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PROJECT REPORT No. 77

GENETICAL AND PHYSIOLOGICAL STUDIES ON SPROUTING IN WHEAT

I. IMPROVEMENT OF RESISTANCE TO SPROUTING IN WHEAT

II. IDENTIFICATION OF A MAJOR FACTOR CONTROLLING SPROUTING IN CEREALS

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Part I

IMPROVEMENT OF RESISTANCE TO SPROUTING IN WHEAT

by

J. FLINTHAM

Final report of a three year project in the IPSR Cambridge Laboratory of the John Innes Institute, Colney Lane, Norwich NR4 7UH. The work commenced in October 1987 and was funded by a grant of £111,099 from the Home-Grown Cereals Authority (Project No. 0046/1/87).

HGCA PROJECT REPORT No. 77

Part II

IDENTIFICATION OF A MAJOR FACTOR CONTROLLING SPROUTING IN CEREALS

by

A.D. EVERS AND J. KRATOCHVIL

Final report of a twelve month project in the Flour Milling and Baking Research Association, Chorleywood, Hertfordshire WD3 5SH. The work commenced in April 1990 and was funded by a grant of £31,700 from the Home-Grown Cereals Authority (Project No. 0006/1/90).

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ABSTRACT, PART I

The aims of this Cambridge Laboratory project were to assess risks associated with sprouting damage and to identify genetic markers for stable, high Hagberg Falling Number. Such genetic markers, together with increased insight into the mechanisms of sprout-damage, will enable plant breeders to select new resistant varieties with consequent benefits to the breadmaking quality and value of the U.K. wheat crop.

Special genetic stocks with known genetic markers have been examined in a series of field trials and artificially controlled tests, to evaluate their potential for controlling α-amylase enzyme activity in the grain and sprouting in the ear. Part of the work has also been directed to produce new genetic stocks, for experiments described in this report and for future investigations.

Three distinct physiological disorders were observed in the 1987 harvest:

(a) Premature α-amylase production in sound, ungerminated grains. This behaviour was first observed in Maris Huntsman, but it has since become clear that many other varieties can be affected. The characteristic grain sample in this case has a low Falling Number without visible evidence of sprouting. The damage occurs early on in ripening, shortly after the onset of grain water loss.

(b) Premature germination during early ripening. Although this results in high α-amylase activities in affected grains, and occurs in the same developmental stage as premature enzyme production, it differs in that the grain sample shows visible evidence of sprouting (embryo growth).

(c) Conventional pre-harvest sprouting can occur in ripe ears exposed to rainfall. Unless the mature grains are dormant, imbibition of water is sufficient to induce embryo germination and α-amylase production. In contrast to the two prematurity risks, this type of damage happens towards the end of (or after) ripening, typically when harvesting is delayed by wet weather.

The *Rht3* dwarfing gene reduces α-amylase levels by some 90% in sprouted grains and in wheats susceptible to premature enzyme production. This gene inhibits enzyme production but does not affect grain dormancy or embryo germination rate. Thus, although *Rht3* can help to maintain high Falling Number, additional sources of resistance are required to avoid sprouting. Other genetic effects on premature enzyme production have proved more difficult to analyze. Specific chromosomes responsible for high α-amylase in special genetic stocks have been identified, but it appears that different mechanism(s) may be involved in more recent U.K. varieties.

Resistance to visible sprouting is largely dependent upon genes controlling grain dormancy. The difference between red-grained and white-grained wheats is the single most important source of variation in artificial tests of sprout-resistance. Amongst the red-grained wheats, increasing the dosage of *R* genes for red pigment from 1 to 2 to 3 copies leads to smaller but significant increases in grain dormancy. The numbers of *R* genes carried by 83 modern varieties and

breeding lines have been determined, allowing breeders to predict the frequencies of different types amongst new varieties bred out of this gene pool.

Other genetic markers for improved resistance include lack of awns (presence of awns is associated with more rapid germination in ears under simulated rainfal). Significant effects of other genes have been detected but these genes have yet to be identified.

Comparisons made between autumn and spring sowings of spring wheats indicate that the generally high sprout-resistance scores of spring versus winter types may be an artefact. When spring lines flower and ripen early they have lower resistance than predicted.

Construction of a Restriction Fragment Length Polymorphism genetic map of the entire wheat genome is underway at the Cambridge Laboratory and is being used to generate new markers for identifying and manipulating sprout-resistance genes.

The physiology and genetics of sprouting are complex. Much useful progress has been made towards understanding the control of this costly problem; much remains to be done, and we are continuing our research into this topic.

ABSTRACT, PART II

An investigation has been carried out into the composition and significance of wax crystals discovered in the embryo cavity of wheat grains. The crystals were found only in grains of red cultivars that had been stored for long periods.

The composition of the wax has now been tentatively defined. A wide range of organic compounds have been detected and details of these are contained in the report. Components with similar composition to the waxes of long-stored grains have been found in freshly hervested examples of both red and white wheats. There are significant differences however in the proportions of certain key components in the two types. Surface properties of the extracts from embryo cavities of red and white grains examined show significant differences, compatible with a water resistant function of the red grain extracts. There is a strong association between surface properties of the extracts and the tendency of the parent grains to sprout. The association now requires to be examined more systematically using breeder's substitution lines and the developmental biochemistry of the different waxes requires to be explored, particularly in regard to seasonal variation.

GENERAL INTRODUCTION

Preharvest sprouting is the premature germination of grains while still borne on the ear in a standing crop. Pre-harvest sprouting is undesirable irrespective of the envisaged use of the wheat, it reduces the ability of grain to germinate after harvest and drying, thus reducing its usefulness for malting or as seed. Grain mass is also reduced due to increased respiration and hydrolysis of stored reserves.

Although sprouting implies the emergence of roots, and more particularly shoots, these aspects of the phenomenon are of less technological importance in some contexts than the accompanying synthesis of high levels of hydrolytic enzymes which mobilise the reserves stored in the endosperm. Although the suite of enzymes includes proteases and enzymes capable of digesting different components of cell walls the enzymes that have been most studied and are considered to be most important are those that digest starch. Of these it is the *alpha*-amylase which increases most as a function of the degree of germination.

High enzyme activity differs from most other quality factors in that its effect on a wheat sample, or flours derived from it, can be disproportionate to the number of grains in which it occurs. Thus, excessive quantities of *alpha*-amylase in a small number of grains can, under suitable conditions, hydrolyse starch from grains in which amylase levels are low, thus rendering the entire sample sub-standard (Stevens *et al,* 1988).

The average loss due to pre-harvest sprouting in the UK has been estimated as £17 million per year (Derera 1990). This figure is for the total cereal crop but it takes account of the fact that serious downgrading of crops occurs only in three years out of ten. The loss is compounded for the nation by the need to import from alternative sources. Although preharvest sprouting is a problem in all cereals this report deals specifically with wheat as conclusive information as to the relevance of the present work to barley and oats has not yet been obtained. In this section of the report we consider the significance of the sprouting problem to major sectors of the grain trade and the baking industry, the main approaches that have been made to alleviating the problem and the background to the experimental work described in the body of the report.

1. The importance of sprouting to the baker

Hydrolytic enzymes of the type produced by grains upon germination, in particular *alpha*-amylase, have a role to play in the production of doughs suitable for baking and some activity during the baking process may also be desirable. However, the level must be controlled as excessive activity can be disastrous. The effect of *alpha*-amylase is to produce from the insoluble starch - the major component of flour - a series of dextrins. These compounds cannot be used as an energy source by yeast, so they accumulate leading to a heavy, sticky crumb of the type enjoyed in malt loaves but not considered desirable in most breads. Even relatively small increases in dextrins, not enough to cause serious deterioration in crumb quality, causes significant problems on slicing machines in which sticky particles adhere to the blades. The adherent material accumulates, rendering the blades virtually useless and causing severe distortion and damage to loaves being sliced. Proteolytic enzymes reduce the quality of the gluten proteins

of wheat endosperm and consequently reduce its ability to support the light cellular structure considered desirable in bread.

Although the various enzymes can be assayed individually the total effects of sprouting are conventionally measured by the Hagberg Falling Number technique (Stevens *et al* 1988). This allows the reduction of hot paste viscosity caused by the activity of enzymes capable of increasing the solubility of macromolecules such as starch and protein, to be monitored. The method has the advantage that to some extent it simulates the baking process and hence determines factors that relate to it. There is little that a baker can do to ameliorate excessive hydrolytic enzyme levels. In consequence he rejects flours with such levels.

2. The importance of sprouting to the flour miller

The flour miller has to produce flour which meets the specification of his customers. By far the greatest use of flour is in the production of bread and hence a level of hydrolytic enzyme activities acceptable to the baker has to be achieved in the majority of flour milled. Other uses of flour also demand an enzyme level below that found in sprouted wheats. Only the lowest-value uses of grain such as stock feeding can tolerate high hydrolytic enzyme levels and these are the concern of feed millers rather than flour millers.

The miller can exercise a number of options in coping with the products of a national harvest in which sprouting is widespread. He can avoid the home-grown harvest altogether by buying wheat from exporting countries less affected by bad weather. If this involves purchase from non-EC countries he will incur tariff penalties, adding considerably to the price of his raw material.

Although the cost penalty is high the miller gains some advantages from importing wheat as he has a very wide choice of the world market, allowing purchase of graded samples of very high milling quality capable of producing outstandingly good flours. The wide range of graded wheat types allows purchase of types suitable for envisaged end-use saving him the trouble of adapting his system to what is, on some occasions, an ill-suited home-grown or EC grist. Any net increase in costs can be passed on to consumers. This, of course, in undesirable and may lead to a reduction in cereal-based foods consumed. However, should this occur to a degree considered undesirable by government a political solution involving reduction in tariffs is available. The option to fail to implement such a reduction or even an elimination of tariffs may be removed should third country pressure in GATT discussions prevail.

Alternatively the miller can purchase samples with unacceptably low Falling Number values and by use of gravity table or similar separators, select the proportion of grains which meet flour milling specifications. It is not yet fully established as to whether this can be done without causing deterioration in baking properties. If it proves to be undesirable, there is no doubt that bakers will devise a test to detect samples that have been thus treated. In any case the additional processing and need to dispose of unselected grains incurs additional costs and consequently influences wheat prices.

3. The importance of sprouting to the grower

The producer is undoubtedly the most vulnerable link in the chain of cereal production and usage when sprouting occurs. One of the effects of premature germination in the field is the decline in test weight through digestion of stored macromolecules in the endosperm (McEwan, 1975). Thus, even if the feed or other relatively undiscriminating markets were to demand the entire national wheat output there would be a reduction in return because of low yields and low prices. In fact there has been a decline in demand for feed wheats in recent years and a huge surplus would result from a widespread failure to meet milling requirements. Unlike the miller the grower, encumbered with a sprouted crop, has no options beyond his own or his merchant's use of a separation technique as already described. The penalties for this are the same as those incurred by millers using the technique.

Neither is there much that a grower can do in terms of agricultural practice to reduce the risk of sprouting. Avoidance of diseases that affect standing ability of plants helps to prevent lodging, and reduction of weed plants that remain green after crop ripening aids rapid drying of crops after rain. Rapid harvesting of just-ripe crops minimises the risk of sprouting caused by continued wetting, but other timetable factors limit the possibilities for this. In general the farmer depends upon breeders to provide cultivars that have short strong straw, good resistance to stem disease, to avoid lodging and to provide ears and grains that are relatively difficult to wet and on wetting are resistant to sprouting. It is in pursuit of this last quality factor that the breeder is at present ill equipped and it is to meeting this requirement that the project described in this report is directed.

4. Breeding strategy in relation to sprouting

Breeding consists essentially of recognising desirable characteristics in different cultivars and crossing these to give a new cultivar in which the desirable characteristics are combined. The ease with which characteristics can be combined varies according to the complexity of the factors controlling them but the breeders' difficulties also vary according to the ease or difficulty with which a character can be assessed. As an illustration plant height is a characteristic that can be easily measured and it is therefore relatively easy for a breeder to evaluate the success of his crossing programme. Tendency to sprout, on the other hand, is a difficult character to measure. The conditions that favour pre-harvest sprouting do not occur every year and, as their exact nature is not fully understood, they can be simulated only with partial success. The performances of cultivars, even in different years in which sprouting conditions prevail, are not consistent, as the variations in scores quoted in Farmers' Leaflets demonstrate. (Anon, annual). Thus between 1973 and 1981 Maris Huntsman scores declined in three stages from 7 to 4. More recently cv Avalon declined by two stages from 7 in 1980 to 4 in 1989.

Identifying a measurable characteristic by which sprouting susceptibility can be reliably assessed has been one of the objectives of the work described in this report. To be useful to a breeder it must be possible to measure the character in a small number of samples as it needs to be recognised in the limited stocks available in early generations of a crossing programme. It is highly desirable that the measurements should be made non-destructively. The results described

herein not only provide a platform for the development of such a test but they also help with progress towards understanding the physical and biochemical mechanisms of sprout resistance.

One characteristic which is fairly consistently associated with sprouting resistance is redness. As a result of this, cultivars of *Triticum aestivum* grown in the UK are almost exclusively red. Inheritance of redness is not simple and work continues to identify those features of redness that specifically influence sprouting susceptibility.

The work described in this report consists of two approaches, considered separately in Parts I & II. In both, genetic markers associated with high activities of *alpha*-amylase, were sought. In Part I attention was concentrated particularly on the influence of *R* genes, associated with grain colour, and with means of establishing the genetic constitution of cultivars with respect to the the three *R* loci. The problem of abnormally high enzyme activities in pre-mature wheats were also addressed. The work in Part II was directed towards investigation of a newly discovered waxy material, found in close proximity to embryos only of long stored red grains and consequently suspected of an association with sprouting susceptibility.

5. Current state of knowledge

The problem of sprouting has been studied with a wide range of approaches. Many reviews have been published dealing with the vast array of findings published in journals and at specialised meetings. There is room here only to provide a skeleton summary of the major areas of research and their findings, particularly stressing their circumstantial relevance to the present study.

Early work, in recognition that water was necessary for germination to occur, concentrated on understanding the mechanism by which water enters the grain, and in particular, how it reaches the embryo. It was well known that, in many seeds of non-cereals, complex mechanisms exist for preventing water from entering and thus ensuring a long and variable period of dormancy, possibly lasting for several years and thus providing a selective advantage through spreading germination of seeds produced in one year over a series of subsequent seasons. Resistance to germination in wheat seeds could be experimentally overcome by removal of the bran layers overlying the embryo. Even a pin prick in these layers was found to be sufficient. The uniqueness of water as a prerequisite for germination was questioned and the apparent impenetrability of the bran layers was envisaged as applying equally to water and gaseous exchange.

Water penetration into ears and leaves has been studied recently in Australian wheats (King and Licis 1990). Contrary to expectation the more glaucous type of leaf showed no greater resistance to penetration than the non-glaucous. There is little information on the nature or function of waxy materials in or on the grains themselves although there is good evidence that impermeable substances associated with the cellular layers and cuticle of the seed coat (testa) control water and gas movements into and out of the grain. The nucellar epidermis and its cuticle are also sometimes invoked as performing a control function. Both testa and nucellar epidermis, together with other bran tissues are thinnest over the embryo and may thus be expected to offer least resistance to penetration at this point. This is in fact found to be the case although it is not clear

whether penetration occurs uniformly over the whole embryo covering. There is good evidence for a highly absorptive tissue (the coleorhyzal papilla) behaving as a wick capable of conducting water, entering via the micropyle to the embryo (Symons *et al,* 1984).

Considerable work has also been done to understand the biochemical chain of events that follows wetting of the embryo. It is established that a series of events triggered by plant hormones is involved. Production of hydrolytic enzymes in the endosperm depends upon production of hormones in the embryo and any reduction in production of, or response to, those compounds clearly offers a potential means of reducing the deleterious effects of enzymic digestion. At the Cambridge Laboratory dwarfing genes have been shown to be associated with reduced synthesis of *alpha*-amylase (Gale and Flintham, 1982). The authors concluded that dwarfing genes may not provide adequate protection in all genetic backgrounds but would undoubtedly be beneficial in combination with other genetic factors for sprouting resistance.

The findings that have emerged from the various approaches show that the problem of sprouting is not a simple one and that a single solution to it is unlikely. The approaches reported here may not, on their own, lead to full control of all elements of sprouting. They embrace advanced breeding techniques built on a foundation of tried and tested principles and observations; and an approach characterised by unusual originality, the combination providing an admirable opportunity to exploit potential interfaces. The fact that the work on waxes addresses the earliest stages of the germination process is an indication of its importance, as control of the first essential stage provides the most effective means of controlling the entire process.

Part of the study continues to explore and define the complex and sometimes capricious relationship between sprout resistance and the genes responsible for wheat grain colour.

The hypothesis that has led to the complementary part is that the crystals found in long-stored samples of sprout resistant wheat types represent the products of aggregation of waxy materials which earlier comprised a water resistant layer lying somewhere between the outer surface of the tissues overlying the embryo and the surface of the embryo itself. The aggregation which occurs over a long period progressively causes the layer to become "holey"! as intercrystalline spaces arise and expand; this is compatible with the observation that sprouting resistance declines with passage of time. The removal or damaging of tissues overlying the embryo removes resistance to germination and suggests that if water and/or gas impermeability is responsible for preventing germination, then the barrier is associated with these tissues rather than the surface of the embryo itself. This is compatible with differences in the bran layers already known to exist between red and white wheats and manifested as colour differences. The challenge is ultimately to separate the resistance factor from the colour factor so that independent control can be achieved.

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PART 1.

OBJECTIVES

The objectives listed below were formulated with a view to identifying, evaluating and manipulating genetic resources for the control of Hagberg Falling Number in U.K. wheats, to facilitate the breeding of new high-quality varieties and to extend our scientific understanding of the crop.

Initiation of this project in the autumn of 1987 coincided with a low Hagberg harvest and the opportunity was taken to explore the physiological mechanisms underlying the resulting losses of grain quality.

The principal objectives of the project were as follows:

(a) Identification of genetic factors controlling high premature α-amylase activity.

(b) Development of tests for determining dosage and identities of *R* (red grain colour) genes in varieties and breeding lines.

(c) Production of isogenic lines carrying different standard *R* genes in adapted winter wheats.

(d) Measurement of the effects of genetic markers on resistance to sprouting damage in field trials and artificial tests.

METHODS

Comparisons made between different commercial varieties are of very limited value for purposes of genetic analysis. The reason for this is that the very large number of genetic differences between varieties are largely unknown, so that differences in their behaviour cannot be attributed to particular markers without additional evidence.

This difficulty can be resolved by comparing special genetic stocks with defined genetic differences. The ideal comparison is between isogenic lines, where differences can be attributed to single marker genes, and this approach was used to measure the effects of different *Rht* dwarfing genes in Maris Huntsman. Where isogenic lines were not available, the effects of single whole chromosomes were evaluated in sets of chromosome substitution lines, each carrying a single chromosome from a donor variety substituted for the equivalent chromosome of a contrasting, recipient variety. A modified approach was used in an attempt to identify the chromosome(s) responsible for premature enzyme synthesis in Maris Huntsman, amongst single-chromosome monosomic F_1 hybrids. A complete set of monosomic F_1 hybrids was produced in which each chromosome in turn was represented by a single copy from Maris Huntsman, all other chromosomes being present in two (hybrid) copies. These special genetic stocks require considerable time and effort to prepare, and are only available in a limited number of wheats. An alternative approach, using related inbred recombinant lines from a single cross, was used to evaluate the effect of increasing *R* gene dosage.

Resistance to sprouting damage was measured in two aspects: α-amylase production in sound grains was determined by enzyme assay; sprouting was scored in ripe ears subjected to simulated rainfall in a purpose-built "sprouting chamber".

The *R* gene dosage in different varieties, breeding lines, and in random recombinant lines was determined by crossing each line to a white-grained wheat, self-pollinating the resulting F_1 hybrid, and scoring the ratio of red- to white-grained plants in each resulting F_2. Although this test-cross method yielded a large array of extremely useful information, the technique is cumbersome and time-consuming, and would not be practicable in a commercial breeder's selection nursery.

A search was therefore initiated to find molecular markers for the *R* genes. DNA from wheat leaves was digested with restriction enzymes and the resulting restriction fragments were screened to identify those lying on the long arms of the group 3 chromosomes (the known location of the *R* genes). Wheats with Restriction Fragment Length Polymorphisms, i.e. showing differences in size of these molecular markers, were crossed to produce random recombinant lines. These lines will be used to identify which of the molecular markers lie closest to the *R* genes, so that in future the *R* dosage of a new wheat might be determined from a small leaf sample, rather than a crossing programme involving three generations and several hundred plants.

RESULTS

Data supporting many of the observations discussed below have been published in cited references. Where previously unpublished data are referred to, they can be found in the tables in the current text.

1. Different sprouting damage risks

Sprouting in ripe ears moistened by rain or dew is not the only source of high α-amylase wheat, indeed the low Hagberg Falling Numbers obtained in 1985 and 1987 were not associated with sprouting in ripe ears but with damage occurring earlier, during ripening (Gale and Flintham, 1988). Three distinct risks have been identified:

(a) Premature synthesis of α-amylase in the absence of embryo germination.

(b) Premature germination during early ripening.

(c) Conventional pre-harvest sprouting, in ripe ears during wet harvest seasons.

Premature α-amylase synthesis in sound, ungerminated grains is responsible for low Falling Numbers in many high-yielding wheats, both in the UK and overseas. Previous investigations indicated that enzyme production occurs during the early stages of ripening and that susceptible wheats are more likely to produce high levels of enzyme when weather conditions lead to slow drying of the grain. Genetic control of premature α-amylase synthesis is discussed in section 2 below.

Premature germination can occur shortly after the onset of ripening desiccation, at a grain water content of approximately 45% and without imbibition of rainfall or dew. This effect was seen in several varieties in 1987. Although premature germination induced α-amylase synthesis it was also observed in Bersee, a variety resistant to premature enzyme production in the absence of sprouting. Premature germination must therefore be considered as a third risk of sprouting damage, in addition to the "Huntsman syndrome" and to sprouting of ripe grain under rainfall (Gale and Flintham 1988).

Conventional pre-harvest sprouting, as distinct from prematurity germination, occurs when grains lose dormancy during the late stages of ripening and imbibe water from rainfall or other sources. This behaviour is seen typically in varieties with low inherent grain dormancy when harvesting is delayed by wet weather. The essential differences between this conventional sprouting and prematurity germination lie in the timing of embryo germination, and in patterns of varietal susceptibility: genetic resistance to sprouting of ripe grain is not neccessarily accompanied by resistance to earlier, premature germination (section 4.1 below).

Genetic susceptibility to each of these risks has been shown to be independent of resistance to the other two risks, e.g. a variety resistant to premature α-amylase production may be susceptible to sprouting and/or premature germination. For the wheat breeder this means that separate resistance genes may have to be employed for adequate protection against all risks.

Genetic markers for resistance to these different risk factors are discussed in subsequent sections of this report.

2. Premature production of α-amylase

Two aspects of the genetic control of premature enzyme production have been investigated. Isogenic lines carrying different *Rht* genes have been used to measure the effectiveness of these dwarfing genes as inhibitors of α-amylase production in Maris Huntsman. A second series of experiments were designed to identify the chromosomes causing inherent susceptibility to this risk.

2.1 Inhibition of enzyme production by *Rht* dwarfing genes

Field trials of inbred isogenic lines of Maris Huntsman have repeatedly shown the inhibitory effects of different *Rht* genes on premature α-amylase synthesis (eg. Table 1).

Table 1a **Endogenous and GA_3-induced α-amylase activity in whole and de-embryonated half-grains from isogenic lines of Maris Huntsman carrying different *Rht* genes.**

	α-Amylase activity			
	Endogenous		GA-induced	
***Rht* Isoline**	**log(mU/grain), (se)**		**log(mU/½-grain), (se)**	
rht (tall control)	1.21	(0.19)	2.93	(0.16)
Rht1	1.06	(0.30)	3.19	(0.27)
Rht2	0.51	(0.15)	3.19	(0.23)
Rht1 + *Rht2*	0.56	(0.09)	3.04	(0.35)
Rht3	0.51	(0.08)	1.37	(0.31)
Rht3 + *Rht2*	0.59	(0.07)	1.47	(0.26)

Note: Enzyme activities are means over 3 replicates of three (half) grains per line.
GA-induced activity after 72 hours in 10μM GA_3 at 25°C.
Grains were from the 1990 harvest, undamaged, well filled and sound.
se = standard error.

Table 1b **α-Amylase activities in flours from bulk plots of isogenic lines of Maris Huntsman carrying different *Rht* genes.**

***Rht* isoline**	**α-amylase activity, mU/g flour (se)**	
rht (tall parent)	2260	(408)
Rht1	542	(219)
Rht2	532	(219)
Rht1 + *Rht2*	166	(157)
Rht3	194	(157)
Rht3 + *Rht2*	278	(419)

Note: Wholemeal flour samples were milled from 4 replicate drilled plots per line, 1988 harvest.
All samples were completely free of visibly sprouted grains.

Inhibition of enzyme synthesis was proportional to the severity of *Rht* dwarfism:

$$rht \text{ (Huntsman parent)} < Rht1 = Rht2 < Rht1+Rht2 = Rht3$$

(increasing order of inhibition = decreasing order of enzyme activity). The most useful effect, a 90% reduction in α-amylase in both sound and sprouted grain, was associated with the *Rht3* gene, however *Rht3* inbreds were severely dwarfed (Flintham and Gale 1982, 1983).

In F_1 hybrids the *Rht/rht* heterozygotes showed intermediate enzyme levels, with the exception of the *Rht3/rht* hybrid, which had α-amylase activity as low as the *Rht3* inbred and a more acceptible semi-dwarf stature (Flintham 1990).

Comparisons between the endogenous enzyme activity in sound grain and that inducible by gibberellin activation of the aleurone are shown in Table 1.a. The general trend to low α-amylase in non-germinated *Rht* wheats was evident, however the effects of the *Rht1* and *Rht2* genes were not due to insensitivity to gibberellic acid, an observation confirmed by other workers (Gold and Duffus 1992). These results suggest that although suppression of enzyme synthesis by *Rht3* is due to genetically dominant insensitivity of *Rht3* aleurone cells to gibberellic acid, a different mechanism must be responsible for the effects of the other *Rht* genes (possibly more rapid drying during ripening of the semi-dwarf grains which are smaller than those of tall *rht* controls). This model could account for the high α-amylase activities detected in semi-dwarf *Rht2* varieties such as Fenman.

2.2 Susceptibility to premature enzyme synthesis in Maris Huntsman and other varieties.

The correlation between slow grain ripening and high α-amylase in Maris Huntsman was established previously (Gale et al. 1983). Attempts to gain more insight into the genetic control of this susceptibility gave complex results. In an analysis of F_1 hybrids between Maris Huntsman and monosomic lines of Chinese Spring (to identify the Huntsman chromosome(s) carrying genes for susceptibility) high enzyme activity was associated with monosomy for each of the chromosomes in turn, while activity in the normal euploid hybrid with Chinese Spring was low (data not shown). The only genetic explanations for this result would be: that recessive genes on every chromosome are critically involved in the control of α-amylase activity; or that any disturbance of the normal chromosome complement carries a risk of premature enzyme synthesis.

In other F_1 hybrids, between Maris Huntsman and Maris Widgeon or Bersee, high α-amylase activity behaved as a dominant trait. Genetic dominance or recessiveness of the gene(s) from Maris Huntsman thus varies according to the particular parents in the hybrid cross.

An attempt to compare genetically determined high α-amylase in Huntsman with similar behaviour in the Australian variety Spica failed due to hybrid necrosis in the Huntsman x Spica F_1.

The Russian variety Bezostaya-I was also found to produce premature α-amylase in ungerminated

grains. A set of lines carrying single Bezostaya-I chromosomes substituted into the breeding line Dwarf-A (a sister line of the variety Hobbit) was therefore screened for premature enzyme production (Flintham 1990). Chromosomes 4D and 7A of Bezostaya-I were identified as sources of susceptibility in this experiment; the 4D effect may have been due to the loss of the *Rht2* dwarfing gene of Dwarf-A, while the 7A effect was associated with a translocation chromosome rearrangement between the two varieties. Even in combination, the effects of these two chromosomes were too small to account for the large difference between enzyme activities of Bezostaya-I and Dwarf-A, indicating that the high level of susceptibility seen in Bezostaya-I is due in part to genetic interactions between different chromosomes.

Genetic susceptibility to premature α-amylase production appears to be more complex than a simple one-gene model would allow. The curious results obtained from Maris Huntsman crosses do not allow predictions beyond saying that any wheat with susceptibility in its ancestry can be considered to be at risk. Susceptibility is not a clear-cut trait: surveys of varieties at the Cambridge Laboratory and elsewhere reveal a continuous spectrum of behaviour from complete susceptibility, through variable and moderate resistance, to apparently complete freedom from this risk (Mares and Gale 1990). Such continuous variation is typical of traits controlled by a large number of genes.

3. Grain dormancy associated with *R* genes for red grain colour

The production of prematurely high α-amylase discussed in the previous sections is independent of visible sprouting, which entails germination and growth of the mature embryo. Resistance to sprouting of the germinative type can be achieved by manipulating grain dormancy, which is known to be associated with red grain colour determined by three independent *R* genes. Wheats carrying one, two or three *R* genes have red grains; white grained wheats carry the *r* genes, *r1* + *r2* + *r3* .

3.1 Detection of *R* genes and their effects on sprout-resistance

The paramount importance of *R* genes and their additive effects on sprout resistance were revealed by a systematic study of grain pigmentation, *R* gene dosage and sprouting resistance in a population of random inbred recombinant lines from the cross RL4137 x Timgalen (RL4137 has extremely strong grain dormancy and carries three *R* genes for red grain colour, Timgalen is a white wheat with little or no grain dormancy). Pure-breeding recombinant lines were developed by single seed descent and scored for resistance to sprouting in three years' field trials. The same lines were also scored for intensity of grain colour (by the sodium hydroxide test, Flintham 1990) and test-crossed to Timgalen to determine how many *R* genes each line carried (see Methods).

The population of random lines was divided into different groups according to *R* dosage (white v red, *Rrr* v *RRr* v *RRR* within the red group, and awned v awnless within each group). Differences in sprouting scores between groups then revealed the effects of presence/absence of red pigment, increasing *R* dosage within the red-grained group, effect of awning, and effects of other unidentified genes (from residual genotypic differences between lines within groups).

Resistance to sprouting was positively correlated with the number of *R* genes in every trial; typical results are shown in Table 2. The main genetic effect in each case is the difference between the white lines and the combined group of red (*Rrr, RRr, RRR*) lines, absence of red pigment being associated with lack of grain dormancy.

Table 2 **Sprouting scores of recombinant lines and parents harvested from winter and spring sowings.**

Recombinant Lines (n)	Colour	Sowing	Sprouting Scores In Test 1	In Test 2
rrr (11)	white	winter	0.46	0.63
		spring	0.25	0.36
Rrr (32)	red	winter	0.05	0.19
		spring	0.03	0.06
RRr (30)	red	winter	0.09	0.18
		spring	0.01	0.03
RRR (11)	red	winter	0.09	0.12
		spring	0.01	0.03
Parents				
Timgalen (*rrr*)	white	winter	0.67	0.70
		spring	0.30	0.60
RL4137 (*RRR*)	red	winter	0.00	0.00
		spring	0.00	0.00

Note: Recombinants were homozygous inbred lines derived by single seed descent from the cross RL4137 x Timgalen.
Sprouting scores: 0 = no sprouting, 1.0 = all ears sprouted after 7 days simulated rainfall at 21±2°C; all differences significant at 5% level.
Winter sowing harvested 12th. August, spring sowing 19th. August 1991. First sprouting test started 29th. August, second test started 12th. September.

Amongst the red-grained lines, small but significant increases in resistance were associated with increases in *R* dosage (from *Rrr* to *RRr* , and from *RRr* to *RRR*, Table 2). The visual intensity of red pigmentation also increased with increasing *R* dosage (Flintham 1992). Thus, on average, selecting for increased *R* dosage, or for increased intensity of grain colour, is expected to lead to limited but significant improvement in sprout-resistance of red-grained breeding lines. This expectation applies to sister lines from a single cross in which more than one *R* gene is

segregating but may not necessarily apply to comparisons between unrelated varieties. The positive correlation between pigment intensity and resistance also lends some support to the idea that *R* genes are directly involved in the control of grain dormancy: if more *R* genes are present more pigment is produced, and increasing pigmentation leads to increased grain dormancy.

In addition to these differences in presence and intensity of grain pigmentation, effects due to presence or absence of awns and to other, unidentified genetic differences were also apparent: lack of awns led to an increase in resistance comparable to the effect of increasing *R* dosage from one to three *R* genes (data not shown). Residual genotypic variation for resistance was detected after the effects of colour, *R* dosage and awns were accounted for. These "non-colour" effects were evident in all *R* dosage classes including the white-grained lines, indicating that a limited amount of grain dormancy can be transferred to white wheats.

3.2 Numbers of *R* genes carried by modern varieties and breeding lines

A total of 83 red-grained varieties and breeding lines were test-crossed to determine the *R* dosages of the different wheats listed in Table 3. The conspicuous absence of white-grained wheats from this list reflects plant breeders' awareness of their poor resistance to sprouting and the consequently rare entry of white wheats into UK agriculture. The *R* dosages of varieties and breeding lines presented in Table 3 will allow breeders to predict the likely frequencies of *R* genes in new lines bred out of this gene pool, either directly or by pedigree analysis.

Table 3 Numbers of R genes in wheat varieties and breeding lines

(a) Wheats with a single R gene (genotypes *R1+r2+r3*, *r1+R2+r3*, or *r1+r2+R3*):

Alexandria, Apollo, Avocet, Axona, Baron, Captor, Copain, Dollar, Dwarf-A, Fortress, Grana, Guardian, Hammer, Hustler, King, Longbow, Luna, Mara, Mardler, Maris Huntsman, Maris Widgeon, Mercia, Motto, NFC75/93/27A, Norton, Peacock, Rapier, Sava, Talent, TJB370/500, Virtue, Voyage, Vuka.

(b) Wheats with two R genes (*R1+R2+r3*, *R1+r2+R3*, or *r1+R2+R3*):

Apostle, Argent, Atlas 66, Bersee, Bezostaya-I, Bounty, Brigand, Brock, Cappelle Desprez, Champlein, Corin, Corinthian, Coxswain, Depot, Desprez 80, Fenman, Feuvert, Flanders, Kanzler, Kudu, Mantle, NFC97/84B, Norman, Pageant, Parade, Pastiche, Poet, Prince, Proton, Renard, Rendezvous, Riband, Squadron, Stetson, Urban.

(c) Wheats with all three R genes (*R1+R2+R3*):

Ambassador, Boxer, Carmen, Cheyenne, Cub, Dauntless, Drum, Favorits, Galahad, Kador, NFC70/84C, Probus, Rannyaya 12, Sentry, Soleil.

Note: 21 other wheats tested gave inconclusive results, due to insufficiently large population sizes and intermediate grain colour segregation ratios in F_2.

The red-grained wheats assayed to date show no evidence of selection for increased *R* dosage. The ratio of 33 *Rrr* to 35 *RRr* to 15 *RRR* varieties in Table 3 is almost identical to the 2 to 2 to 1 Mendelian ratio expected from random segregation within a gene pool carrying all three *R* genes and all three *r* genes in equal frequencies (given that the resulting white-grained lines are discarded). This means that there is considerable scope for increasing the *R* dosage of cultivated wheats, since more than 80% of those surveyed had less than the maximum three *R* genes.

Although the mean sprouting score of these red wheats showed the expected reduction relative to white-grained controls, mean scores for the *Rrr* v *RRr* v *RRR* groups were similar and showed no evidence of *R* dosage effects (data not shown). This is in contrast to the dosage effects seen in the random recombinant lines (section 3.1 above). This failure to detect *R* dosage effects amongst varieties was probably due to the small size of the expected effects and to the wider genetic variation amongst varieties compared to that between sibling lines from a single cross.

Wide variation for resistance to sprouting in the ear was evident within each dosage group of varieties (as in the recombinant lines, section 3.1), indicating the presence of additional important resistance genes independent of variation for *R* dosage. These independent effects are being investigated further, in crosses between highly susceptible and highly resistant *RRR* varieties.

3.3 Production of isogenic white and red wheat lines

Three important questions concerning the relationship between *R* grain colour genes and resistance to sprouting remain unanswered:

(a) Is the resistance associated with red pigmentation a direct effect of the *R* genes, or are the *R* genes acting as markers for linked but otherwise unidentified genes conferring grain dormancy?

(b) How does the physiological mechanism of grain dormancy in red wheats differ from that in white wheats?

(c) Do the different *R* genes, carried on different chromosomes, have equivalent effects on sprout-resistance, or are some *R* genes or combinations more effective than others?

These questions can only be answered by comparing isogenic lines carrying defined *r* and *R* gene combinations in uniform genetic backgrounds, so that differences can be attributed to specific single genes with confidence. The relevant lines were not available at the start of this project and production of isogenic stocks for the *R1/r1, R2/r2* and *R3/r3* genes in adapted winter wheat backgrounds was initiated during 1988.

Lack of a standard single-*R* gene source for the locus on chromosome 3B (*r1+r2+R3*) has necessitated a complex approach to this objective. Although introgression of *R1* from Chinese Spring and *R2* from Red Bobs into the white-grained winter wheats Holdfast, Wilma, Abele and JBW (a PBI breeding line) is relatively straightforward, *R3* has had to be introduced from Cappelle Desprez (*r1+R2+R3*) and from RL4137 (*R1+R2+R3*).

In order to be reasonably certain of recovering the target gene, either 6 (Cappelle) or 12 (RL4137) lines are being maintained through 6 successive backcrosses to each of the 4 white parents. Since maintenance of each line requires a minimum of 4 independent crosses per generation, this exercise demands a major breeding effort. Additional problems were encountered during the early stages of the backcross programme due to wide differences in flowering time, resulting in the loss of two generations of material. Despite the ensuing delay, this breeding programme is continuing at the rate of two generations per year. Isogenic lines will be extracted from each backcross line at the end of a new HGCA/MAFF LINK project, allowing precise analyses of *R* gene effects for the first time in adapted winter wheats.

4. Other genetic effects on sprouting

Rht3, and the *R* genes (*R1, R2, R3*) are examples of genetic markers associated with potentially useful resistance to sprout-damage. Effects of other genetic markers, notably inhibitors of awn development, have been demonstrated but are less relevant in the U.K. context. These markers cannot account for all of the detected genetic variation in resistance; other as yet unmarked genes are responsible for significant and potentially valuable effects.

Evidence for unidentified genes affecting dormancy but not grain colour has been obtained from various sources, e.g. residual genotypic variation within *R* dosage groups (section 3.1), effects seen in chromosome substitution series (section 4.1), dormancy in white wheats from overseas. Perhaps the most telling example for U.K. breeders is the observation of extremes of sprout-resistance and susceptibility within the *R1+R2+R3* group of varieties in Table 3. In particular, the varieties Boxer and Galahad have shown consistently low resistance, almost as low as some white controls, while the varieties Sentry and Soleil have resistance comparable to that of RL4137 (an internationally acknowledged standard of extreme dormancy). Boxer, Galahad, Sentry and Soleil all behaved as *R1+R2+R3* wheats in test-crosses, so that these differences in resistance must be due to some other genetic factor(s). These four UK varieties do not differ as regards awning or any other known resistance marker; identification of the factors responsible for differences between them has therefore been adopted as one goal of a new breeding programme.

4.1 Evidence from chromosome substitution lines

In the Dwarf-A(Bezostaya-I) chromosome substitution lines, chromosomes 4D and 7A of Bezostaya-I were associated with susceptibility both to premature α-amylase synthesis in sound grain and to premature germination (Table 4, Flintham 1990). Chromosome 5B of Bezostaya-I was also implicated in premature germination. These results contrasted with the pattern of sprouting in ripe ears in 1988, which was most severe when Bezostaya-I chromosomes 1D or 3B were present. Chromosome 1B appeared to carry a resistance factor. These three different results for a single set of lines reinforce the conclusion reached in section 1 above: that different genes are responsible for resistance to different risks.

Results from other substitution series showed no consistent pattern of resistance to sprouting, with the exception of recurrent effects of group 3 chromosomes possibly associated with *R* gene differences (Table 4). One difficulty with these chromosome substitution studies has been the relatively small variation in resistance between the parents involved. The long time needed to produce a chromosome substitution series, and the fact that the available series were produced for reasons unrelated to sprouting, has limited the usefulness but not the validity of this approach. It is apparent that different chromosomes can influence resistance to pre-harvest sprouting depending on the variety from which they are derived and on the variety into which they are transferred, and that in most cases more than one chromosome is implicated in resistance differences between pairs of varieties.

Table 4: **Chromosomes identified as conferring partial resistance or susceptibility to different sprouting damage risks in single chromosome substitution series.**

Recipient variety	Donor variety	Donor chromosomes for: Resistance	Susceptibility	Risk[1]
Dwarf-A	Bezostaya-I	None	4D,7A	PMA
Dwarf-A	Bezostaya-I	None	4D,7A,5B	PMG
Dwarf-A	Bezostaya-I	1B	1D,3B	PHS
Bersee	Champlein	3B	2A,3D,4B,7D	PHS
Bersee	Desprez-80	None	2B,4B,6B	PHS

[1]Note: Abbreviations for different sprouting damage risks:
PMA = Premature α-amylase production
PMG = Premature germination
PHS = Sprouting in ripe ears exposed to artificial rainfall.
These different risks are explained in detail in section 1.

4.2 Endogenous protein inhibitors of α-amylase enzyme activity

Variation in the content/activity of endogenous α-amylase inhibitors is shown in comparisons between wheat, triticale and rye in Table 5. Rye extracts showed most inhibition against a standard α-amylase preparation, followed by triticale then wheat. Evidently the inhibitor genes from rye were partially effective when introduced into the wheat genetic background (as in Triticale), however the inhibitory effects were small compared to the levels of enzyme present in sprout-damaged harvests (compare inhibitor activities with α-amylase levels shown in Table 1.b). In addition it is not clear whether the inhibitors would be stable during processing.

Table 5 **Endogenous α-amylase inhibitor activity in flours from different rye, wheat and Triticale lines.**

Species/cultivar/line	Inhibitor activity[1]	Group mean activity
Rye cv Kustro	112	
Rye cv Animo	105	Rye cultivars = 113
Rye cv Otello	140	
Rye cv Ehokurz	95	
Rye line RXL	125	
Rye line 541	190	Rye lines = 145
Rye line DS2	123	
Rye line Ot1-3	143	
Secale montanum	70	
Secale kuprijanovii	140	
Secale dighoricum	100	
Secale africanum	200	Rye species = 119
Secale chaldicum	100	
Secale anatolicum	80	
Secale silvestre	145	
Wheat, Chinese Spring	3	
Wheat, Bersee	5	
Wheat, Maris Huntsman	80	Wheats = 16
Wheat, RL4137	5	
Wheat, Timgalen	0	
Wheat, Aus 1490	0	
Triticale, AD-767	75	
Triticale, BR-2	85	
Triticale, Lukas	90	Triticales = 73
Triticale, Bokolo	35	
Triticale, Newton	80	

[1]Note: Inhibitor activity is expressed as mU barley α-amylase inhibited per gram flour.

4.3 Sprout-resistance of spring wheats

NIAB sprout-resistance scores show spring wheats to be significantly more resistant than most winter varieties (Morgan 1988). This effect was investigated by including spring varieties in an autumn sowing of winter wheats (10th. October 1990). Additional sowings, of inbred spring recombinant lines and their parents Timgalen and RL4137 were also made, in October 1990 and again on 18th. March 1991.

Following harvest on 19th August 1991 sample ears of winter and spring varieties were subjected to an artificial sprouting test over seven days from 11th October 1991. Sprouting scores of spring varieties were more severe than expected on the basis of NIAB resistance scores (Table 6). One reason for this anomaly may have been early ripening of the spring varieties relative to winter wheats in the same test, however the results from split sowings of spring recombinants, described below, suggest that additional factors were involved.

Table 6 Sprouting scores of spring and winter wheat samples from a single sowing in autumn 1990.

Variety	Habit	NIAB[1] score	Sprouting in [2] test
Apollo	Winter	7	1.00
Avalon	"	4	0.80
Brigand	"	5	0.20
Brimstone	"	5	0.10
Brock	"	7	0.40
Fenman	"	7	0.60
Galahad	"	4	0.90
Haven	"	2	0.80
Hornet	"	7	0.80
Longbow	"	8	0.10
Mercia	"	6	0.80
Norman	"	6	0.00
Parade	"	7	0.00
Pastiche	"	7	0.40
Rendezvous	"	6	0.50
Riband	"	7	0.40
Slejpner	"	6	0.00
Mean for winter wheats		5.9	0.46
Alexandria	Spring	8	0.40
Axona	"	8	0.80
Minaret	"	8	0.20
Tonic	"	8	0.70
Wembley	"	7	1.00
Mean for spring wheats		7.8	0.62

[1]Note: Resistance scores, inversely related to sprouting.

[2]Note: Sprouting scores: 0 = no sprouting, 1.0 = all ears sprouted, after 7 days in simulated rainfall at 21±2°C.

Ripening in the two sowings of recombinant lines was monitored twice weekly; sheaves were cut as soon as green colour disappeared from the straw and were stored under glass prior to sprout-testing. Two successive sprouting tests were carried out on all lines (Table 2). The differences between sprouting scores of winter versus spring sowings of the same lines were larger than could be accounted for by the seven days difference in ripening date, since sprouting of the spring-sown samples in the second test (after a further 14 days storage) was still less than that of winter-sown samples in the first test. The higher resistance (lower sprouting scores) of spring-sown material must have been due to an interaction between stage of maturity and environment prior to harvest. A similar effect might explain the high resistance ratings of spring-sown commercial varieties. If this is so, then early sowings of spring varieties may have less sprout-resistance than would be expected from their NIAB scores.

5. New markers for sprout-resistance

The limited number of morphological markers (dwarfism, grain colour, awns) in wheat has limited the resolution of genetic analyses in the past. Since the mid 1980's new biochemical and molecular techniques have increased the number of markers available, generating an exponential increase in the power of analytical techniques. Of these new developments, perhaps the most promising is the genetic mapping of DNA Restriction Fragment Legth Polymorphisms (RFLPs, Gale, Flintham and Mares 1990). The RFLP maps of wheat and related cereals have now been developed to the extent that traits controlled by multiple genes can now be analysed for linkage to DNA markers distributed throughout the cereal genomes.

RFLP mapping of DNA markers for the *R* genes is in progress. Polymorphisms between parental lines for markers on the long arms of group 3 chromosomes have been detected and DNA has been extracted from recombinants out of crosses between white-grained lines and red lines carrying single, known *R* genes. These recombinants will be used to identify DNA markers for the *R* genes, and to test for the presence of a gene on chromosome 6B for susceptibility to premature α-amylase synthesis in the variety Spica (Mares and Gale 1990). One advantage of DNA markers is that they can be detected in a small sample of leaf tissue from a single immature plant. This is in contrast to the determination of *R* genotype by conventional test-crossing, involving three test-crosses, three generations of plants, and scoring grain colours of hundreds of F_2 segregants for each plant tested.

A possible alternative to the molecular mapping of genetic resistance effects involves the analysis of physiological/morphological traits for which an influence on germination behaviour can logically be inferred. An opportunity for this type of approach is offered by the observation of waxy deposits over the embryo, discussed in the F.M.B.R.A. part of this report. The potential importance of new markers has been made clear by the discovery of wide variation for grain dormancy amongst some commercial varieties carrying optimal combinations of conventional markers, as in the case of the *R1 + R2 + R3* wheats discussed in sections 3.2 and 4 above.

DISCUSSION

When the mature aleurone layer is exposed to gibberellic acid, either from the germinating

embryo, some other source within the plant, or by exogenous supply, it responds with massive synthesis of hydrolases, primarily by transcription and translation of the *α-Amy1* gene family.

If it were possible to design a single gene for maintaining high Hagberg Falling Number and introduce it into wheat, perhaps the most attractive option would be to disable this aleurone response to gibberellins.

The *Rht3* dwarfing gene does exactly this, by strongly inhibiting all known gibberellin responses in wheat and in triticale. Another consequence of *Rht3* insensitivity to gibberellins is the inhibition of stem elongation growth, resulting in extreme dwarfism. Because of biomass yield penalties associated with the extreme dwarf habit, breadwheat breeders have rejected *Rht3*. Breeders in Germany and elsewhere have exploited *Rht3* to produce high Hagberg triticales; the higher biomass of triticale compared to wheat and the severity of the Hagberg problem have been sufficient to justify use of *Rht3* in this crop.

Rht3 is also effective (dominant) in its inhibition of α-amylase synthesis when in heterozygous condition, in F_1 hybrid (semi-dwarf) wheat. In addition, *Rht3/rht* hybrids have been shown to yield as much as or more than tall or semi-dwarf controls (Flintham and Gale 1991). Exploitability of *Rht3* thus depends upon achieving sufficiently high biomass by manipulating *Rht3* dosage and/or the genetic background in which the dwarfing gene is placed.

In view of the complexity of the physiology and genetics of sprouting and other causes of low Hagbergs, use of *Rht3* combined with breeding for increased biomass may be a viable alternative to other genetic strategies for reducing sprout-damage. However it would not be effective against losses of germination vigour observed in sprouted grains after drying. Whether *Rht3* is employed or not, some control of grain dormancy will still be essential if damage from sprouting is to be avoided.

Red grain coat pigmentation is the only marker for grain dormancy currently available for discriminating between commercially viable UK breeding lines. This is well known by wheat breeders and white-grained varieties rarely enter Classified Lists. This project has demonstrated the association between increased dosage of *R* genes and increased dormancy. This effect, and the finding that most modern varieties carry less than three *R* genes, suggest that some improvement in resistance can be made by selecting for wheats carrying *R1 + R2 + R3* . Until molecular markers for these *R* genes become available the most practicable selection index will be depth of grain pigmentation, given the positive correlations between colour intensity, *R* dosage, and resistance to sprouting in the ear (Flintham 1992).

The mechanism of *R* gene action in conferring grain dormancy, the relative merits of different *R* genes, and the identities of genes carried by varieties have yet to be established. Research into these topics is continuing at the Cambridge Laboratory, with the production of isogenic lines (for precise analysis of *R* gene effects) and extended test-crossing (to identify *R* genes in varieties).

This project has made it clear that genetic resistance to sprouting damage is complex, partly due to a complexity of risks, but also due to unexpectedly complex inheritance of resistance. The

effects of available markers have been measured and new techniques are now being developed to identify and evaluate new sources of resistance. Recent advances in molecular biology are particularly promising in this respect, however much remains to be done: much of this new potential has yet to be realised and transferred onto the scientific record; some areas of the cereal genomes are poorly represented on the genetic maps; polymorphisms for some markers are infrequent amongst the narrow gene pool used by many commercial breeders; the modes of action and control of expression of key developmental events are often obscure. It has become evident that currently available markers cannot account for all of the observed variation in resistance. The search for new markers and sources of resistance continues.

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PART II **Page**

PART II Identification of a major factor controlling sprouting in cereals

OBJECTIVE

To investigate the composition and function of waxy crystals found in the embryo cavity of long-stored red wheats and to provide for breeders a readily detectable marker for distinguishing phenotypes with strong resistance to sprouting.

MATERIALS

Wheats

The wheats used were from small "museum" stocks of authentic cultivars maintained by the Milling Section at FMBRA.

Chromatography standards

Authenticated pure standard compounds were purchased from Sigma Chemical Company Ltd. The include hydrocarbons, fatty acids, alcohols, wax esters (all saturated and straight chain), cholesterol and tristearin.

Reference surface waxes were also prepared from wheat plants and leaves of *Eucalyptus gunii*. The plant parts were washed for 15sec in three aliquots of chloroform. after filtration, the solvent was evaporated under vacuum. The yields were 0.29% and 2.2% (dry basis) from wheat and Eucalyptus respectively. The composition of these waxes is well documented (Tulloch & Hoffman 1973, Horn *et al* 1964).

Thin layer chromatography (TLC) plates and solvents

Pre-coated TLC plates 5, 10 or 20 x 20cm with Silica gel 60, 0.25mm thick without indicator (Merck) were used.

Solvents were all of analytical or HPLC grade and supplied by Aldrich Chem. Co. Ltd., Absolute ethanol (A.R. Quality) was supplied by James Burrough (FAD) Ltd.

METHODS

Scanning electron microscopy

Scanning electron microscopy was carried out with a Cambridge S600 SEM. Photographs were recorded on Polaroid Type 55 positive/negative film.

Embryo waxes

Waxes occuring in the embryo cavities of wheat grains were exposed by dissection. Grains were held fast to an adhesive surface with dorsal side uppermost so that the loose outer pericarp 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 which can then be removed and stuck, inner side uppermost, on an adhesive surface. The crystals thus exposed could be photographed or carefully removed for analysis.

For collecting wax material for analysis two methods were used. The first method was used when an assured high degree of purity was required. It consisted of careful transfer with a clean mounted needle, of crystalline aggregates from exposed cavity and inner pericarp, to redistilled chloroform. Material collected in this way was required for definitively establishing the composition, and material from 1000 grains of cv Yeoman has been collected for this purpose. It is to be used for detailed analyses by preparative TLC followed by gas chromatography - mass spectroscopy (GC-MS) of individual fractions. From 1000 grains approximately 1mg has been collected. Smaller quantities, dissected from fewer grains have been used throughout the preliminary studies.

In the second more convenient method the seed coats overlying the embryo were not completely removed. An incision was made only half way round the cavity so that access could be gained to its contents. The embryo end of the grain with exposed cavity was dipped 5 times in chloroform for 1 sec. TLC analysis of these washings showed no difference when compared with TLC of manually collected crystals from the same variety. The method provided a means of comparison with grains containing no visible crystals. It was used for analytical TLC, GC and surface pressure measurements.

Thin Layer Chromatography

For TLC various solvents and solvent mixtures were tested as developing systems. These ranged from low-polarity systems (toluene, ethanol-free chloroform, trichloroethylene and chloroform 3:1) to medium polarity systems e.g. chloroform with 1% ethanol. The best separation of embryo-wax components was achieved with chloroform (Aldrich HPLC) cotnaining 0.3% absolute ethanol.

Plates were conditioned at 105°C for 1 hr. Chloroform washings from 20 grains were concentrated in 10μl and applied in this way. Solvent run path was 170mm. After development the solvent was dried from the plates in a fume cupboard.

Two general detection methods were applied (Holloway, 1984; Jones et al, 1966). The first, consisting of exposing the plates to iodine vapours for 1-3h, was used in working out the method, and testing developing systems etc. The second method was used in final analyses and for documentation purposes. Here the plates were exposed to vapours of sulphuryl dichloride for 5 mins, then held over a steaming water bath for 30 sec and heated to 200°C for 10-30 mins. In addition to these general detection systems specific reagents were applied to detect particular

components (Holloway, 1984, Holloway and Challen, 1966). Vanillin spray (3% ethanol solution containing 1% sulphuric acid) followed by heating at 120°C was applied to detect hydroxy compounds and sterols. Fast Blue B spray (0.1% solution in cold 3N-sodium hydroxide) followed by heating at 100°C was used to detect β-diketones and hydroxy β-diketones.

Gas chromatography

Measurements were made on a Perkin-Elmer 8500 gas chromatograph. The column, 1m x 3mm ID, was packed with 1% Dixsil 300 on Chromosorb WHP 100-120 mesh. A linear temperature gradient 6° m in^{-1} from 130 to 370°C was used. Due to temporary technical problems a program for compensation of base-line shift could not be applied. Carrier gas (nitrogen) flow rate was 25ml min^{-1}, temperatures of injection and FID detector 390°C.

The chloroform washings from 20 grains were concentrated to 10μl, 5μl were injected. In the case of derivatised samples the washings of 20 grains were evaporated to dryness, 8μl of BSA (N, O-bistrimethylsilylacetamide) and 3μl of pyridine were added. The mixture was heated to 50°C for 1h (Holloway 1984), 5μl of the mixture was injected. By this procedure of "silylation" free acids and alcohols were converted to their trimethylsilyl (TMS) derivatives, while triglycerides and monoesters remained unchanged.

Force-Area Isotherms

The films of embryo wax material from 20 grains dissolved in chloroform were spread on the surface of de-ionised, doubly distilled water. The waxy material was spread using a microsyringe, the monolayer spreading being achieved by direct application to the surface of 100μl of solution in the form of numerous small drops. Measurements were made on a Joyce Loebl Langmuir - Blodgett surface balance. The constant perimeter trough avoids the problems of monolayer leakage at barriers. The temperature of the water subphase was maintained at 25°C $\pm$ 0.5°C. A standard procedure was adopted for determining Force-Area (π-A) isotherms. The waxy material was spread on the maximum available area (560cm^2) and the speed of the barrier chosen to give a constant compression rate of 1.1cm^2s^{-1} and compressed to the minimum available area, 110cm^2.

Atomic Force Microscopy

Wax crystals dissected from the embryo of Yeoman wheat grains were used to form a spread monolayer which was compressed to a surface pressure of 15mNm^{-1} to form a cohesive film. The film was transferred on to a glass microscope cover slip (approx 10mm dia) previously rendered hydrophobic by silyation. The cover slips were dipped in and out of the water subphase, at a constant speed of 5mm per minute. Film was transferred on both the down and upstroke and each cover slp was coated by two film layers. The film layers were inspected by a Nanoscope II Atomic force Microscope, by courtesy of Environmental Monitoring Systems Ltd., Smarts Heath Road, Woking, Surrey.

RESULTS

Microscopy

Examples of the crystals found in long-stored red wheats are shown in Figs 1 and 2. The crystals are needle-like and occur in tufts of various numbers. On closer inspection the crystals appear flattened and ribbed. They may be hollow but it has not been possible to demonstrate this from examination of broken ends.

Fig 1. Scanning electron micrograph of crystals in the embryo cavity of cv. Yeoman.

Fig 2. Higher magnification micrograph of wax crystals from cv Yeoman.

In examining the endosperm cavity of wheat types in search of wax crystals, or to expose the areas to solvent for preparation of washings, we have noted a striking variation in the amount of fungal infestation. White grains have tended to suffer far more than red in this way although it should be borne in mind that the number of white wheats examined is very small. The differences noted, if substantiated, may be indicative of an additional important function of the embryo cavity-wax. At the very least the observation is compatible with the suggestion that water penetration - necessary for the development of fungal mycelium - occurs more readily in white than in red wheats.

Some variation in morphology has been noted but no systematic survey has been carried out to detect varietal differences. Such a survey, related to composition, when known, may be approprate.

Thin Layer Chromatography

A Typical TLC analysis of wheat embryo waxes, standards and reference mixtures is shown in Fig 3. The embryo waxes separated into seven fractions, however, more spots can be seen on TLC plates as some classes of compounds were apparently further separated according to chain Length.

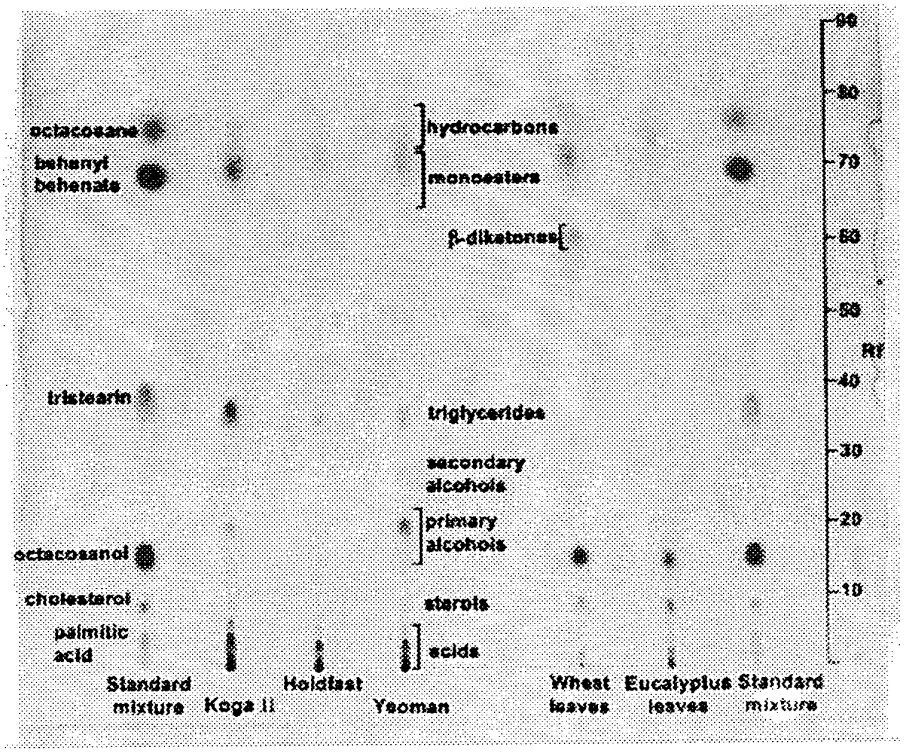

Fig 3. Thin layer chromatographic separation of embryo waxes from three wheat varieties, surface waxes from wheat and Eucalyptus leaves, and a standard mixture. Silica Gel 60 (0.25mm, without indicator). Chloroform with 0.3% ethanol.

Identification of the individual spots was made by comparison of Rf value with those of standards and reference mixtures in developing systems of different polarities. Literature data of Rf values were also taken into account (Holloway, 1984, Holloway and Challen, 1966, Tulloch, 1976).

Fraction 1. Acids: The lowest Rf values in low-to-medium polar systems are typical for free fatty acids. The exact position of free acid spots was verified on disappearance of those spots after treatment of the mixture with sodium methoxide.

Fraction 2. Sterols: This spot stained blue on treatment with vanillin reagent, but the reagent proved to be only semi-specific, as some other substances gave similar responses. The presence of hydroxy-β-diketones cannot be completely excluded as they are present in wheat surface waxes (Tulloch and Hoffman, 1973) and their Rf coincidces with the Rf of sterols in the given developing systems (see spots of Rf 8 in patterns of wheat and Eucalyptus samples in Fig 3). However a Fast Blue B reagent did not show a positive reaction to hydroxy-β-diketones.

Fractions 3 and 4: Primary and secondary alcohols: The spots of free primary and secondary alcohols stained blue after treatment with vanillin reagent. No standards of secondary alcohols with apropriate chain length were available from suppliers.

Fraction 5: Triglycerides: The spot of this fraction disappeared after treatment of the sample with sodium methoxide and the spot of fatty acid methyl esters appeared instead.

Fraction 6 and 7: Monesters and Hydrocarbons: No further verification was applied to these fractions as the fastest mobilities (highest Rf) are typical of monoesters and hydrocarbons in the devloping system used.

Semiquantitative assesment of TLC analyses was made by comparison of relative intensities of the spots of embryo waxes from three wheat varieties (Fig 3). Cv Holdfast (white, less sprout resistant) showed markedly lower concentration of primary and secondary alcohols in comparison to cv Yeoman and cv Koga II. The concentraton of sterols and hydrocarbons were also less in cv Holdfast. Koga II contained significantly higher amounts of triglycerides.

The most marked difference between the composition of wheat embryo waxes and green wheat epicuticular waxes is the absence of β-diketones in the embryo waxes (cf Rf - 59 in Fig 3). It is possible that the less stable β-diketones are present in freshly harvested grains but decompose on storage. Wheat embryo waxes contained relatively high proportions of triglycerides which are virtually absent in wheat leaf waxes. Possible contaimination of embryo cavity washings with triglycerides extracted from the embryo itself can be almost ruled out as the same spot appeared on the TLC patterns of manually collected, and hence pure, crystalline material. Briggs (1974) found triglycerides present on barley testa but arbitrarily attributed this finding to contamination.

Gas Chromatography

Examples of chromatograms obtained by GC analyses of embryo waxes from cv Yeoman and cv Holdfast before and after TMS derivatisation are shown in Figs 4-7. Approximate relative contributions of classes of compounds present are shown in Table 1.

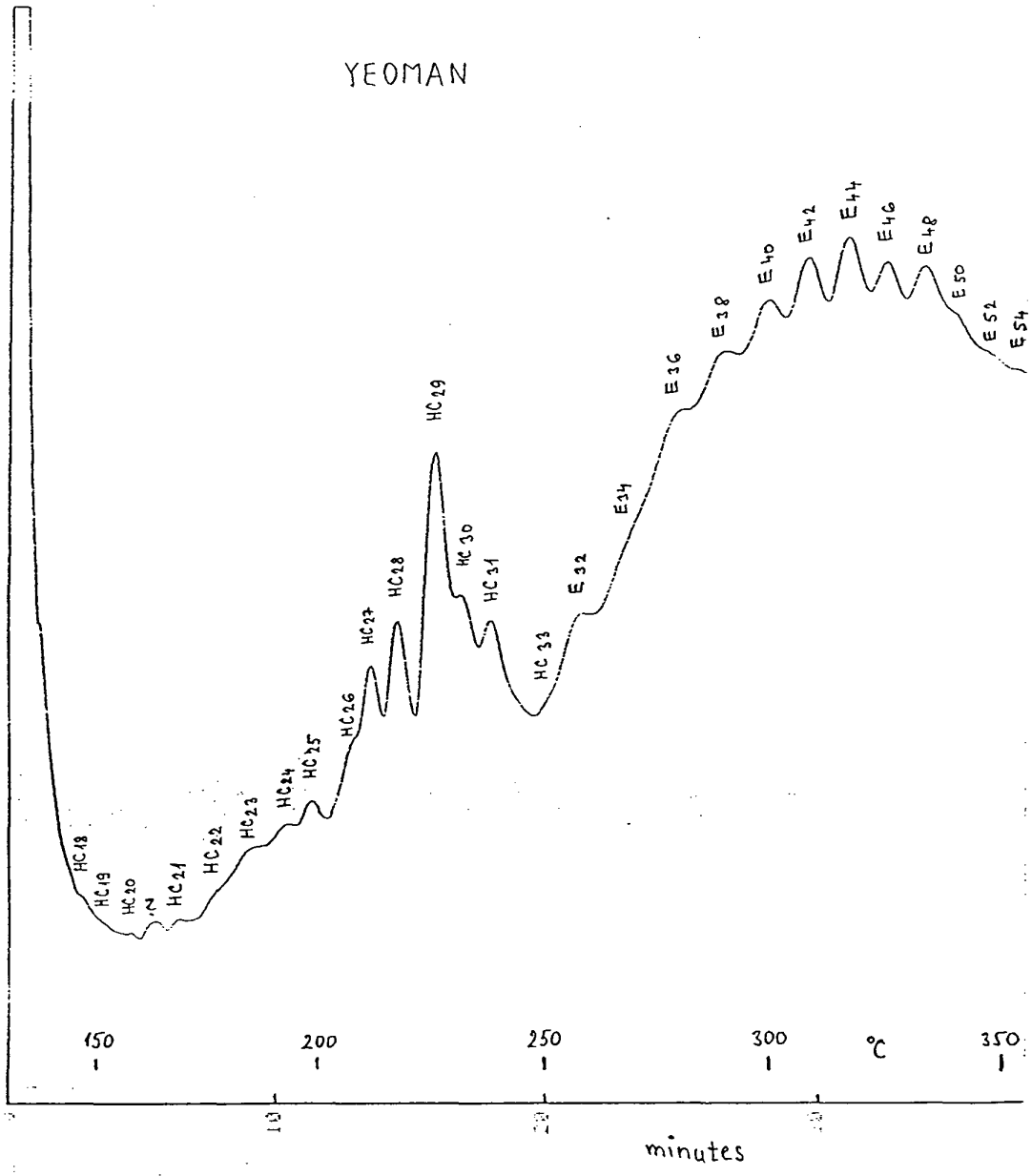

Fig. 4. GC chromatogram of underivatised embryo wax from variety Yeoman. HC = hydrocarbons, E = monoesters, numbers correspond to the carbon chain length. Column 1m x 3mm ID, 1% Dexsil 300 on Chromsorb WHP, t-program 6°C/min 130-370°C, N_2=25ml/min

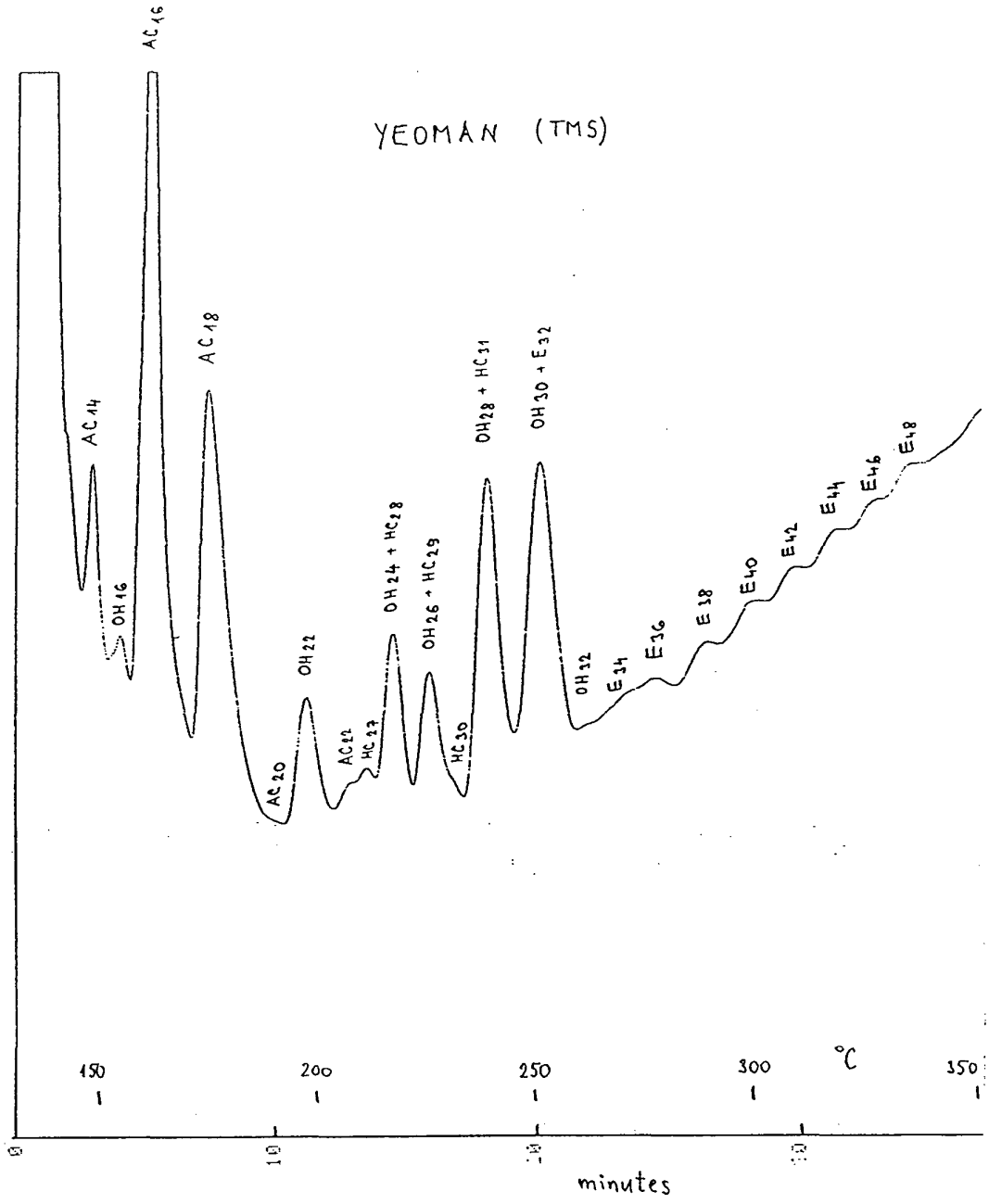

Fig. 5. GC chromatogram of TMS-derivatised embryo wax from variety Yeoman. AC = free fatty acids, OH = free primary alcohols, HC = Hydrocarbons, E = monoesters. Further see legend of Fig. 4.

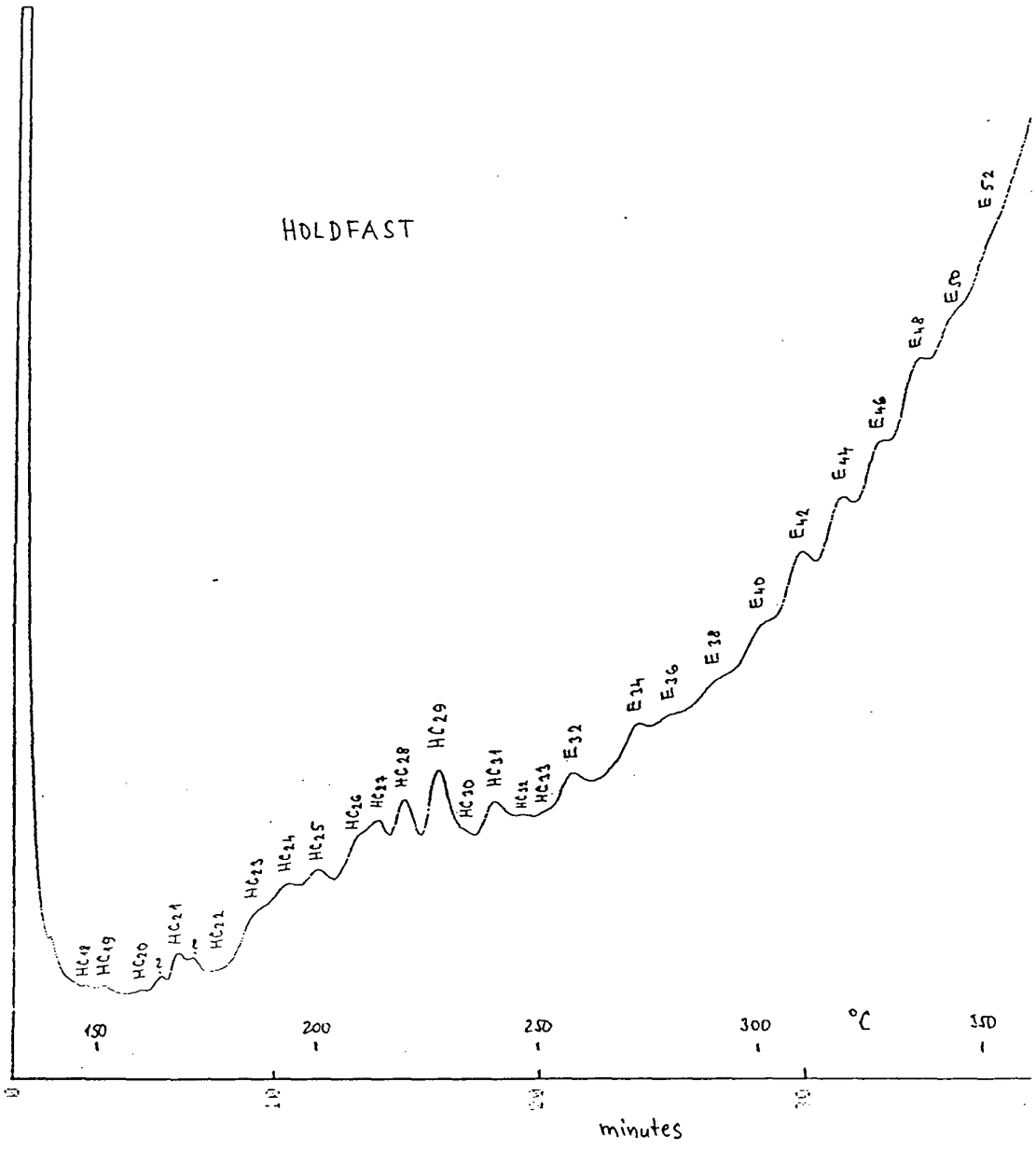

Fig. 6. GC chromatogram for underivatised embryo wax from variety Holdfast. Further see legend of Fig. 4.

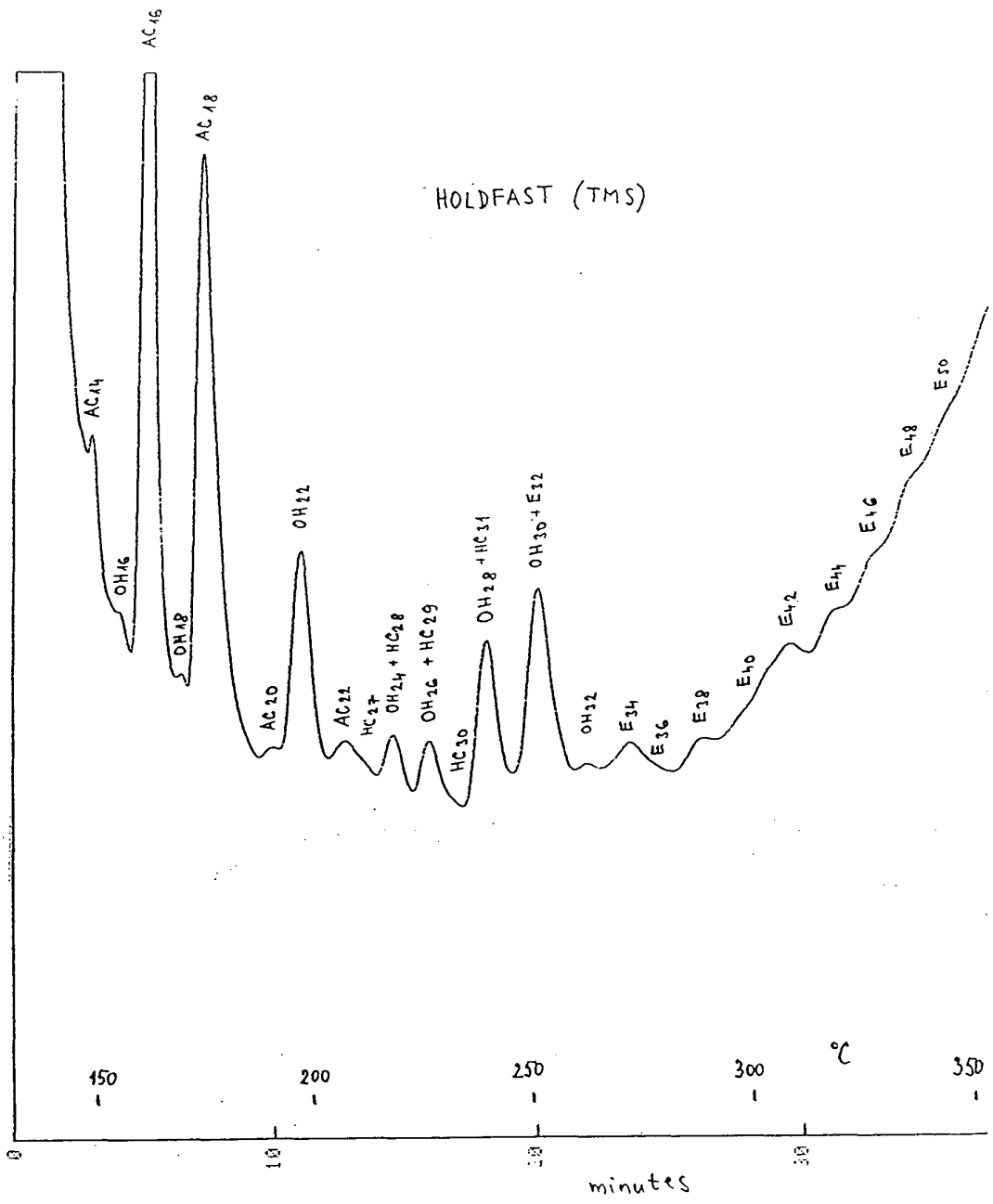

Fig. 7. GC chromatogram of TMS - derivatised embryo wax from variety Holdfast. Further see legend of Figs 4 and 5.

The identification of peaks was made on the basis of analyses of standard mixtures under the same conditions. Retention times of standard components were verified every day. The identification was made under the following presumptions: straight chain saturated hydrocarbons and straight-chain saturated even-carbon-number acids, primary alcohols and monoesters were taken into account. No attempt was made towards identification of any secondary alcohols possibly present, nor sterols, due to the lack of appropriate standards. Triglycerides did not show a response in underivatised or TMS-derivatised samples.

It is quite obvious that no more detailed qualitative results could be obtained with a packed column and FID detection on a complex mixture containing perhapos as many as 100 individual compoundcs. GC-MS with a capilliary column is the only method to solve this.

Under the above presumptions the hydrocarbons present in cv Yeoman embryo wax were found to be between C18-C33 with a maximum in the range C27-C54 with no significantly predominating compound.

Among the free fatty acids (Fig 5) the highly predominating species, palmitic and stearic acids contributed about 90% of the total free fatty acid content. Fatty acids ranged from C14-C22, although peaks of some higher-chain-length acids could be hidden under the large peaks of major compounds of alcohol and hydrocarbon fractions. Free alcohols ranged between C16-C32. Peaks of octadecanol C18 and eicosanol C20 are perhaps hidden under the large peaks of palmitic acid and stearic acid. Maximum contents were found for alcohols C28 and C30. It should be noted that free underivatised alcohols have some response on FID but this response is more than 20 times less than that of the same compounds after TMS treatment. Therefore no peaks of free alcohols could be detected in the underivatised samples (Figs 4, 6).

Comparison of the relative composition of the classes of compounds in embryo waxes, cv Yeoman and cv Holdfast (Table 1) did not show any major differences. However, the relative contributions of the classes were very different between the two varieties (cf chromatograms in Fig 4, 5 to 6, 7). The greatest difference was found in the hydrocarbon fraction. The ratio of hydrocarbon total peak areas in cv Yeoman to cv Holdfast was 3:1 and to primary alcohols 1.6:1. The embryo waxes of both varieties contained approximately the same amount of monoesters (1.2:1) and free fatty acids (1:1).

Force-area isotherms

The force-surface area curves of embryo waxes from the wheat varieties investigated appear to fall into two categories (Fig 8).

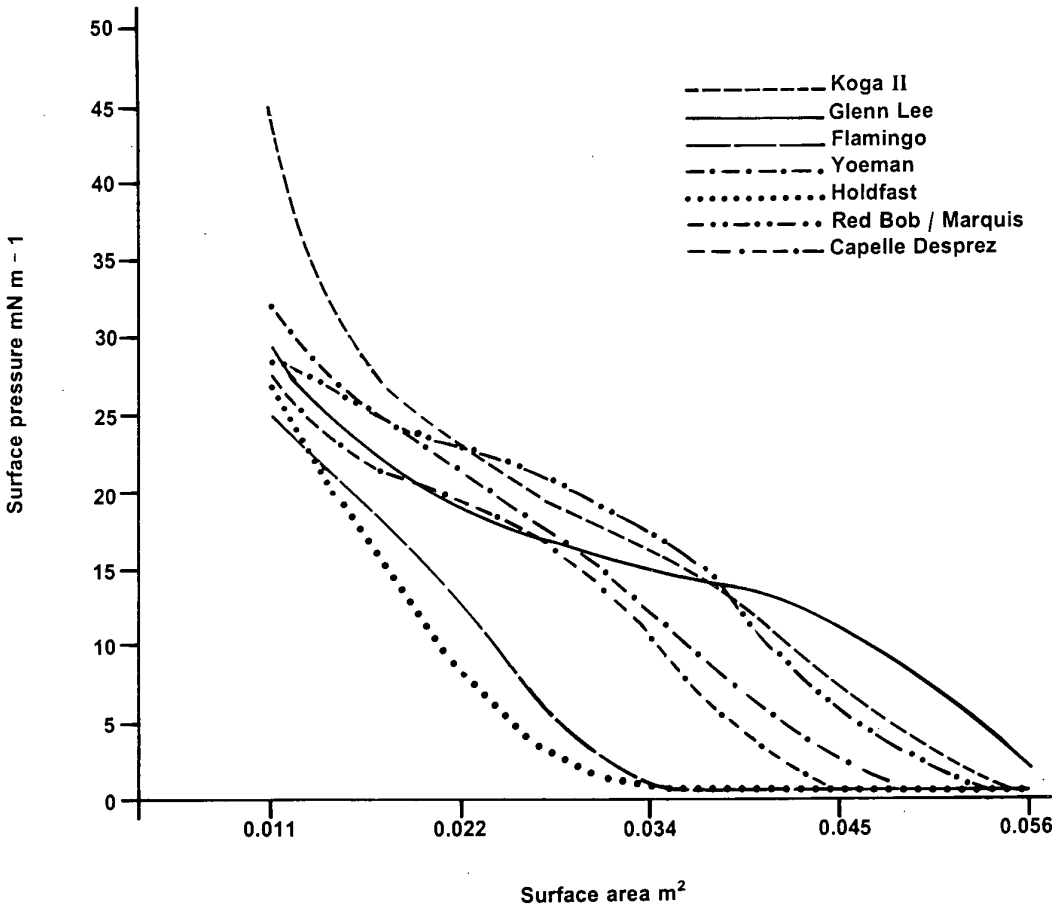

Fig 8. Force area isotherms of embryo waxes isolated from wheat varieties.

The two white varieties, Flamingo and Holdfast appear to form cohesive films at a much smaller surface area than the red wheat varieties. This suggests that the quantity of material extracted from the white wheats is lower than that collected form the red wheats. The characteristics of the curves from both red and white varieties are of the classification "liquid-expanded" (See Fig 9), suggesting that the hydrocarbon chains of the molecules are in a random rather than regular orientation, with only the polar groups being forced into contact with the subphase.

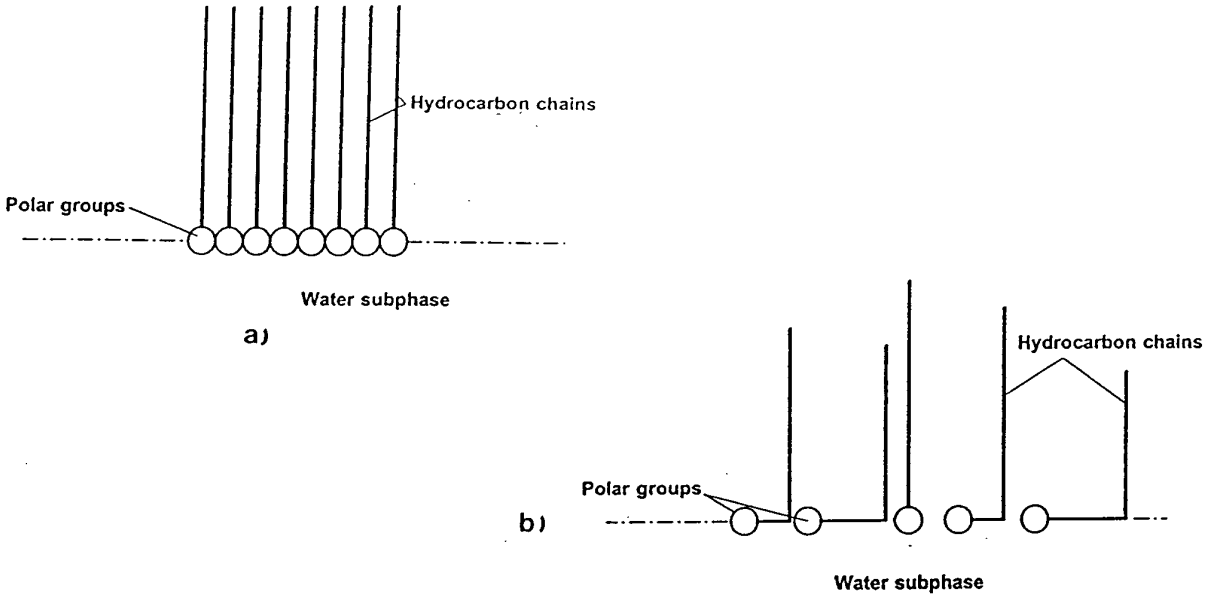

Fig 9. Schematic representation of a monolayer in a) liquid condensed state, and b) liquid expanded state.

In the case of Koga II there is additional evidence for the formation of a condensed type of monolayer at the smaller surface area. The molecules in this type of film are closely packed and steeply oriented towards the surface, the film being thought of as a two-dimensional crystal. The findings may be explained by higher content of triglycerides found in embryo wax of Koga II.

The physical state of the monolayer depends on lateral cohesive forces between the constituent molecules, lateral cohesion also depending on the geometry and orientaton of the film molecules. Factors such as bulky head groups, more than one polar group, bent chains and branched chains favour the formation of expanded films as these factors interfere with close packing of neighbouring molecules. This behaviour is also reflected in the bulk crystallisation properties of such compounds, branched/unsaturated materials crystallise with difficulty compared with saturated counterparts.

In general, the liquid-condensed type of film with its very close packing of hydrocarbon chain would be expected to form a better water repellant barrier than the liquid-expanded film where the hydrocarbon chains are randomly aligned (see Fig 9).

These interpretations apply to the film produced on a water surface. Hydrocarbons are known as the most hydrophobic among the compounds present in plant waxes (Holloway, 1970). In the solid state thee compounds form several monomolecular layers with chains placed perpendicularly on a given plant surface. Hence in cv Yeoman these compounds may protect the embryo against water penetration more effectively than in the case of cv. Holdfast. The value of the force area isotherms lies mainly in providing a means of detecting functional compounds in very small quantities of material.

Atomic Force Microscopy

The overall view of the surface feature observed by the AFM technique is shown in Fig 10.

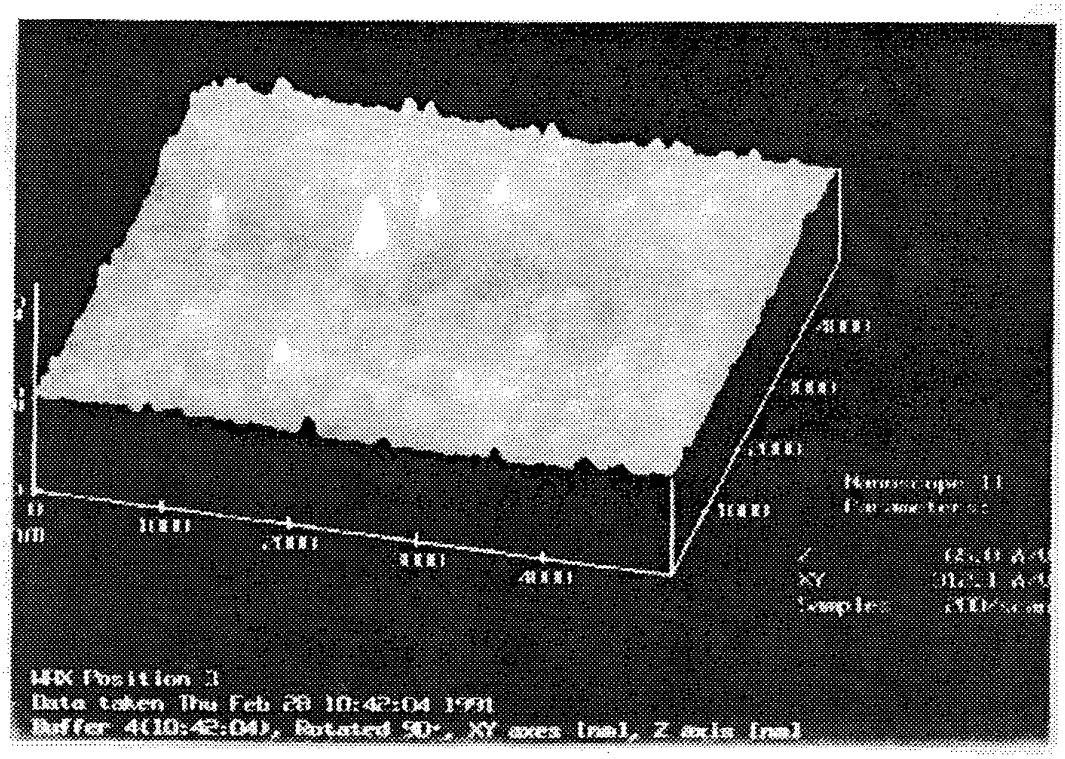

Fig 10. Atomic force microscopy pattern of the monolayer formed by embryo wax from cv Yeoman.

A large number of projections in the size range of 2-20nm are seen to be originating from the surface. A tentative explanation for the appearance of the surface features may be possible by considering the following argument. The material used for forming the film on the surface of water was originally present as crystals in the embryo cavity of wheat grains. On compression of the surface film the molecules are forced together to form a cohesive film. Previous studies have shown that the integrity of such films is retained on transfer from the surface of water to suitable solid substrates. On evaporation of water molecules associated with the polar head groups of the film-forming molecules, the closeness of the hydrocarbon chain may lead to initial crystallisation of the constituent molecules in the same manner the crystals form in the grain. The appearance of the numerous surface features may represent centres of crystallisation at various stages of growth. Further work is necessary to confirm this explanation, and the AFM technique needs to be explored further in this respect.

DISCUSSION

In the embryo cavity of wheat grain hydrophobic substances are present that are supposed to have a protective role against water and air penetration to the embryo. These substances may control the sprouting resistance of wheat grain by forming a solid hydrophobic film around the embryo cavity.

These substances are present in the embryo cavity in a crystalline and/or amorphous state. The visible crystals were discovered only in red cultivars stored for long periods. Two methods, i.e. manual collecting or washing, have been developed for isolation of these substances from wheat grain.

Chromatographic analyses (TLC, GC) showed that substances isolated from the wheat embryo cavity were of a waxy nature and their overall composition was to some extent similar to that of surface waxes of the wheat plant. Thin layer chromatography showed the presence of seven compound fractions in the embryo waxes. Four of these fractions were further characterised by gas chromatography.

The hydrocarbons ranged in chain length between C18-C 33 with a maximum in the range C27-C31. The monoesters ranged between C32-C54. In the free fatty acid fraction highly predominating were palmitic and stearic acids. Free primary alcohols ranged between C16-C32 with a maximum of octacosanol C28 and triacontanol C30. The remaining three fractions were characterised as sterols, secondary alcohols and triglycerides.

The analysis of waxes has been completed as far as this is possible with available techniques. Preparative TLC followed by capillary GC with mass-spectrometric detection has to be applied to completely solving the composition of the complex mixture that comprises perhaps as many as 100 individual compounds.

Comparison of three wheat varieties was made in chromatographic experiments. These were two red varieties Yeoman and Koga II with a higher sprouting resistance and white variety Holdfast with a lower sprouting resistance. The analyses showed a markedly higher content of hydrocarbons and free alcohols in the embryo waxes isolated from cv Yeoman and Koga II in comparison to cv Holdfast. This implies a higher amount and overall shift towards a higher hydrophobicity of embryo waxes isolated from higher sprout-resistant varieties.

These findings are supported by results of the surface pressure measurements. The curves of surface pressure vs surface area of embryo waxes from red wheats differed substantially from those of white wheats. The waxes from red wheats formed a cohesive film on a large area which implies that more surface active hydrophobic material was obtained form these samples.

All these findings support the original idea of a close relation between the amount and composition of waxy substances present in the embryo cavity and the sprouting resistance of wheat grain.

Of great importance for practical applications is the fact that sensitive and rapid methods are now available for characterising factors closely related to the sprouting resistance of wheat grain. The TLC and surface pressure measurements require material from 20 grains. For GC analysis 10 grains are sufficient and by using a capillary GC the sensitivity can be further increased. Moreover, all these tests are virtually nondestructive.

Further research on this project is envisaged to continue in two major directions. The first direction will be towards a more detailed analysis of the composition of embryo wax substances. The material (*circa* 1mg) obtained by manual collection of crystals from 1000 grains of cv Yeoman will be used for this purpose. The main steps of the procedure will be preparative TLC, chemical processing of isolated fractions (hydrolysis, derivitisation) and capillary GC with MS detection.

The second direction of research will include a targeted survey of wheat varieties with a sufficiently broad range of sprouting susceptibilities. Simpler methods of surface pressure measurements and TLC and/orGC will be used to find out an easily accessible factor closely related to sprouting resistance. A simple nondestructive test on small numbers of grains would be of great benefit to breeders for monitoring sprouting resistance at early stages of crossing.

CONCLUSIONS

The overriding problems associated with the work to date has been the relationship between the small amount of crystalline wax in grains and the limited stock of grains in which the crystals might occur. The conflicting pressures to extract relatively large quantities of material and to conserve stocks combined to slow down initial progress and they have continued to present difficulties almost to the present time. We are now confident that it is possible to extract by a simple solvent washing technique, material that is indistinguishable (by TLC) from that found in the crystals. The absence of obvious crystals no longer presents a barrier to valid comparisons among wheats of different propensity to sprouting.

The chromatographic analyses completed to date have demonstrated consistent differences between varieties with differing sprouting potential. These differences are totally consistent with surface properties of compounds washed from the embryo cavity, and the differences found between those in red and white wheats.
While it is not yet possible categorically to demonstrate a direct association between sprouting and the nature of the embryo wax, that which was a tenuous hypothesis has now gained a high level of credibility and has generated considerable interest among breeders and plant physiologists. The time has now come to pursue the hypothesis in conjunction with colleagues who have the experience and experimental material which will allow a systematic comparison of wax composition with sprouting behaviour and with redness. Completion of the analysis using sophisticated analytical techniques will inevitably provide a useful input into this programme but the simple tests like surface pressure measurements and/or TLC, GC will contribute a means of collecting rapid survey data.

ACKNOWLEDGEMENTS

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