically diverse (U.K., French) gene pools. Synchronous parents were selected with a view to minimising variation for flowering time amongst recombinant lines, while genetic diversity was maximised (as far as possible within the defined experimental constraints) to increase the likelihood of marker polymorphism. Out of approximately 5,000 F_1 hybrid florets pollinated with maize, over 200 haploid wheat embryos were recovered, however most of these did not develop into plantlets and only forty survived colchicine treatment to double chromosome numbers. A total of 34 doubled-haploid recombinant lines are currently available from this cross and are being multiplied for analysis of the "non-*R*" genetic control of grain dormancy. This number of lines is considered sufficient to determine whether variation in dormancy is controlled by a small number of easily selectable genes, or is due to the combined effects of a large number of genes which would not be amenable to marker-aided selection.

Ripe ears and grains from the dialled experiment were tested for both germination in the ear and for rates of germination of isolated grains. Germination curves for grains from field-grown samples of Boxer, Galahad, Sentry, Soleil, and their F_1 hybrids are depicted in Figure 3.3

The germination tests commenced some four weeks after harvest ripeness, and a comparison with the curves in Figure 3.1 reveals that dormancy was both more profound and more slowly lost than in the white and red-grained isolines. Ranking of the parents was the same as had been observed in a pilot study of pot-grown material, which also demonstrated a sequential loss of dormancy from two to four to six weeks following harvest ripeness (data not shown). In order to make quantitative comparisons between the different genotypes, each was assigned a germination score equal to the integral of the area under its germination curve up to the fourth day after imbibition. This metric was selected as a realistic summary of the germinability of each genotype, given that the lag period was the same for each curve and that the chosen integral gave a higher ratio of genotypic/error variances than alternative instantaneous, rate, or integral functions.

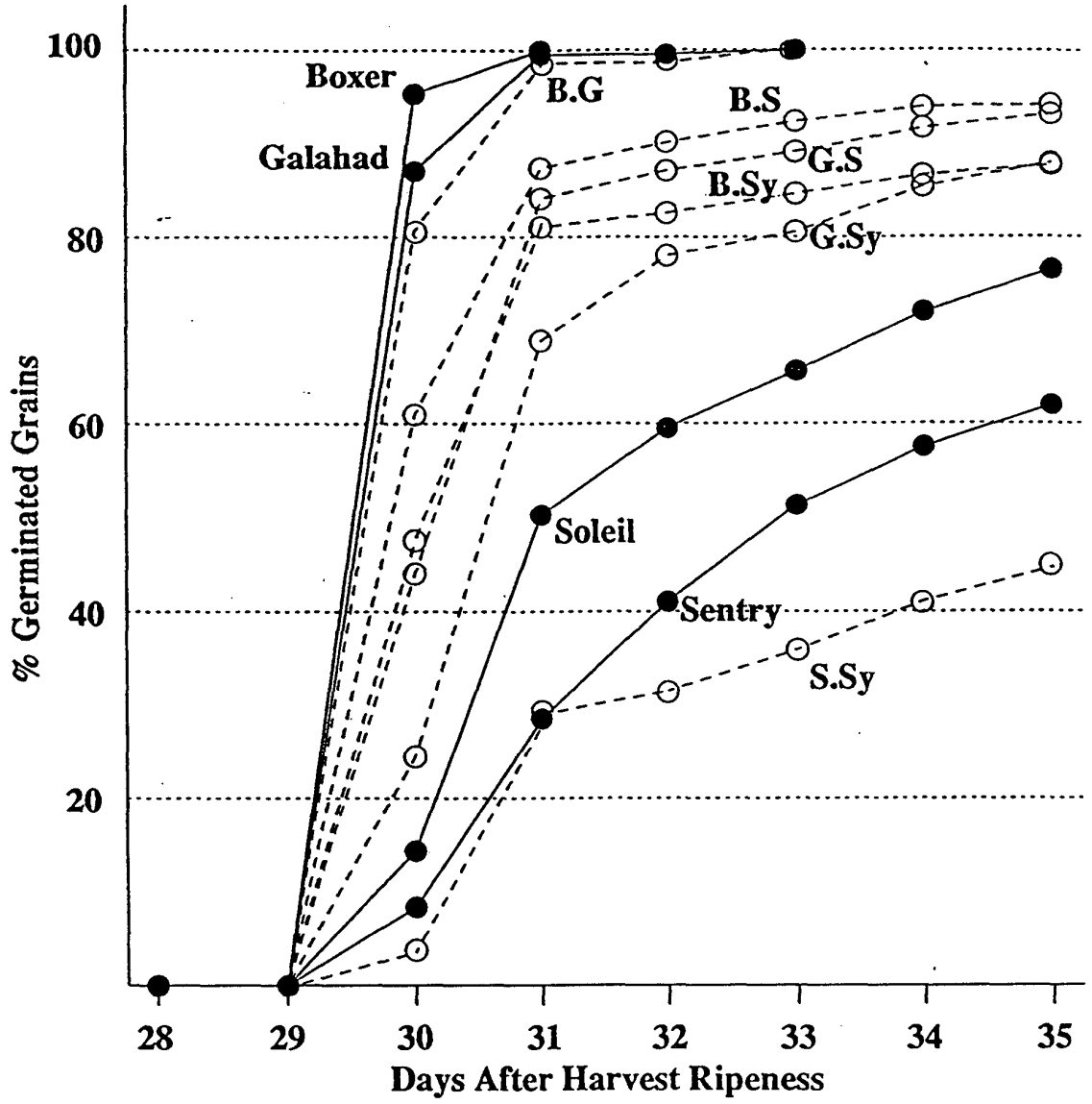


Figure 3.3 Cumulative germination curves for the *R1 R2 R3* varieties Boxer, Galahad, Sentry and Soleil and their F_1 hybrids.

Note: Curves for the pure-breeding varieties are shown as solid lines between filled circles, F_1 curves as dashed lines between open circles. The hybrids are denoted as follows:

B.G = Boxer x Galahad and the reciprocal cross,
B.S = Boxer x Soleil and reciprocal,
B.Sy = Boxer x Sentry and reciprocal,
G.S = Galahad x Soleil and reciprocal,
G.Sy = Galahad x Sentry and reciprocal,
S.Sy = Soleil x Sentry and reciprocal.

Figure 3.4 shows the four-day integrals for each of the F_1 hybrids plotted against the mean of its parents, which are also shown plotted on the line predicting the loci of hybrids with scores equal to the mean of their parents. There is a very close correlation between the scores of the six hybrids and their respective mid-parent values (r_{5df}=0.9523, $P < 0.001$) and the deviation of each hybrid from mid-parent was in no case significant compared to replication error. Hybrid scores are expected to equal mid-parent scores when the variation is due to genes with simple additive effects. The effect of genetic dominance will be to shift a hybrid score towards the score of the parent carrying more dominant genes (unless both parents carry different dominant genes in effectively equal measure - an unlikely situation given that the parental varieties for this experiment were selected for the extremes of high or low dormancy). Variation in dormancy among the F_1 hybrids is therefore attributed to predominantly additive gene action, such that the cross Sentry x Soleil yields a highly dormant hybrid, the Boxer x Galahad hybrid has very low dormancy, and the hybrids between low- and high-dormancy varieties have intermediate dormancies which can be predicted, within the limits of experimental error, as the mean of their parents.

The conformity of the hybrids to the simple additive genetic model was confirmed by analyses of alternative metrics from the germination test, and from the percentage of grains germinated in ears subjected to a sprouting test (data not shown). Results from the sprouting test were closely correlated with grain germination scores (r_{9df}=0.9515, $P < 0.001$), supporting previous evidence that resistance to sprouting in the ear is due to grain dormancy rather than to ear characteristics. Ears from Novosibirskaya 67 and from the ANK isolines grown in a neighbouring spring-sown trial and subjected to the same sprouting test all contained more than 90% sprouted grains, confirming that dormancy among the *R1 R2 R3* genotypes was significantly more profound than amongst the isolines carrying single *R* genes.

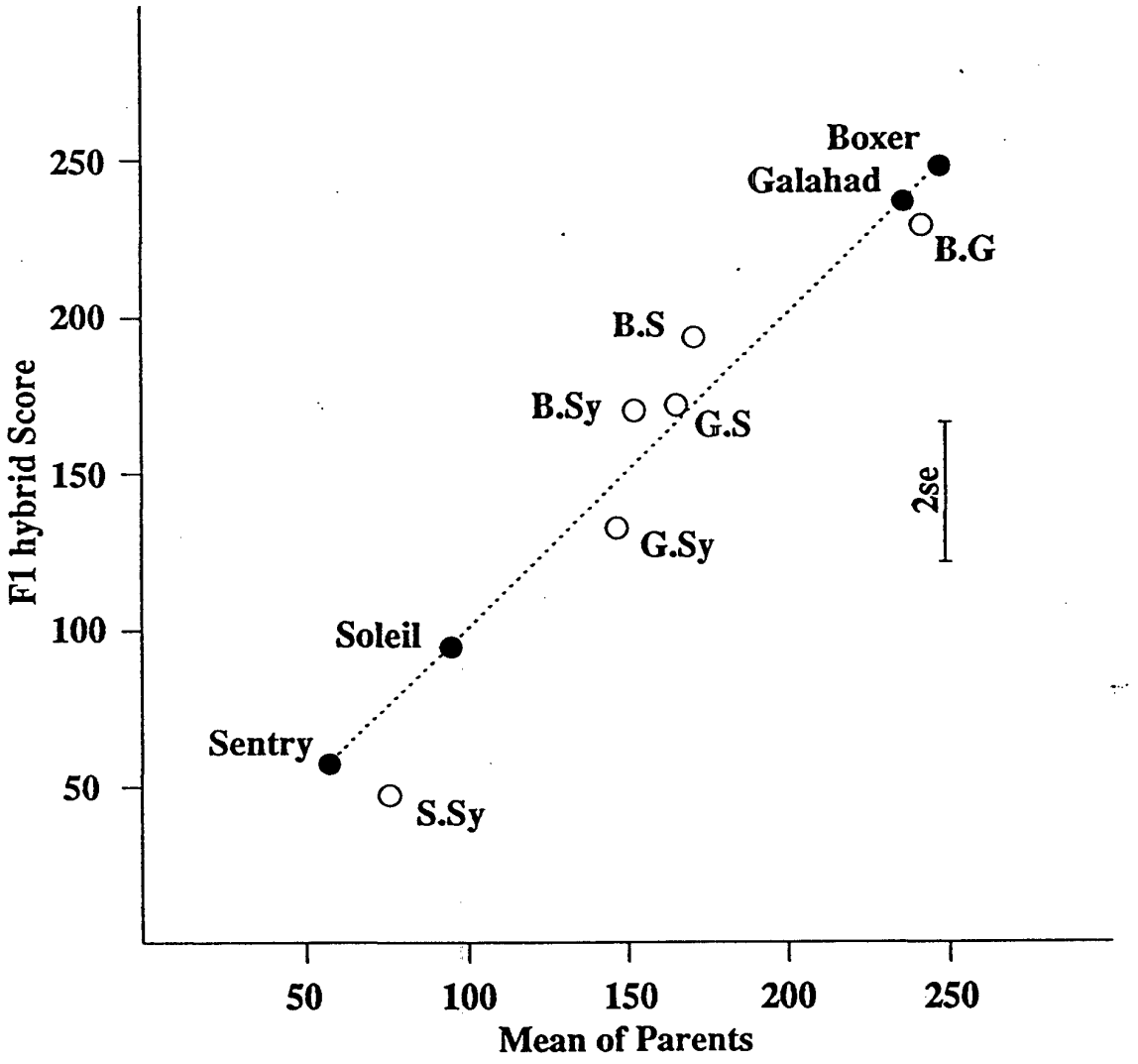


Figure 3.4 Cumulative areas under germination curves of F_1 hybrids over the first four days of imbibition, plotted against expectations on the basis of simple additive gene effects.

Note: Hybrid data (open circles, labelled as in Figure 3.3.) are plotted on the vertical axis, against the mean of their parental scores. The parental varieties are shown as filled circles and the dotted line represents the expected scores of hybrids in the absence of dominant gene effects. The vertical bar labelled "2se" depicts twice standard error of genotype means.

3.3) Embryo cavity waxes

3.3.1 Sprout-susceptibility of wheat varieties

The sprout resistance of 7 selected wheat varieties from the 1992 harvest are shown in Fig 3.5 Differences were greatest 1 week after harvest.

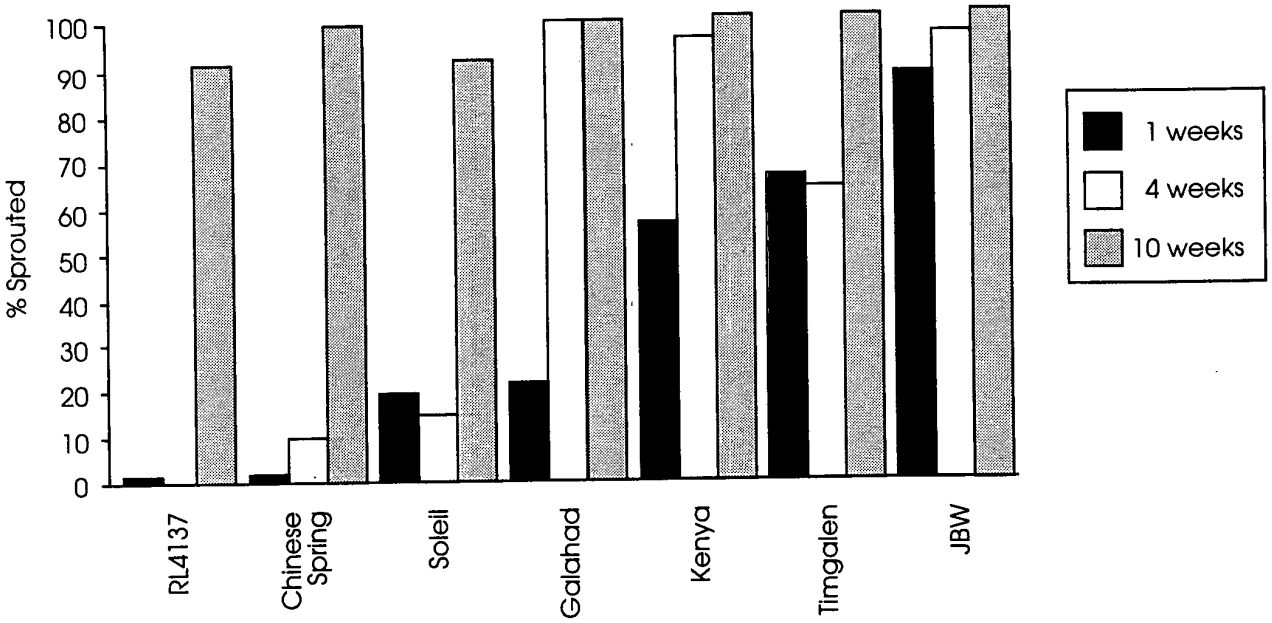


Fig 3.5 Sprout resistance of 1992 crop wheats from which extracts were analysed by GC

3.3.2 Composition of extracts

Embryo cavity wax extracts were subjected to chemical analysis by chromatographic methods and mass spectroscopy, in search of differences in composition between waxes from red/white, dormant/non-dormant grains and varieties. This process was intended to provide fundamental information to substantiate the theory that had been proposed in an earlier report (Flintham *et al.*, 1993). It was recognised that, because of the time requirement of chromatographic methods, they were not suitable for routine comparisons. For that purpose, a rapid test involving the measurement of surface activity had been developed and could be used on extracts prepared in as for analytical purposes. In early trials they had shown some promise (Flintham *et al.*, 1993).

Thin-layer chromatography (TLC)

In *Eucalyptus gunii* and wheat leaf extracts, groups of compounds present were fatty acids, sterols, primary and secondary alcohols, triglycerides, beta-diketones and hydrocarbons.

No beta-diketones could be detected in extracts from wheat grains, but in a recent more concentrated extract, a spot running just below beta-diketones and probably aldehydes, was observed. This constituent was also present in a beta-diketone fraction from eucalyptus.

TLC is primarily a qualitative technique, although it can be used preparatively, to isolate constituents that are subsequently quantitated by other means. Different lipid classes and lipids of different fatty acid composition stain differently. Whilst densitometry across a charred plate is possible, quantitative spotting is difficult unless internal standards can be used for each group and differences need to be comparatively large to be significant. Embryo cavity waxes have so far only shown subtle differences between varieties. Greater scatter would result from separating groups of compounds by TLC before GC, although it could be a useful technique for identification purposes.

Capillary gas chromatography - Comparison of duplicate samples of 2wheats of different sprout resistance

Blanks showed that the HPLC grade chloroform (Aldrich, stabilised with amylenes) was fairly satisfactory, though some small contaminant peaks occurred, which differed between batches. Pyridine contained no contaminants and a fresh batch of (bis)-trimethylacetamide overcame a problem with that reagent.

Once techniques had been improved to eliminate contaminants from old silylating reagents, glassware and general handling, only a few small peaks were found in chromatograms of extracts of embryo covers from 10 grains (Fig. 3.6). After increasing the number of grains to 100 and obtaining more peaks well clear of the baseline, the analysis of RL 4137 and Timgalen 1992 wheat wax extracts relative to heneicosane is shown in Table 3.IV

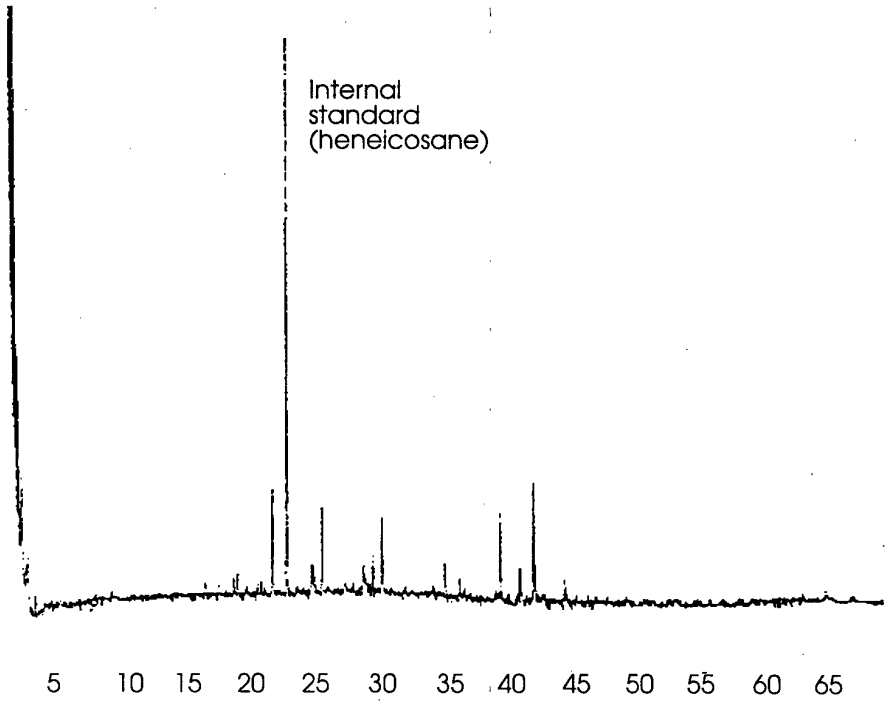


Fig 3.6 Analysis of extracts of embryo covers from 10 grains of RL4137

Table 3.IV Comparison of waxes in embryo cover extracts of RL4137 and Timgalen wheats

Analysis of 100 grains

PEAK NO.	UKELY CONSTITUENT	RT	RL4 137 MEAN	RL4 137 RANGE+/-	TIMG ALEN MEAN	TIMG ALEN RANGE+/-	BLA NKS MEAN	BLA NKS RANGE+/-	PEAK NO.
1	AC16	22:32.7	78.94	6.17	80.73	2.02	10.67	0.82	1
2	HC21	23:40.1	0.00	0.00	0.00	0.00	0.00	0.00	2
3	UB	23:52.1	23.22	10.19	5.80	5.80	0.00	0.00	3
4	OH18	25:35.0	142.20	5.29	150.80	3.76	1.54	1.54	4
5	HC22	25:44.8	33.66	0.68	36.11	0.24	0.25	0.25	5
6	AC18	26:22.5	19.53	2.77	8.31	8.31	4.80	4.80	6
7	OH20	28:41.2	13.08	0.85	26.99	16.44	10.51	4.89	7
8	HC24	29:29.7	0.38	0.38	9.09	4.25	15.84	11.21	8
9	UB	30:56.9	46.23	2.92	64.54	14.34	39.33	8.50	9
10	HC27?	34:50.3	20.64	16.48	20.83	16.27	2.22	2.06	10
11	HC28	35:47.0	42.21	16.45	55.77	6.56	1.16	1.30	11
12	HC29	37:16.8	47.73	4.92	78.97	14.33	0.90	0.31	12
13	UA	39:37.8	23.57	2.22	32.46	3.60	1.77	0.78	13
14	UA?AAE	39:57.0	306.22	32.03	199.74	130.90	2.28	0.01	14
15	UA	40:44.2	20.34	5.16	30.76	1.71	0.98	1.95	15
16	HC32	41:40.2	72.64	1.84	80.82	7.83	1.96	1.96	16
17	HC33?	42:36.8	407.10	4.56	472.42	130.32	0.00	0.00	17
18	UA?AAE	42:46.9	107.84	2.53	107.35	12.30	0.00	0.00	18
19	UB	43:24.7	43.06	4.37	55.43	27.58	1.60	0.20	19
20	UB	45:10.4	66.46	10.19	96.73	31.36	1.14	0.54	20
21	UA	47:29.1	17.77	2.48	15.08	15.08	0.56	0.31	21
22	HC40	51:32.3	17.70	0.23	14.91	0.17	0.42	0.42	22
23	UA	53:19.7	39.03	2.53	50.59	7.37	0.48	0.48	23
24	UB	57:23.8	17.15	7.16	10.59	6.36	0.89	0.89	24
25	?TG	63:53.5	80.52	8.51	94.48	35.41	0.88	1.34	25
26	?TG	65:48.1	312.24	33.50	422.19	178.90	2.09	1.12	26
27	?TG	67:58.3	262.43	55.83	389.44	202.07	0.76	1.51	27
	TOTAL ALL PEAKS		2261.87		2610.94		103.03		
	TOTAL HCS		642.06		768.92		22.76		
	TOTAL WAX ESTERS								
	TOTAL ALCOHOLS		155.27		177.79		12.05		
	TOTAL ACIDS		98.47		89.05		15.48		
	TOTAL TRIGLYCERIDES		655.19		906.11		3.72		
	TOTAL UNKNOWNS		296.82		361.88		46.74		
	UNKNOWNS ?AAE		414.06		307.09		2.28		

Note: In constituent designations: AC = acid, OH = alcohol, HC = hydrocarbon, TG = triglyceride, ?AEE = possible ferulic acid ester, UB = unknown, before silylation, UA = unknown, appearing on silylation; numbers indicate chain length. Later evidence showed that TG25 and 26 are sterol esters and the major peak 17 is likely to be β-sitosterol.

Replication of these results was poor. For this reason, extraction time and temperature were increased in the following study.

- Comparison of waxes from embryo covers of six 1992 wheat varieties

Single samples of 100 embryo covers dissected after removal of beeswing from 6 varieties of varying sprout resistance did not show any clear relation between sprout resistance and total wax (Fig 3.7) or any single wax component of 150 examined. Neither was there any relation between peaks of similar retention time groups and sprouting behaviour (Fig 3.8). When amounts of groups of similar chemical entities were determined, using the best available peak identifications, simple correlations fell well short of significance. Hydrocarbons and alcohols-wax esters, plotted against sprout-resistance had slight positive gradients for lines of best fit, whereas the corresponding fatty acids *vs* sprout resistance graph had a slight negative slope (Fig.3.9).

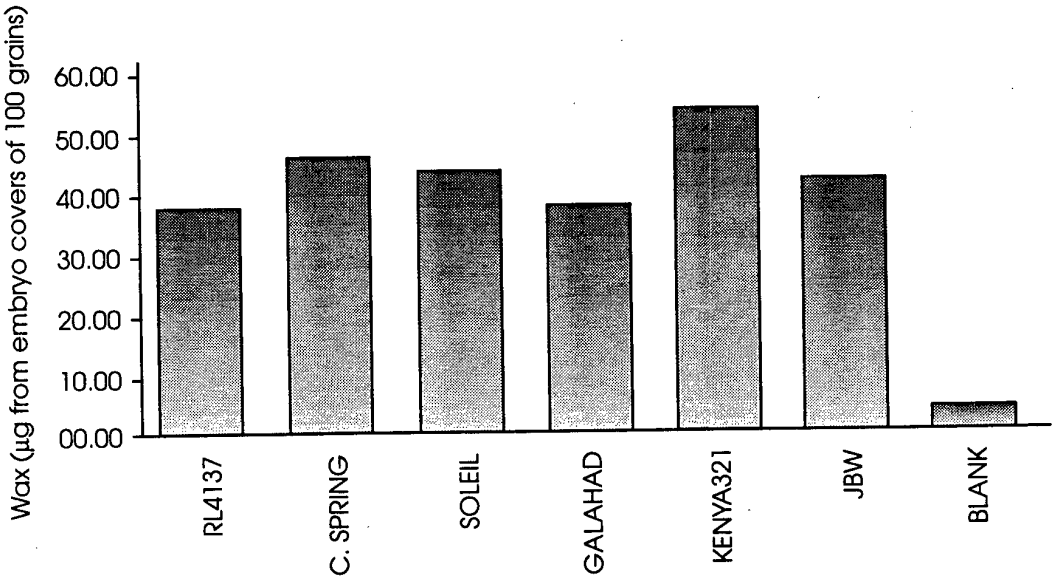


Fig 3.7 **Total wax (sum of 150 peaks) determined in 6 wheat varieties of varying sprout resistance**

In many cases sprout-susceptible varieties contained more of particular constituents than sprout-resistant ones. Thus, Galahad contained the most fatty acid, Kenya more hydrocarbon and JBW more alcohol or wax ester than the others. Individual peaks also differed among varieties. Timgalen was omitted from the analysis due to computer error. Its chromatograms were rather different from those of the other wheats.

- Recovery of standards following derivatisation and gas chromatography

Using a similar technique to that for the above analysis, with alcohol and fatty acid standards at concentrations relating to large (mixture volume 1:1 with IS) and small (mixture 0.2:1 with IS) peaks, recoveries relative to heneicosane were low and variable. In the case of fatty acids recovery decreased with increasing molecular weight. Even if response factors vary at 1:1 and 0.2:1 ratios (Peter Holloway, private communication), this clearly indicates a problem.

Similar quantitation problems may be assumed to have occurred in the analysis of embryo covers of 6 wheats. Wax constituents are relatively insoluble. In order to inject sufficient wax for GC analysis without unduly large solvent peaks, the residue from 2ml solvent is only dissolved in 10µl solvent as efficiently as possible by rotating and warming.

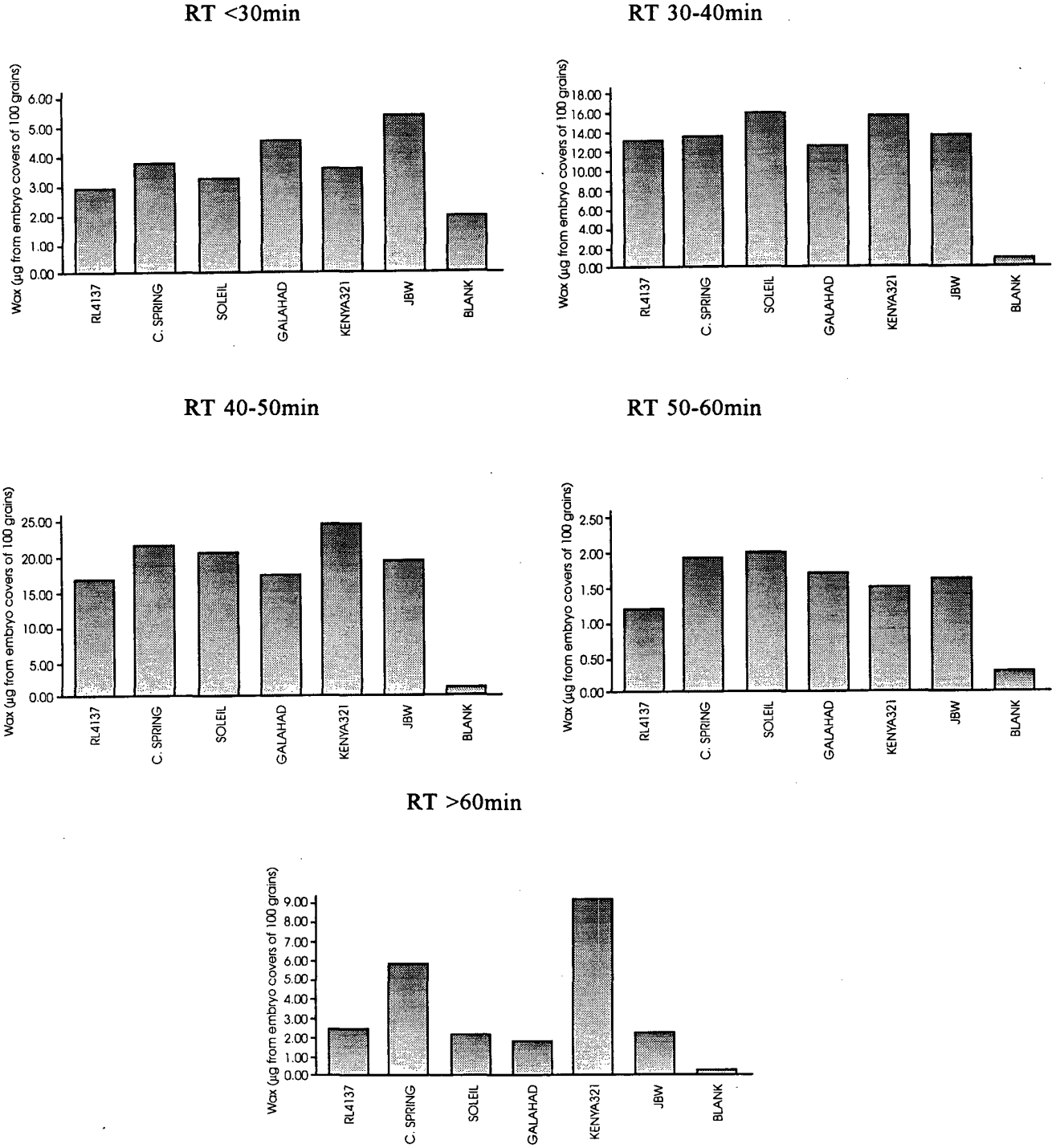


Fig 3.8 Wax constituents of varying retention times from embryo covers of 6 wheats of varying sprout resistance

This was an extremely small volume with which to wash the vial walls, but a relatively concentrated solution was needed for injection. Also problems with wax remaining on glass surfaces have recurred through this study, unless extremely stringent cleaning techniques are used. It was appreciated that compounds of higher molecular weight are likely to be less soluble than similar ones of lower molecular weight, but thought that if poor solubility affected the results, all samples would be influenced similarly. However, this is not true in the case of the alcohols.

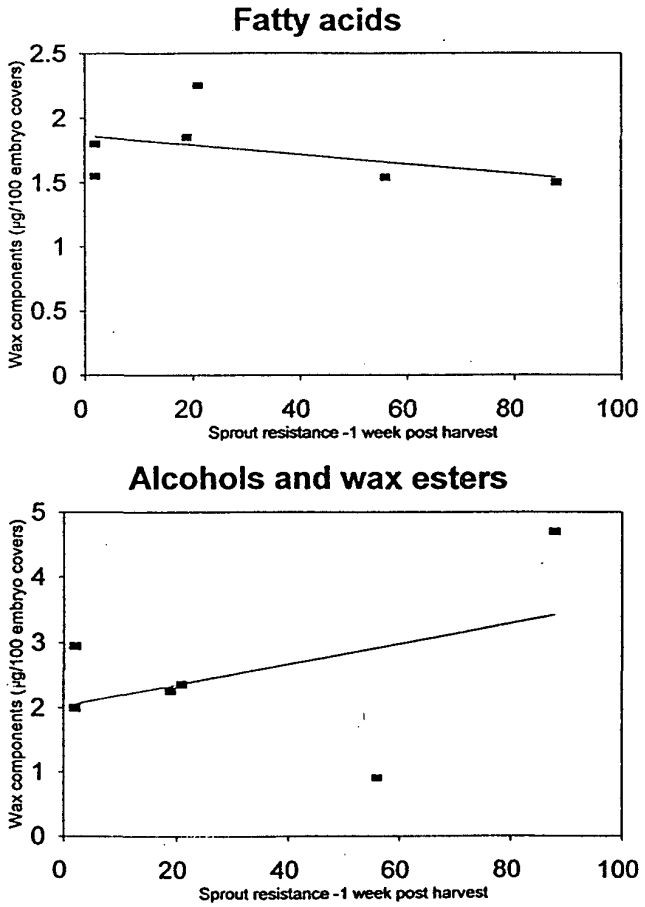


Fig 3.9 **Relation between polar lipids in embryo cover waxes and sprout resistance as measured one week after harvest.**

Table 3.V Percent recovery of standards after GC

Standards	Alcohol	Alcohol	Acid	Acid
Volume:IS volume	1:1	0.2:1	1:1	0.2:1
C12:0, C14:0	73	48	80	93
C24:0	97	60	44	27
C30:0	80	74	13	-

Identification of wax constituents: a) by comparison with standards

Comparison of RTs with those of standards likely to be present, whilst being a useful guide, is not entirely reliable. Peaks identified relative to a standard on a single column may or may not be entirely that compound. The chromatograms of RL4127 before and after silylation, in which the heights of the heneicosane internal standard peaks are similar, illustrate this point (Fig.3.10).

Some peaks decrease in height after silylation (eg. a peak RT ca. 20 min and those coinciding with hydrocarbons C27:0 and C29:0). Hydrocarbons should not be affected by silylation. Aldehydes and beta-diketones may decrease and in any case do not survive GC well. The peak coinciding with fatty acid C16:0 is almost as large before silylation as after. Fatty acids should only appear after silylation.

The 2 strongest peaks, previously designated as an unknown: UA and hydrocarbon C34:0, appear to shift retention times by about 1min. extra on silylation. Sterols change in retention time upon silylation and the UA peak coincides with beta-sitosterol both before and after. It is suggested that the other major peak may be one of the ferulic acid esters. The peaks for campesterol are comparatively large in some varieties and fall between beta-sitosterol and this peak.

The beta-diketone fraction prepared from *Eucalyptus gunnii* gave a number of peaks on GC, two of which with retention times of 45 and 47.3 min. approximately coincided with peaks from wheat embryo cavity extracts and may be aldehydes, which also appear to be present in thin-layer chromatograms of this fraction.

Identification of wax constituents: b) by GC-MS

In addition to commercially available straight-chain saturated fatty acids, alcohols, hydrocarbons and wax esters, branched chain and unsaturated fatty acids, a range of compounds differing by 2 methylene groups which were suggested to be ferulic acid esters and squalene were found. The contaminants BHT, dioctyl phthalate and several compounds derived from (bis) trimethylacetamide were identified and measures have since been taken to minimise them.

Clearly identification of peaks is incomplete. It was not considered essential to establish the identity of every peak, unless it is shown to be important in relation to sprout resistance It was necessary to identify the type of compound represented in order to ensure correct grouping. However, to establish differences between wheats was accorded highest priority.

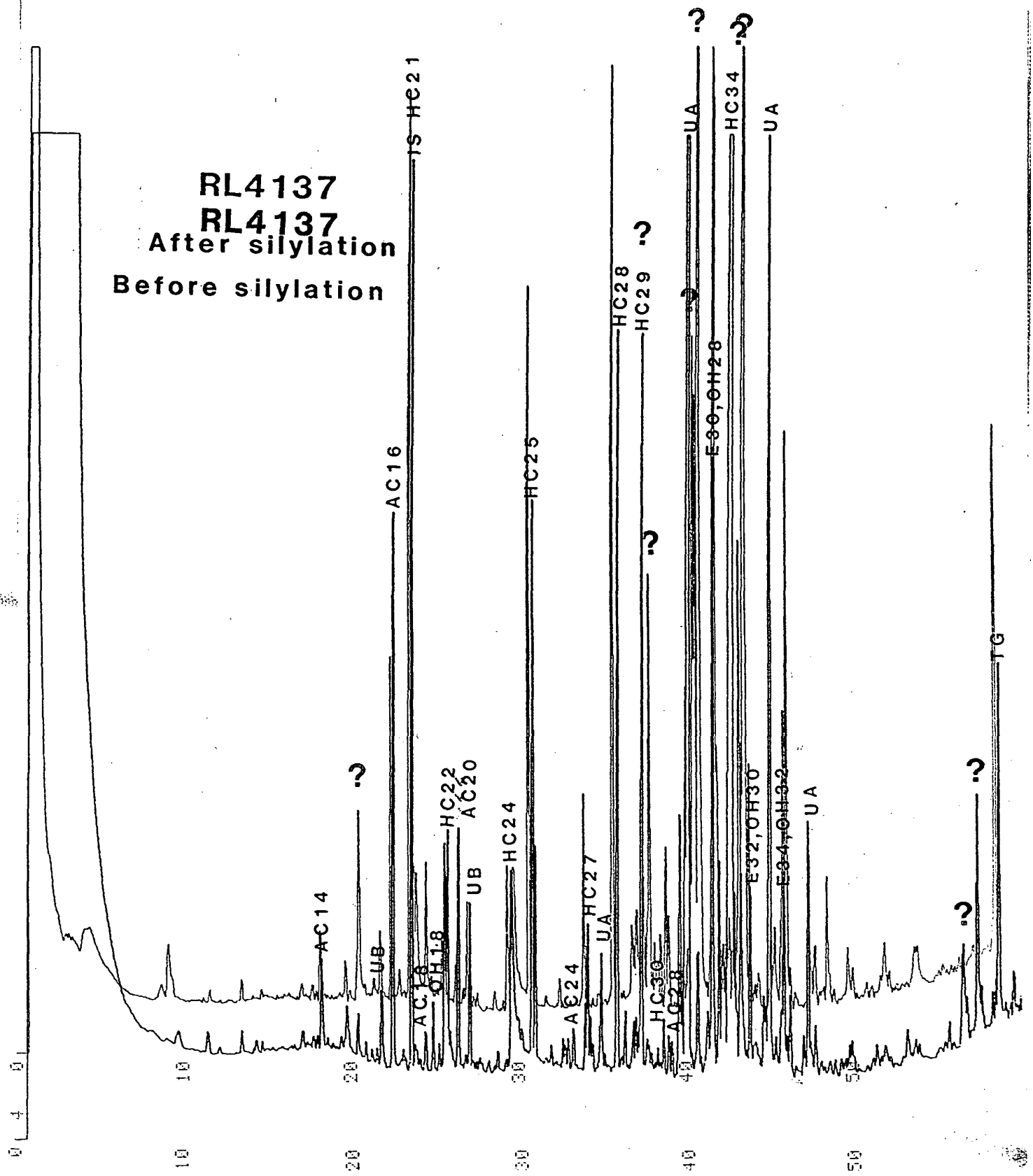


Fig 3.10 Chromatograms of RL4137 before and after silylation

3.3.3) Surface pressures

Standard curves from waxes from dissection

Curves obtained from aliquots of a solution of wax accumulated from dissections showed surface activity and, while reproducibility was not perfect, a reasonably compact set of five replicate curves was obtained. Fig 3.11

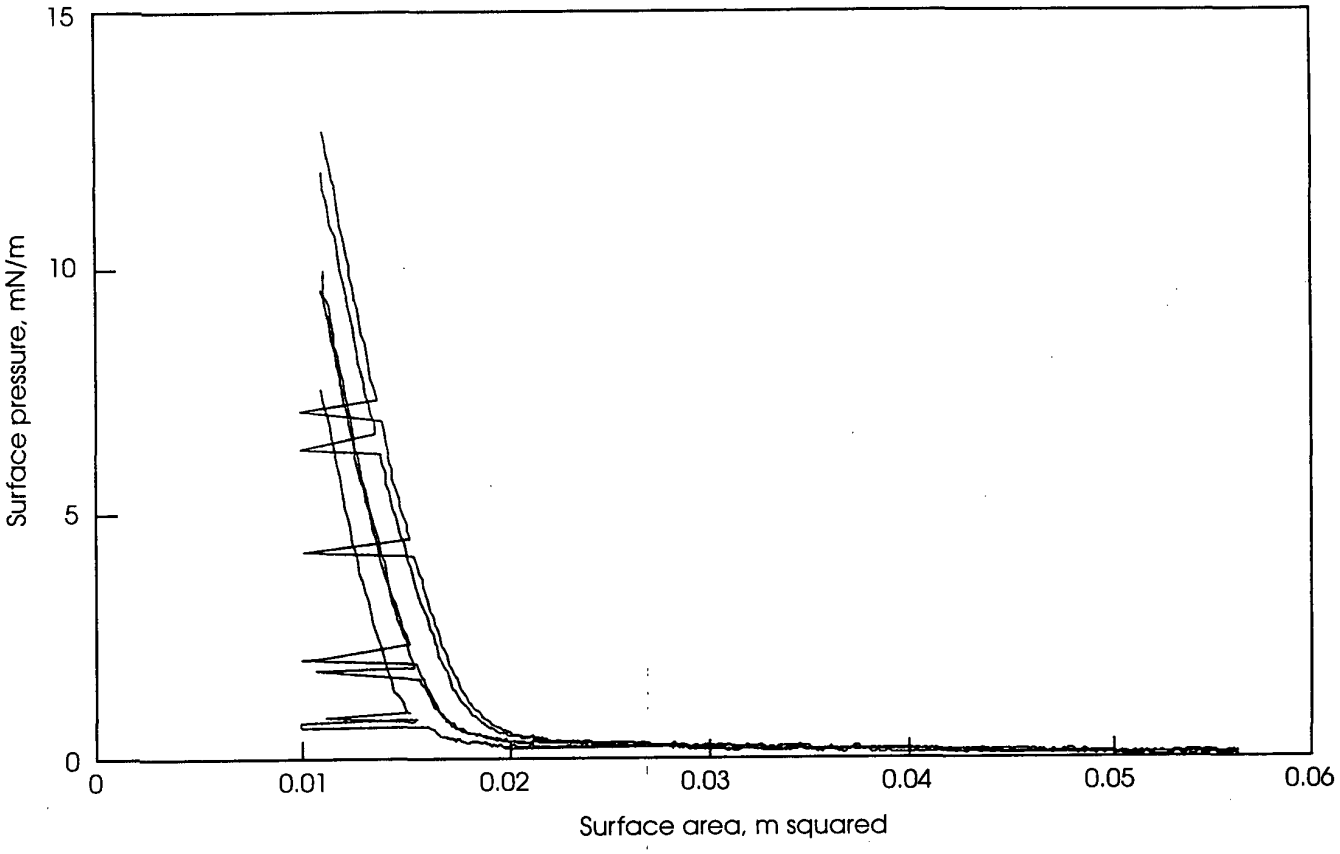


Fig 3.11 Replicate curves obtained from aliquots of embryo cavity wax solution.

From each curve the following parameters can be derived for the purposes of numerical comparison:

- The surface area (A_L) at which surface pressure starts to increase, i.e. the surface area corresponding to the end of an initial lag phase
- The rate of increase in surface pressure as a function of the decrease in surface area following the lag phase - given by the slope.
- The surface pressure at maximum compression (i.e. minimum surface area) of the surface film . The range and variability of results suggested that use of a log transformation of this parameter was appropriate.

In the replicate wax curves above it can be seen that the most consistent characteristic is the slope.

Curves from extracts of recently harvested grains

As with the analytical methods described above, results were obtained on many series of extracts, as improvements in extraction procedures were sought. Extracts from 60 grains, divided into 3 aliquots gave even less satisfactory reproducibility than replicated extracts from 20 grains of the same sample set. While this result is unexpected and inexplicable, a pragmatic approach was taken, and the latter method was thus adopted for much of the comparative work. The results below were obtained using extracts from beeswing-free embryo covers from 20 grains, submerged in chloroform and vortex mixed for 30 s. Initial washings (1ml) were filtered and supplemented by subsequent washings (1ml) filtered after 10 s vortex mixing.

Triplicate measurements were made on the complete sample set of 7 varieties and comparisons were made between resistant and susceptible sub-sets.

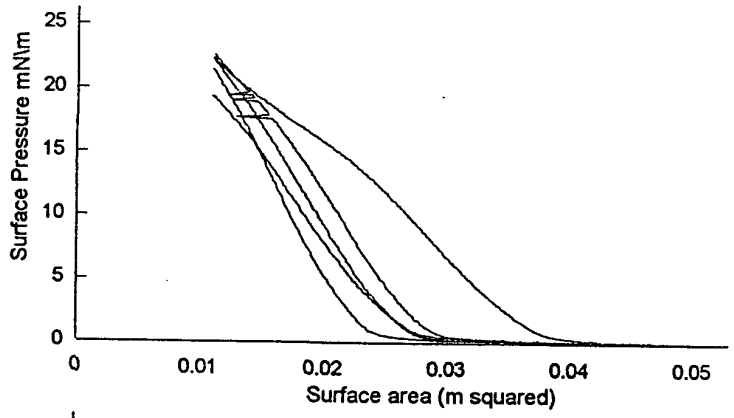
Table 3.V1 Surface pressure parameters for three resistant and four susceptible wheats.

Variety	A_l	Slope	log(P_{min})
RL4137 (r,res)	0.0168	1714	1.924
Soleil (r,res)	0.0173	1667	2.094
Chinese Spring (r,res)	0.0217	1557	2.598
Galahad (r,sus)	0.0198	1450	2.131
JBW (w,sus)	0.0224	1471	2.563
Timgalen (w,sus)	0.0226	1622	2.763
Kenya 321 (w,sus)	0.0257	1307	2.735
Min sig diff	0.0073	560.3	1.0186

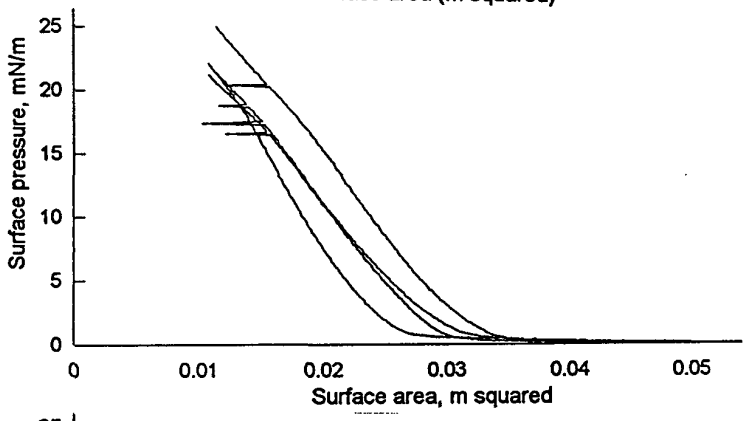
r=red, w=white, res=sprout resistant,sus=sprout susceptible

Significant differences in A_L (p=0.01) and slope (p=0.05) were found between susceptible and resistant groups but differences in log P_{min} were not significant .

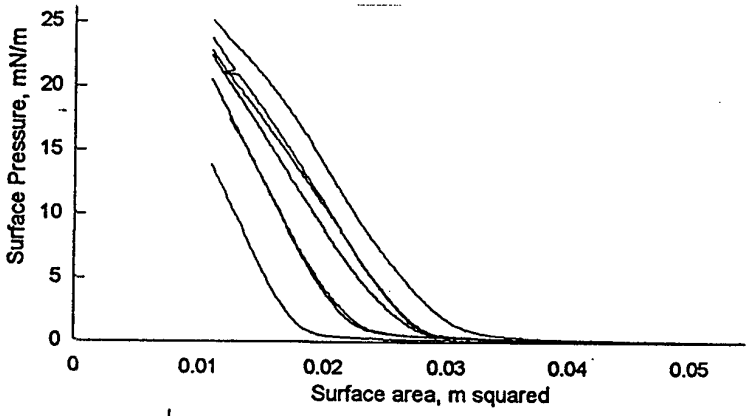
Replication on individual extracts was good for two of the triplicate analyses but the third did not accord well with these. A re-examination of the results with the outlying set omitted introduced a highly significant difference for P_{min}, (p=0.001) but the significance of the other parameters was not altered. The inconclusive results obtained pointed to the need for a definitive simultaneous comparison of red and white wheats showing as much variation as possible in sprouting resistance. A comparison of 12 samples, including some replication and some varieties grown in two different years, was carried out. The results are shown in Fig 3.12.



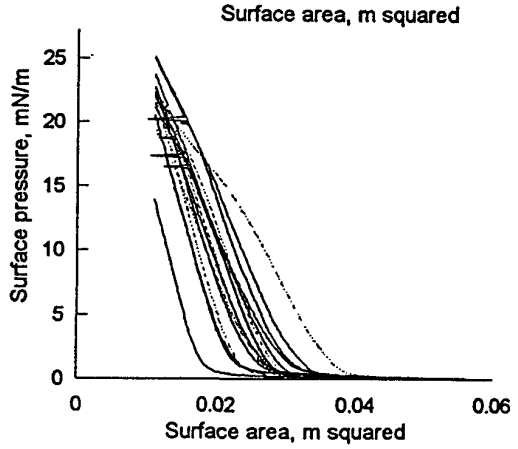
a) Sprout susceptible varieties (l to r: Bounty, JBW 4, Voyage, Kenya - duplicates)



b) Sprout resistant varieties 1991 harvest: (l to r: Parade, Pageant, Longbow, Pastiche)



c) Sprout resistant varieties 1992 (l to r: Fortress -duplicates, Parade - replicate 1, Pastiche, Pageant - duplicates, Parade - replicate 2)



d) All types (continuous lines represent resistant types, broken lines represent susceptible types).

Fig 3.12. Curves showing surface pressure as a function of surface area for extracts from sixteen wheat samples.

Where replication was performed the results were somewhat variable: Pastiche and Pageant in c) gave almost superimposable curves, Kenya in a) produced adjacent curves while Parade produced curves that were widely separated. All samples had been prepared and treated in an identical fashion throughout, and no explanation can be offered for the discrepancies in behaviour.

From d) it can be seen that no clear distinction can be drawn between the characteristics of curves produced from resistant and susceptible types.

The lack of consistency among the behaviour patterns of replicate extracts and of aliquots of the same extract, is reminiscent of the experience encountered in relation to the chromatographic analysis of the same extracts. The problems associated with the two types of measurement thus probably derive from the same source, namely the difficulties of dealing with minute quantities of substances capable of interacting with surfaces with which they come into contact during essential handling. While this problem was recognised at an early stage, and all possible steps were taken to minimise it, it did not prove possible to reduce it to a level at which a distinction could be made between real and artifactual differences related to sprouting potential. As no further improvements in handling techniques could be conceived, the work on this aspect of the project was reluctantly terminated coincidentally with abandoning wax analysis.

3.3.4) Physical characteristics of embryocovers

During the course of dissection on a wide range of wheat types it became clear that the thickness and brittleness of embryo covers varied systematically. An impression was gained that brittle tissues were characteristic of sprout susceptible types, and as this accorded with a notion of sprouting susceptibility being influenced by permeability to water or gas, the relationship was explored in a test in which a brittleness assessment was made on a set of covers whose origins were not known to the assessor. The scores attributed did not accord with sprouting susceptibility to any greater degree than would be expected to occur by chance.

3.4) Assessment by NMR imaging of water uptake by dormant and non-dormant grains

Resonance imaging of water uptake by imbibing grains from the same sets

The facility was available for one day and, following the preparation of materials and apparatus, it was possible to observe at 0, 1, 3, and 6.5 hours of imbibition. Viewing was conducted on whole grains but images are of optical sections. Attempts were made to view sections of comparable thickness, orientation and position in grains from dormant and non-dormant samples. In the images, bright areas are indicative of water.

Fig 3.13 shows the distribution of water in the non-dormant grain at resting moisture content (approx 14% m/c). The two images are of adjacent 0.5mm near-sagittal optical sections. The embryo is the only site where water is indicated clearly, although the outline of the grain section can just be discerned by a lighter outline, probably corresponding to the aleurone layer.

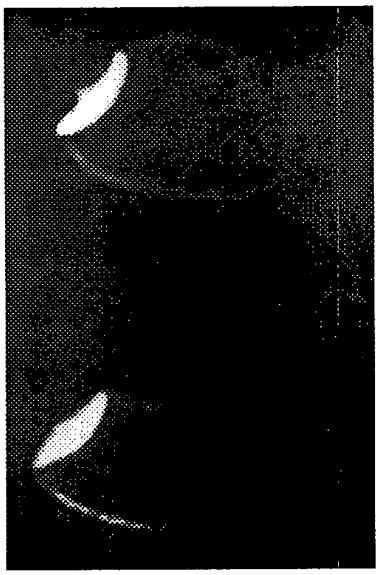


Fig 3.13 Non-dormant grain - resting moisture content. Near sagittal sections

Fig 3.14 shows a mid-grain optical section of dorso-ventrally orientated dry, dormant grain. Once again, the embryo is brightest with a less distinct peripheral outline, but because of the orientation and plane, a faint indication of the crease can also be detected.

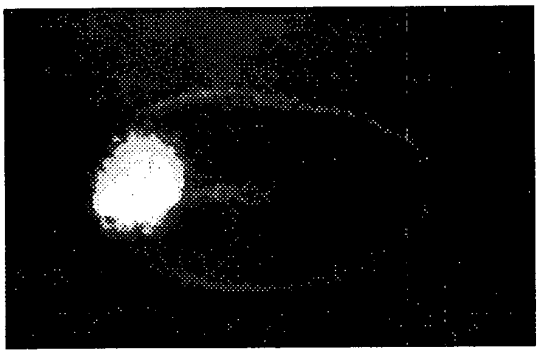


Fig 3.14 Dormant grain - resting moisture content. Mid dorso-ventral section

Fig 3.15 shows a sagittal section of a dormant grain after a steep of 6.5 h. Because the water concentration is higher than in Figs a and b the resolution is reduced, and although the endosperm may contain more water than previously, the image of the periphery is less clear. The imaging conditions were chosen to provide maximum detail in the embryo, as this is assumed to be the site of greatest importance in relation to germination control.

Fig 3.15 Dormant grain after 6.5 h steep. Sagittal section

Although minor differences were noted between grains from dormant and non-dormant samples, none was consistent and longer imbibed samples tended to show greater similarity than dryer ones. On the basis of the limited study it was concluded that no evidence of penetration rate or distribution of water could be claimed. As the technique is very costly, it was considered that further examinations were not justified by the results. The absence of differences lent no support to an explanation of dormancy involving denial of access by water to the embryo or even a specific region of it.

All grains of the "aged" (non-dormant) set germinated within two days of imbibition, whereas none of the "deep-frozen" (dormant) grains germinated after two days. Thereafter, their germination rate was very slow and typical of freshly harvested material of the same variety (eg. Figure 3.3). Viability of the dormant grains was proven by their complete germination following cold treatment.

3.5) Genetic mapping of *R* loci on long arms of wheat group 3 chromosomes

Table 3.VII lists 26 DNA clones which have been screened for locations on the long arms of group 3 chromosomes and for RFLP's amongst the mapping parents Chinese Spring, Spica, Red Bobs, and Kenya 321. These probes identified 558 fragments over six different genomic digests of Chinese Spring, and 122 polymorphisms (absence of bands, additional bands, or size shifts) were observed between these fragments and their homologues in the other parents. A subset of the clones were also scored for polymorphism in the variety Dollar and the artificial hexaploid Synthetic. Only seven clones yielded polymorphisms in appropriate linkage groups and co-dominant markers were selected wherever possible. The clone/enzyme combinations used for linkage analysis are shown in Table 3.VIII.

The mapping populations segregated for each marker according to Mendelian expectations; 1 homozygous AA : 2 heterozygous AB : 1 homozygous BB for *R1*, *R2* and RFLP's based

on size differences; 3:1 in the case of null alleles and the *R3* allele for which only F_2 scores were available. Each *R* allele was mapped from a different cross (Table 3.VIII) and wheat homoeologues to barley clones BCD131 and WG110 were mapped to chromosomes 3A and 3D respectively from the cross Chinese Spring x Synthetic, in collaboration with M.D.Gale and K.M.Devos.

For any pair of genetic markers, the frequency of recombination varies according to their relative locations on the chromosomes: for markers on different chromosomes, or for markers widely separate on the same chromosome, half of the gametes from F_1 hybrids are expected to carry the same combination of genes as one or other of the parents, while the other half are expected to carry recombinant (non-parental) combinations. These gametic expectations yield derivative expectations for frequencies of different genotypes amongst F_2 plants, so that recombination frequencies can be estimated from phenotypic frequencies in F_2 or F_3. The smaller the separation between marker genes on the same chromosome, the smaller the probability of recombination, and the genetic map distance between any two markers is a function of the probability of recombination between them. For independent, freely dissociating loci recombination frequency equals 50%. For loci which are so close that recombination is never observed the corresponding map distance is zero; intermediate recombination frequencies yield proportional map distances after correction for double recombination events (Kosambi 1944).

Mapping of chromosome 3A yielded the clearest result, placing *R2* in the interval between the DNA markers *XCxp1* and *Xpsr904*, approximately 28 centiMorgans (cM) from the former and 7cM from the latter. Mapping data for chromosome 3B are incomplete at the time of writing, however the preliminary analysis placed *R3* very close to *Xabc174*, in the interval between *XBbcd131* and *Xpsr1205*. Further analysis of this cross should elucidate the map order within this segment of 3B. Analysis of chromosome 3D showed *R1* to be tightly linked to *Xbcd131*, some 15cM from *Xabc174* and effectively independent of *Xabg377*. It was not possible to determine the order of *R1* and *Xbcd131* from the mapping data, however a consideration of the available maps for chromosome 3H of barley, wheat chromosomes 3A, 3B and 3D in the cross Chinese Spring x Synthetic, and the crosses described above, suggests that the order of genes is conserved over each wheat chromosome as indicated in Figure 3.16.

Table 3.VII. DNA clones screened for wheat genomic restriction fragment length polymorphisms

Clone	Source	No. of bands	No. polymorphic
psr 36	JIC, wheat gDNA	29	4
psr 78	JIC, wheat cDNA	17	5
psr 170	" "	37	8
psr 904	JIC, wheat gDNA	25	8
psr 931	" "	18	3
psr 1203	" "	20	11
psr 1205	" "	17	8
ABC 161	WSU, barley cDNA	22	4
ABC 166	" "	41	10
ABC 174	" "	18	14
ABC 176	" "	14	1
ABG 4	WSU, barley gDNA	18	3
ABG 377	" "	11	2
ABG 389	" "	16	1
BCD 131	CU, barley cDNA	30	11
CDO 474	CU, oat cDNA	20	2
WG 110	CU, wheat gDNA	19	0
BG 131	CSU, barley gDNA	20	0
MWG 41	FBR, barley gDNA	9	1
MWG 546	" "	28	8
G 048	KSU, diploid wheat gDNA	30	3
G 059	" "	7	0
Cxp 1	LTU, barley cDNA	10	3
Glb 33	" "	44	2
Glb 35	" "	35	9
per 1	RNL, barley cDNA	3	1
Totals	26	558	122 (mean 40.7 per variety)

Note: DNA clones were kindly provided as follows:
JIC - M.D.Gale, John Innes Centre,
WSU - A.Kleinhofs, Washington State University, USA,
CU - M.Sorrells, Cornell University, USA,
CSU - N.Lapitan, Colorado State University, USA,
FBR - A.Graner, Federal Biological Research Centre for Agriculture and Forestry, Germany,
KSU - B.Gill, Kansas State University, USA,
LTU - G.B.Fincher, La Trobe University, Australia,
RNL - S.K.Rasmussen, Riso National Laboratory, Denmark.
Species of origin of each clone is indicated, gDNA = genomic fragment, cDNA = reverse transcript from mRNA. All clones were screened against genomic DNA of the varieties Chinese Spring (standard reference), Spica, Kenya 321, and Red Bobs, digested with each of the six restriction enzymes Eco RI, Eco RV, Hind III, Dra I, Bam HI, and Sst I. The number of bands is the total number of restriction fragments observed over all digests of Chinese Spring and the number polymorphic is the total number of fragments observed over the other three varieties which differed from Chinese Spring.

Table 3.VIII. DNA clone / restriction enzyme combinations used to map wheat and barley genes and *R* genes to wheat chromosomes

Chromosome (*R* gene)		Clone	Restriction Enzyme	Polymorphism Type
3A (CSxSyn)	-	BCD131	DraI	Null allele (Syn)
3D (CSxSyn)	-	WG110	EcoRV	Null allele (CS)
3A	*(R2)*	psr904	DraI	Size difference
3A	*(R2)*	Cxp1	EcoRI	Size difference
3B	*(R3)*	psr1205	DraI	Null allele (Dollar)
3B	*(R3)*	ABC174	DraI	Null allele (Dollar)
3B	*(R3)*	BCD131	SstI	Null allele (Dollar)
3D	*(R1)*	ABC174	BamHI	Size difference
3D	*(R1)*	ABG377	EcoRV	Size difference
3D	*(R1)*	BCD131	BamHI	Null allele (Spica)

Note: The first two columns denote the chromosome on which the polymorphism was mapped, and the *R* gene targeted where applicable. The first two rows were not directed at particular *R* genes but were used to align the wheat map from the cross Chinese Spring x Synthetic (CSxSyn, Devos and Gale 1993) with the barley map published by Kleinhofs *et al.* (1993). Each *R* gene was mapped from a different, dedicated cross to avoid phenotypic masking by duplicate dominant genes, *R2* from Red Bobs x Kenya321, *R3* from Dollar x Kenya321, *R1* from Spica x Chinese Spring.

This is the first report of molecular markers for the *R* genes, and the first genetic map for the long arm of chromosome 3B to include *R3*. The genetic maps derived for chromosomes 3A, 3B and 3D in this study are consistent with previously published wheat and barley maps, allowing for minor differences in recombination frequencies between different experiments. All of the markers are present as homoeologous copies on each of the three wheat chromosomes, with the exception of *Xpsr904* which has not been detected on 3B and the possible exception of *Xabg377* for which the 3A location has yet to be confirmed. These maps support the growing evidence for conservation of genetic linkages over a wide range of cereal species (Moore *et al.* 1993), allowing the transfer of genetic information between different crops.

The RFLP mapping approach described above can only detect polymorphisms between genes which differ between specific restriction enzyme recognition sites and it is clear that an RFLP which is useful for analysis of one recombinant chromosome may not necessarily be directly applicable to a different cross, due to lack of polymorphism. The marker *Xbcd131* is in close linkage with the *R* loci (Figure 3.2.1) and the (non-polymorphic) marker *Xglb33* is expected to lie in the same interval from a consideration of its location.

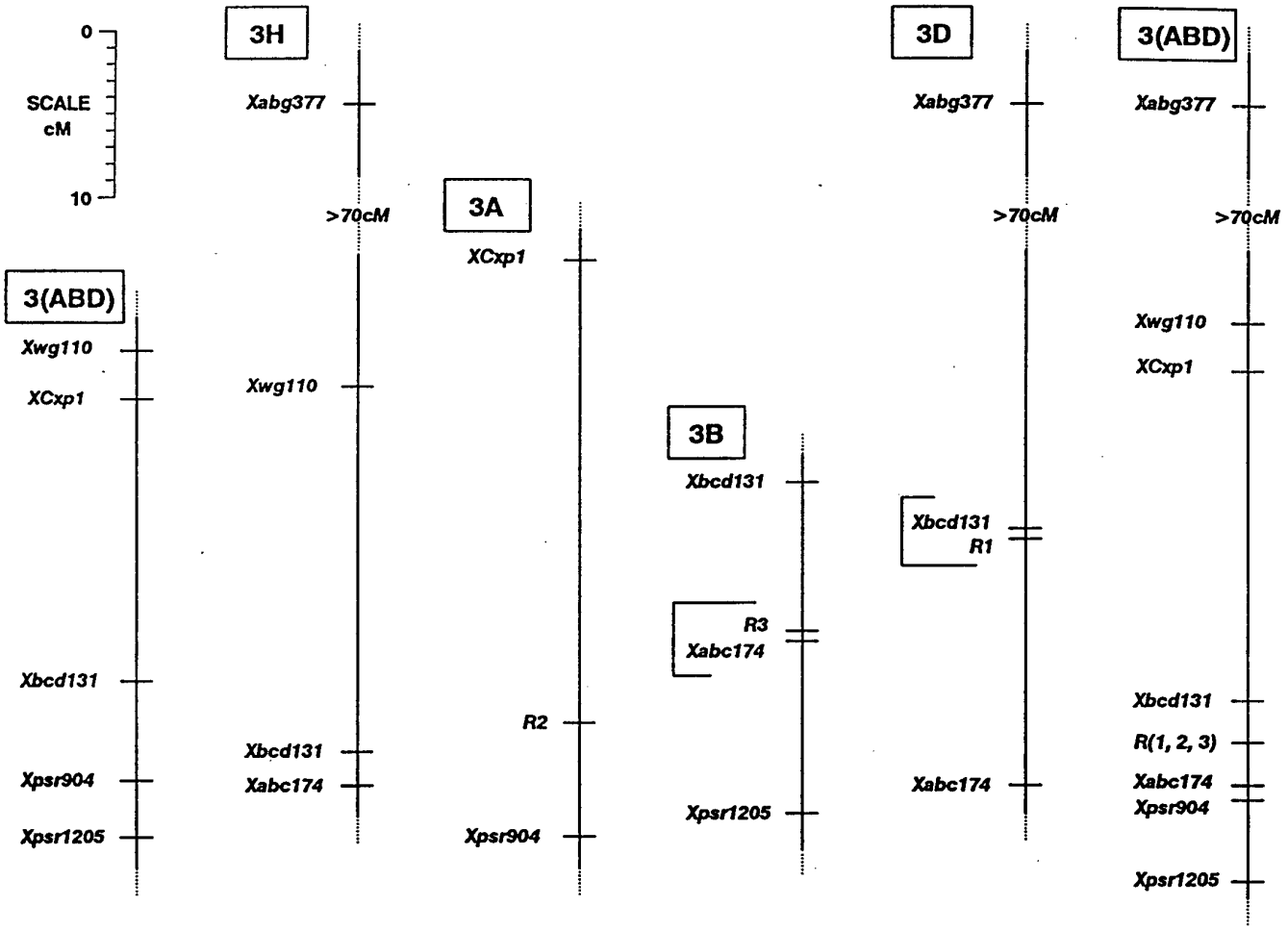


Figure 3.16.Genetic maps of wheat and barley showing the locations of cloned DNA fragments and the *R* loci on long arms of group 3 chromosomes.

Note: From left to right, all to the same scale,
3(ABD) = detail of the consensus 3A/3B/3D map from Devos and Gale (1993) with addition of loci *Xwg110* and *Xbcd131*,
3H = detail of barley chromosome 3 from Kleinhofs et al. (1993),
3A, 3B, 3D = maps from Red Bobs x Kenya321, Dollar x Keny321, Spica x Chinese Spring,
3(ABD) = consensus map showing probable conserved linkages (derived by integrating the other five maps).

on the barley map of Devos and Gale (1993). DNA base sequences of these two barley clones were used to design oligonucleotide primers for amplification of wheat homoeologues by PCR (Methods) in an attempt to raise polymorphic clones of the equivalent genes from wheat. The aim of this exercise was to clone wheat genes lying close to the *R* loci which could then be used to design rapid, non-radioactive markers for the *R* genes, however when the barley primers were used to amplify genomic templates from the set of mapping parents, no repeatable polymorphisms were observed. In addition to the lack of polymorphism, considerable difficulties were encountered with lack of reproducibility of amplification products, most probably due to base sequence differences between the barley primers and their wheat targets. Future work in this area will be based upon wheat rather than barley clones for primer sequences; although this will mean using less closely linked markers it will avoid the problem of sequence mismatching.

3.6) Variation for *alpha*-amylase in the absence of germination

Out of 130 recombinant F_3 families from the cross Spica x Chinese Spring, only five produced grain with *alpha*-amylase contents equal to or greater than that of the high-amylase parent Spica (Figure 3.17). This result rules out the possibility of simple monogenic inheritance and suggests that the high-amylase trait is determined by two, or possibly more than two recessive genes. Of the five high-amylase recombinants four were white-grained and carried Spica genes at the *Xbcd131* and *Xabc174* loci adjacent to the *r1* gene. This strongly suggests that one of the genetic factors leading to high amylase activity is closely linked to the *r1* gene, since the probability of four out of five random lines being white-grained is 0.015. The fifth, red-grained line was segregating for the *R1/r1* genes, and for the other linked genes and it is possible that high amylase in this sample may have been due to presence of white-grained *r1* homozygotes as in the other four lines. This association between white grain colour and high *alpha*-amylase activity suggests that one of the factors responsible for enzyme accumulation during ripening maps to the linkage group on the long arm of chromosome 3D carrying the *r1* gene, possibly *via* the same mechanism as the reduced embryo dormancy associated with *r1*. Whether or not *r1* is itself directly involved in the control of amylase activity, at least one other factor must be involved since the majority of white-grained lines in the population produced only low levels of activity.

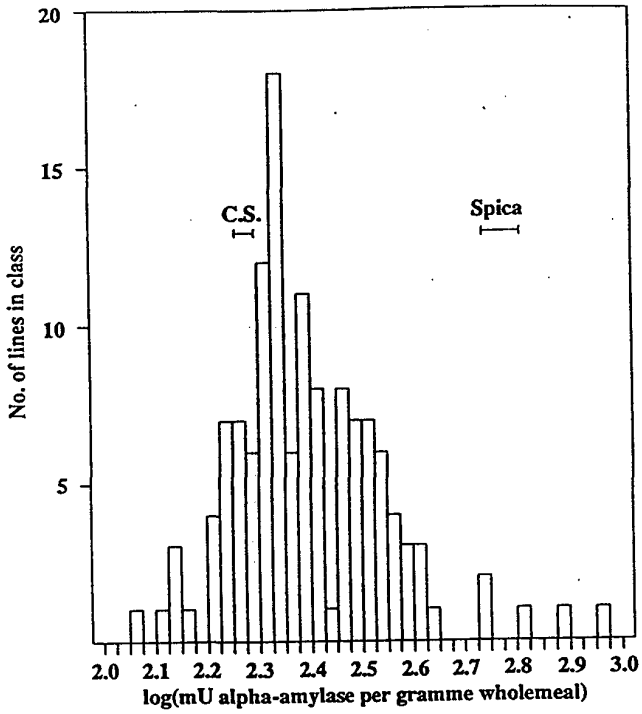


Figure 3.17. Frequency distribution for grain *alpha*-amylase content amongst recombinant lines from the cross Spica x Chinese Spring.

Note: Grain samples were taken from glasshouse-grown F_3 families completely free of germination and *alpha*-amylase activity in these grains is attributed to enzyme synthesis during the latter stages of grain maturation. The horizontal bars indicate the ranges of activities found in grains of the parents Chinese Spring (C.S.) and Spica in the same trial.

3.6.1) Distribution of non-germination enzyme within the grain

This set of samples was used both to investigate the nature and sources of *alpha*-amylases present and at the same time to evaluate gel and microtitre assays against the established Phadebas method.

Examination of microtome sections probed with fluorescein dibutyrate, consistently indicated most or all esterase activity of the grain in the aleurone tissue of the crease region. This novel observation was confirmed in similar investigations on cut faces of half grains and dissected aleurone layers.

With the gel assay it was possible to quantify crease activity separately from *alpha*-amylases produced elsewhere in the grain, when these were present. Initially grains were cut transversely as this showed clearly whether the activity lay in the crease region or around the periphery of the grain (the latter being typical of both aleurone germination enzyme and pericarp activity - although it is possible to distinguish them visually in most cases). A more than three-fold increase was found between grains less than 5.2mm long and those longer than 7.1mm. The trend was continuous throughout the intermediate size classes.

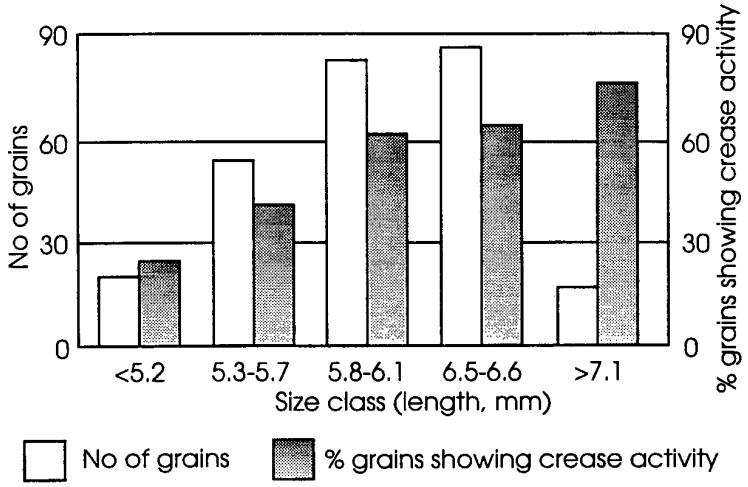


Fig 3.18. Percentage of grains in which *alpha*-amylase was detected in the endosperm cavity by gel print

Fairly good agreement was established between microtitre plate and Phadebas results (Fig 3.19) allowing the microtitre plate test to be used with confidence, either alone or in tandem with the gel assay.

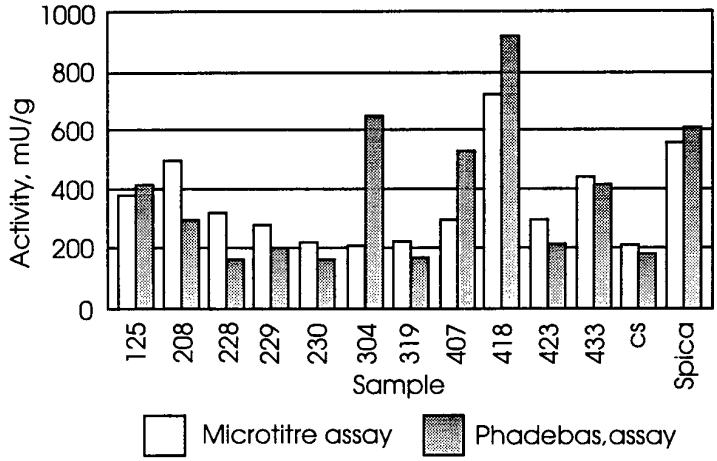


Fig 3.19 Comparison between results of microtitre and Phadebas assays (Spica and Chinese Spring)

By using the gel assay on one half grain and microtitre assay on the other, a semi-quantitative location assay and a fully quantitative assay could be made on the same grain providing confidence in the identification of the enzyme type being quantified.

3.6.2) Archival evidence

An ideal archive for the comparison of performances of wheat varieties would contain consistently gathered information on the same varieties over a number of years. However, because of the frequent introduction of new successful wheat varieties into recommended lists, annual trials reports do not contain information on the same varieties over a long period. As the number of varieties on which reports are made remains fairly constant, it is inevitable that new cultivars entering trial displace those that become outclassed. Nevertheless it is possible to make comparisons of types or classes, and table 3.IX shows the result of a comparison of varieties described as "larger than medium" (Anon)with those described as "medium" or smaller. For each year between 1986 and 1993 the four varieties with the lowest Hagberg Falling Number were noted and a record made of the frequency with which each variety fell into the "worst four" category in relation to its frequency of occurrence in the trials.

Table 3.IX Relative frequencies of grains of different putative sizes among the "worst four"

Larger than medium-grain varieties

Brimstone (**'86, '87,**[a] no results in '88 *et seq*) ie 2 of 2
Fenman (**'86, '87**, no results in '88 *et seq*) ie 2 of 2
Haven (**'90, '91, '92** no results before '90) ie 3 of 4
Hornet (**'86, '87, '88, '89, '90, '92,** no results in '91 & '93) ie 6 of 6
Norman (**'86, '87, '88, '89, '92, '93**, no results '90, '91) ie 6 of 6
Beaver [b](**'89, '90, '91, '92, '93**, no results before '89)) ie 5 of 5

Medium and small-grained varieties

Buster (**'93,** no results before '93) ie 1 of 1
Estica (**'91**, no results before '91) ie 1 of 3
Galahad (**'86**, no results '92, '93,) ie 1 of 6
Longbow (**'88**, no results '89 *et seq*) ie 1 of 1
Brock (**'88** no results in '91 only) ie 1 of 7
Riband (**'89, '90,** no results before '88) ie 2 of 6
Tara (**'91**, results for '91 and '92 only) ie 1 of 2

[a]Figures in bold indicate years when variety occurred in "worst four " category
[b]the size of Beaver grains is not described but its most similar variety is Haven

The table presents a convincing picture of an association between low HFN and grain size as the relative frequency with which larger grained varieties appeared among the worst four was dramatically higher than that of other types. The true picture is even more convincing as the table shows all the large grained varieties to appear in trials but only those five smaller grained types that appeared at all in the worst four category, out of a

total of more than 50. In fact, out of 25 appearances in trials, large grained varieties appeared in the worst four 24 times, while the equivalent performance of smaller grained types was 8 out of 127.

3.6.3) Relationship between size and alpha-amylase activity

Table 3.IX above comprises results obtained over a period of years in which harvest conditions were relatively dry, and hence the predominant type of amylase present was of the late maturity endosperm type. However, even in 1987 and 1992, the worst years during the period covered, it was still the large grained varieties that occupied the "worst four" positions. This may arise from a greater tendency of these types to germinate or from the presence of a high level of activity before germination, effectively raising the base line to which germination activity is added.

Evidence for a genuine relationship between size and tendency to germinate comes from trials conducted in the 1987 when sprouting was common, by the Agricultural Development and Advisory Service (Cambridge, UK), in which only one variety (Brimstone) is of a large grained type. These showed that, when samples were separated into different size categories by use of slotted sieves, those passing through a 2.75mm sieve had a higher Hagberg Falling Number (HFN) than the larger grains (Table 3.X)

Table 3.X. Hagberg Falling Numbers of sub-samples prepared by sieving wheats from the 1987 harvest

SAMPLE	<2.75mm	2.75-3.75mm
Avalon	97	63
Avalon	187	151
Avalon	79	62
Avalon	117	83
Avalon	139	101
Avalon	131	99
Brimstone	157	68
Brimstone	177	149
Brock	110	67
Galahad	107	71
Galahad	96	67
Mercia	144	83
Mercia	124	77

As three out of four of the varieties fractionated here typically bear medium or small grains it is reasonable to suppose that the low HFN values arose from germination enzymes. From this we may deduce that grain size is an important factor in relation to this type of enzyme also.

It should be pointed out that the observations recorded in Table 3.X indicate a relationship that is the reverse of that previously reported by Tkachuk *et al.* (1991) in which smaller grains had higher activity than large ones. While complete resolution of this apparent paradox is not currently possible, it should be borne in mind that Tkachuk and his co-workers were dealing with samples of Canadian wheats, which are characteristically small-grained. Further the samples which they examined were mixtures of many samples blended into Canadian grades. It is therefore unclear whether the gravity table was separating one sample from another, or different types of grain from the bulk. The samples considered in this work were authentic products of a single plot or field, grown under experimental conditions.

3.6.4) Further observations with fluorescein dibutyrate

In addition to applying staining for esterase activity to microtome sections as described above, some observations were also made on aleurone layers exposed on the surface of the non-endosperm layers, from which endosperm had been removed by soaking and gentle scraping. The preponderance of activity in the crease region was confirmed but another interesting observation was also made on the enzyme activity present in other regions. Whereas all cells surrounding the endosperm cavity rendered the stain fluorescent,a less regular pattern was experienced elsewhere. Fluorescence was detected in pockets of cells that were surrounded by cells in which no activity was detected. This finding is compatible with observations by Hillmer *et al* (1992) who showed that aleurone protoplasts varied in their ability to render fluorescein diacetate fluorescent. Although this staining technique has been employed as an indicator of viability (Heslop-Harrison and Heslop-Harrison 1970), absence of fluorescence was, in this case, interpreted as indicating only absence of synthetic activity. This inconsistency in interpretation by different authors is worthy of further investigation as it offers a means of relating cell condition and micro-environment to transcription capabilities.

Although the observation on aleurone layers was considered interesting, it was not accorded high priority in this study and no quantitative comparisons were conducted between grains differing in size or genetic background.

4) DISCUSSION

Markers for resistance to sprouting

Because preharvest deterioration in Hagberg Falling Number can occur through a variety of different risks, which may or may not affect a particular crop, resistance to "sprouting" damage is not an easy character to measure. A new wheat variety may go through several years of trials without showing symptoms of susceptibility before it encounters the particular conditions which reveal its lack of adequate resistance. When the additional, logistical difficulties of conducting sprouting tests on large numbers of new candidates for release as varieties are considered, the value of simple markers which can be used to identify potentially susceptible samples is clear. In view of the limited scope for remedial action once susceptibility is detected in a commercial crop, the most cost effective application of markers will be in the breeding of new varieties, with improved sprout-resistance.

Markers may either be resistance factors in their own right (as with the enhancement of dormancy caused by red grain colour), or may simply act as labels with no direct effects (eg DNA markers in tight genetic linkage with resistance factors). Markers having intrinsic effects, although preferable, are not available for several cases in which the existence of resistance/susceptibility factors is apparent but in which we have little or no information as to how they exert their effects. In such cases markers are chosen which are simply correlated with observed differences in sprout-damage.

Ideally, a useful marker will be easy to detect in a small sample, be uniform and stable over different environments and generations, and be a reliable indicator of resistance. For simple characters such as grain colour, single gene differences fulfil these ideals most completely. However for more complex traits, such as the production of *alpha*-amylase during ripening, an understanding of the physiological mechanisms underlying the problem is a prerequisite for its solution.

Genetic maps and the conservation of chromosome structure across different cereal genomes

The genetic maps in Figure 3.14 depict the linear arrangement of genetic markers along the group three chromosomes of wheat and barley. The distance between any pair of markers on the map is directly derived from the frequency of recombination observed between them, i.e. the probability that a plant carrying one of the markers also carries the other. For markers which lie close together this probability is large, and when a target (*R*) gene is carried between two closely linked flanking markers this probability effectively becomes a certainty. This behaviour can be exploited to select target genes indirectly, according to the presence of flanking markers. Thus for example it would be possible to screen leaf samples from F_2 seedlings out of the cross Spica x Chinese Spring for the markers *XBCD131* and *XABC174*, in order to detect the presence of the *r1* gene. Within a population of 130 F_2's from this cross, the 29 lines which carried Spica versions of the two DNA markers were white-grained (*r1/r1*). One advantage of using the DNA markers derives from the fact that they are present in every cell of the plant, so that the presence of a target gene can be predicted early enough to allow selection for cross-breeding (this would otherwise be impossible for a trait such as grain colour which does not develop until flowering has ceased).

Another advantage of DNA markers is that they can be used to detect equivalent homologous genes across different species. In the case of the *R* genes it was already known that *R1*, *R2* and *R3* were carried on the long arms of chromosomes 3D, 3A and 3B respectively; wheat DNA clones from these chromosome arms were therefore used to find the map locations of the grain colour effects. The similarity of gene orders along the different wheat chromosomes seen in previously published maps is regarded as evidence of their common origin from a primitive ancestral grass. A similar conservation of chromosome structure is apparent in other cereal crops (Devos *et al.* 1992, Moore *et al.* 1993) and this was exploited by screening additional clones from the equivalent chromosome arm of barley in this study. Although further work needs to be done to confirm gene orders and to improve the resolution of linkage estimates, it is clear that the long arms of chromosomes arms 3A, 3B and 3D of wheat and 3H of barley share a common arrangement of genes. It will be interesting to see whether there is a barley gene corresponding to the wheat *R* series, or whether this locus is absent from barley. Even wider comparisons have revealed conservation of chromosome structure between wheat, rice and maize, with the implication that these widely divergent cereals share a basic set of genetic "building blocks" carrying genes which in most cases have homologous functions in different crops. In addition to the scope for extrapolating genetic and physiological studies from one species to another, this concept also raises the possibility of transferring genes controlling economic traits between different species. A preliminary investigation of the feasibility of using the maize gene *viviparous 1*, which controls sprouting in the cob, to locate homologous genes in wheat was initiated at the end of this project.

Grain dormancy - effects of red grain colour genes

The production of *alpha*-amylase in sprouting grains is known to occur as a response to gibberellin hormones released by the germinating embryo. The control of embryo germinability by genes for enhanced dormancy is thus an important component of resistance to sprouting damage, giving protection against both the visible symptoms of shoot and root growth, and against the accompanying deterioration in Hagberg Falling Number caused by enzymic degradation of starch. Several genetic markers for grain dormancy have been described previously, however the only trait relevant to the U.K. context is the red grain colour conferred by *R* genes.

Prior to this project it had been established that white-grained varieties have only low levels of grain dormancy, although a few lines showing limited improvements had been bred in the white wheat growing areas of Australia, Canada and the U.S.A. Red-grained varieties, while generally more dormant, exhibit wide variation in dormancy and some of this variation was associated with increasing the number of *R* genes carried from one, to two, to three copies amongst random lines from a resistant x susceptible cross (Flintham *et al.*, 1993). It was not known whether the *R* genes themselves were responsible for the observed variation in grain dormancy, or whether they were simply acting as genetic markers for other dormancy genes lying on the same chromosomes. Whether adequate grain dormancy could be obtained by accumulating all three *R* genes together, and whether one particular *R* gene was more efficient than the others, were additional questions to be investigated.

The direct involvement of *R* genes in enhancing grain dormancy is demonstrated by the

contrast between the germination of the white-grained wheat Novosibirskaya 67 and its red-grained isolines in Figure 3.1. Because the red isolines were produced from repeated backcrossing to the white parent, all of their genes will be identical except for those at the genetic loci which control grain colour. The dormancy differences between red and white lines must therefore be due to the *R* genes.The five different red-grained isolines, each carrying a different *R* gene, all showed similar germinability and it is concluded that each of the *R* genes has an equivalent dormancy-enhancing effect. The degree of protection offered by an *R* gene is initially strong, consisting of a marked reduction in the number of grains which will germinate when wetted. This enhancement of dormancy decays gradually with time after harvest ripeness, with a half-life of approximately one week in dry storage at 25°C.

When dormant grains from red-grained isolines are imbibed they germinate at a very low, more or less constant rate. Dormancy in wet, non-germinated grains can evidently be maintained to a high degree, in contrast to matching grains stored dry over the same time period. This is most clearly seen in the comparison between red-grained samples imbibed one week after harvest ripeness and parallel samples which were stored dry for a further week before being imbibed: less than twenty percent of grains in the first sample had germinated after ten days' incubation while fifty percent of the second sample had germinated by the same time, after only three days' imbibition (Fig 3.1.). There is obviously a fundamental difference between wet as opposed to dry conditions, such that dormancy is maintained to some degree in imbibed grains but is rapidly lost in the dry. This is in accord with other evidence of active synthesis of a set of proteins in dormant imbibed grains which are rapidly degraded when dormancy is lost (Ried and Walker-Simmons 1990).

A survey of the *R* and *r* genes carried by a wide range of commercial varieties revealed no systematic differences in the frequency of different genes. Although U.K. breeders routinely discard white-grained lines due to their inadequate resistance to sprouting, this has not resulted in any detectable decrease in the frequency of the susceptible (*r*) genes in the gene pool. One result of this random distribution of genes is that one out of every eight new lines bred can be expected to be a white-grained discard. The information generated by the present *R* gene survey is intended to be of use to breeders in planning crosses to avoid this problem. For crosses in which this cannot be avoided, the DNA markers identified in Figure 3.14 can be used to select red-grained recombinants at an early stage of elite line selection.

Grain dormancy - effects of other genes

The original observation that grain dormancy in red wheats increased in proportion to the number of *R* genes present raised the possibility that resistance to sprouting might be maximised by designing breeding programmes to accumulate all three *R* genes, alternatively that one particular *R* gene might be more effective than the others. In order to see whether either of these hypotheses might lead to adequate resistance in commercial crops under U.K. conditions, the *R* genes carried by a large sample of varieties were compared to their sprouting scores derived from Flintham *et al.* (1993). The results indicate that no single *R* gene is associated with greater dormancy than are the others, confirming the result obtained from near-isogenic lines. Furthermore there was no detectable effect of increasing *R* dosage from one to two to three genes, possibly because the limited improvement expected was

masked by the presence of many other genetic differences between the varieties studied.

Taken together these observations indicate that only limited improvements can be expected from manipulating the *R* gene complement of our red-grained wheats. The additional discovery of wide variation in sprout-resistance amongst wheats carrying identical *R* genes points to the presence of other genes, not controlling grain colour but having profound effects on grain dormancy through some unknown mechanism. Such genes could account for the range in resistance among *R1 R2 R3* varieties from extremely resistant types such as Sentry and Soleil down to the extremely susceptible varieties Boxer, Galahad and Haven. They may also be involved in the partial dormancy of some white-grained breeding lines from overseas. A comparison between the germination curves shown in Figures 3.1 and 3.3 suggests that the effects of such "non-colour" dormancy genes are at least of comparable magnitude to the red grain effect. It would clearly be of considerable value to identify markers for these as yet anonymous genes.

One clue as to the location of some of the "non-colour" dormancy genes is obtained from a comparison of group three chromosome substitution effects reported previously (Flintham, Evers and Kratochvil 1993) with the subsequent information regarding *R* genotypes. Chromosomes 3B of Dwarf A, 3B of Champlein, and 3D of Bersée have all been shown to carry dormancy factors which are definitely not *R* genes. Thus, although the *R* genes have direct effects on grain dormancy, they are also linked to other dormancy genes carried on the same chromosomes. The DNA markers mapped onto the group three chromosomes may thus have applications in breeding for improved sprout-resistance beyond their use as markers for the *R* genes. Previous chromosome substitution experiments also detected effects in other groups and it is not anticipated that dormancy genes will prove to be confined to group three.

The presence of crystal-like waxes in embryo-cavities of long stored resistant grains appeared to offer the possibility of another marker, for which a simple test might have been developed. In the event, its characterisation and ready recognition proved too difficult. Evidence for its validity was considered substantial in the early stages of the project. However, the problems of successfully extracting and manipulating such small amounts of material in the presence of so many potential contaminants, which dogged the reliable establishment of wax composition and variability, ultimately led to termination of work on proving a significant association of wax with dormancy.

Embryo cavity waxes

Although the further survey, conducted in this study on long-stored grains, largely validated earlier findings that waxy tufts of embryo cavity waxes are characteristic of red grains, it has not been possible to substantiate the theory that these differences reflect differences in composition. Nor has it been possible to detect differences in composition or behaviour of solvent extracts from embryo cavities of freshly harvested grains, corresponding to differences in sprouting resistance.

Results of examination of extract composition and behaviour were both characterised by lack of consistency. This was recognised at an early stage and considerable efforts were

made to eliminate the variability or to apply statistical methods capable of taking account of it. Failure to do so in both cases probably resulted from the same causes, which reflect both technique and inherent properties. Dealing with very small amounts of material clearly presents special difficulties, but it is felt that good methods were developed to overcome most of the problems associated with scale. The problem that could not be overcome was manifested in the lack of consistency among aliquots of the same extract and this could only be attributed to differential adsorption of some of the functional components, on to the surface of the glass.

The hypothesis that waxes in sprout-susceptible and -resistant types of wheat might prevent access by water to critical parts of the embryo was explored by use of NMR imaging. This technique allows tracking of water ingress previously only dreamt of by researchers. Images taken at intervals as comparable as possible, in the limited time available, of dormant and non-dormant grains of the same variety showed indistinguishable paths for water passing into the grain. Because of the limitations on resolution of the technique, the results of this exercise cannot support a categorical denial of the possibility that parts of the embryos of dormant grains are inaccessible to water; they certainly provide no support for it. The one remaining possibility of a hydrophobic layer denying access to an essential element of germination now appears to relate to oxygen access. The concept of local oxygen inadequacy as a barrier to germination is not a new one but its exploration has always presented even greater difficulties than an equivalent enquiry concerning water. The fact that dormancy can be overcome completely if tissues covering the embryo are removed, has yet to be explained physiologically or biochemically and the reduction in the significance of water, as the victim of denial, which the NMR evidence suggests, shifts emphasis on to oxygen in this context.

Different risks

Whether or not sprouting is involved, the central problem in preharvest deterioration in HFN is *alpha*-amylase. For some cultivars, enzyme activity is strictly correlated with embryo growth, and virtually no enzyme is present in dry-ripened grain; for others enzyme activity varies or is high even in the absence of detectable sprouting. In the former, *alpha*-amylase synthesis is dependent upon an embryonic gibberellin signal produced during germination, but in the absence of embryo germination some other signal, either gibberellin or possibly failure of inhibition, must be responsible for initiating enzyme synthesis. Termination of enzyme synthesis during ripening is probably caused by desiccation, and in this connection it is interesting to speculate that the concentration of enzyme around the crease may reflect the pattern of grain dehydration at the end of ripening.

Non-germination enzymes

The relationship between size and dormancy can be substantiated by evidence of a correspondence between the two factors by virtue of their coincident relationship with position of occurrence on the ear. Evers and Ferguson (1980) reported a relationship among enzyme activities of grains within a spikelet that corresponded to their weight distribution but they were unable to demonstrate a simple general relationship between weight and *alpha*-amylase activity using the available stocks. Gale *et al.* (1987) confirmed the

distribution of activities within spikelets and the relationship they found along the length of the spike also coincided with weight distribution.

The evidence for an association between grain size and hydrolytic enzyme activity, coming as it does from a number of different sources, is compelling. Evidence cited here suggests that the relationship between grain size and hydrolytic enzyme production cannot be detected as a linear correlation, but that grains above a certain threshold size have anatomical abnormalities that can lead to failure of the control mechanisms operating in smaller grains. It is difficult at this stage, to be certain if large grain size inevitably leads to problems with enzyme activity and more detailed studies are now in progress. The current evidence justifies awareness by others who may monitor the apparent association, however, hopefully increasing the experimental base from which deductions can be made.

The observation by Radley (1978) that large grains are characterised by larger than normal endosperm cavities has been confirmed statistically, though not universally, both by manual measurements and by counting cells in grain section images. Unusually the cavities are open in mature grains, as described by Radley. However it is more common for the crease to have become eclipsed, presumably because of distortions during ripening as a result of desiccation. Distortions of several types can be recognised and some of the observed patterns are shown in Fig 4.1.

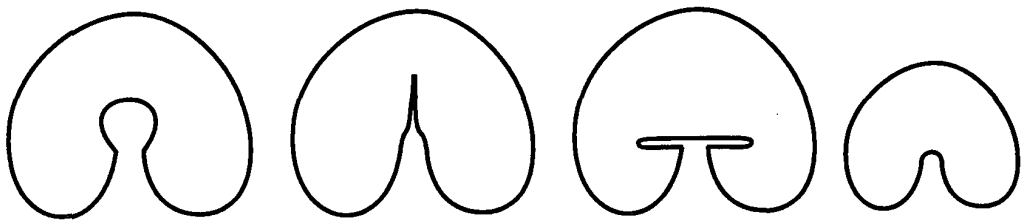


Figure 4.1. Diagram of some of the types of endosperm cavities encountered in wheat grain transverse sections: 1) Large cavity in an open condition, 2) and 3) Frequently occurring distortions of the cavity through crushing, 4) small cavity typical of small grains

While non-germination enzymes have never been reported to attain the highest levels of activity experienced as a result of sprouting in the ear, they are not dependent upon weather conditions at harvest and can therefore reach unacceptably high levels even in the absence of adverse conditions. Further, by contributing a background of activity they reduce the threshold level that germination enzymes must reach before downgrading occurs. Although some details of their distribution, such as its irregular and non-polar nature, have been known for sometime, our findings in this project have shown for the first time, that activity is primarily or exclusively located in the aleurone tissue surrounding the endosperm cavity.

The finding that late maturation endosperm *alpha*-amylase activity is located in the endosperm cavity is surprising since, during germination, little enzyme appears to be produced by the aleurone cells in the region. The evidence for this is that starchy

endosperm digestion tends to occur as a result of local hydrolytic enzyme production and that in the crease region is the last to be digested. The coincidence of its location with two other observations reported here, namely that, compared with small and medium sized grains, large grains have bigger endosperm cavities and that the same have higher levels of late maturation endosperm *alpha*-amylase, signals the possibility of a causal relationship. Consideration should also perhaps be given to the other known incidence of *alpha*-amylase production in cereals in the absence of germination, ie. in some of the early triticales that were bred. Many were characterised by seriously shrivelled grains. With the microscope, pockets of cells were seen to have undergone auto-digestion (Simmonds 1974). Cells within the pockets had failed to complete cytokinesis due to the variation in doubling time of chromatin in the slower diploid rye and the faster polyploid wheat genomes present (Bennett, 1974). The pockets occurred mainly around the periphery of the endosperm, where cell division was most active in the layer that matures as aleurone. Endoreduplicate cells are known to occur also in wheat endosperm (Cattley, 1988) but their location has not been specified. Indeed it is possible that they occur throughout the tissue. However it is well established that the aleurone cells in the crease region behave differently from the remainder during the cell division phase of development, in fact it is as a result of their failure to divide that the crease is formed. Whether their failure to divide is the result of a failure of mitosis or cytokinesis is not known, but their condition and that of the known endoreduplicate cells of some triticales may repay study, since it appears that in both, enzymes are produced in the absence of a gibberellin or similar stimulus, and light may thus be shed on regular and irregular control mechanisms. It is conceivable that the increased number of cells surrounding the endosperm cavity results from a generally greater number of cell divisions having occurred in larger grains. While this has not been followed up specifically, it is reasonable to expect a larger endosperm to comprise more cells, and the widely varying estimates of endosperm cell number (Evers, 1988) could arise from counts having been performed on endosperms of different sizes.

Other factors affecting enzyme levels

Although not formally part of the work of this project, attention has been drawn to the occurrence of wheat orange blossom midge damage during grain development. Germination in the ear during late grain-fill in 1987, referred to as premature germination in a previous HGCA report, has been identified as being associated with midge attack (Flintham *et al.*, 1993; J.N.Oakley, ADAS Reading, pers. comm.).

Applications and benefits

The coordination of work at two different laboratories has resulted in significant progress in our understanding of the complexities of "sprouting" damage, which would not have been achieved so rapidly by isolated projects (if at all). In the case of embryo cavity waxes, the synergy between the biochemical expertise at CCFRA and the availability of special genetic stocks at JIC enabled the hypothesis of an association between wax components and grain dormancy factors to be tested rigorously at an early stage of the investigation. Although this did not result in the identification of practical resistance markers, due to the technical difficulty of purifying a very small component of the whole grain, some results of this nature must be expected when testing new ideas. Indeed it is essential to know that a postulated breeding strategy (such as accumulating all three *R* genes) is unlikely to result in

adequate resistance, in order to avoid wasting time and resources which would be better allocated in other directions.

Collaboration has also extended beyond the LINK partnership, notably with the inception of an HGCA/ITCF consortium investigating the feasibility of predicting the onset of sprouting damage in field crops. Regular consultations between the different U.K. research groups have ensured a co-ordinated and systematic approach to the planning and interpretation of experiments. The use of medical NMR facilities to demonstrate the common pattern of water uptake into imbibing dormant and nondormant grains is yet another demonstration of the value of cooperation between laboratories. At an international level, much of the work carried out during this project has been founded upon the experience of groups working on the sprouting problem in different laboratories around the world. The free exchange of information and materials with laboratories in Europe, Russia and the U.S.A. has been particularly valuable for the demonstration of *R* gene effects in isolines and for their genetic mapping using DNA markers.

Once sprouting damage has occurred there is very little scope for remedial action and any practical solution to the sprouting problem will be aimed at preventing the damage from arising in the first place. The most appropriate way of achieving this will be to incorporate resistance mechanisms into the crop itself, and the feasibility of this is demonstrated by the availability of a number of varieties which have shown consistently low levels of damage over the years. The difficulty is to identify the mechanisms underlying the resistance of such cultivars, so that resistance can be incorporated into new varieties by design rather than by chance. It is intrinsic to the system of variety breeding, certification and release that benefits ensuing from improved insights into crop performance will be of a long term nature.

During this LINK project a series of marker systems have been tested for associations with resistance to sprouting damage, in order to identify markers which may be useful in screening selection nurseries and in designing new crop ideotypes. The corollary, the identification of markers of insignificant or limited effects, is an inevitable result of rigorous testing of new hypotheses and is worthwhile in its own right. The clarification of the distribution of different *R* genes within U.K. breeding stock has important implications for wheat breeders, allowing them to predict the outcome of particular cross combinations and to minimise their production of white-grained, sprout-susceptible lines. The finding that presence of *R1* + *R2* + *R3* does not guarantee maximum grain dormancy casts doubt on the adequacy of breeding programmes designed to accumulate all three genes for red grain colour.

Genetic mapping of the long arms of the group three chromosomes now allows breeders to select wheats carrying specific *R* genes prior to flowering, so that appropriate cross combinations can be made by design instead of by chance. The detection of other group three effects on grain dormancy, and the demonstration of their independence of the grain colour effect, lends additional value to the genetic maps, especially when it is considered that there are no morphological markers for these "non-colour" effects. An extension of the DNA mapping technique, applied to new genetic stocks produced as part of this project, will be used to search for markers for "non-colour" effects as a priority in further work.

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