DORMANCY IN WHEAT GRAIN

(*Triticum aestivum* L.)

Studies on Grain-coat Pigment Formation
and Abscisic Acid Content During the
Development of Wheat Grain of Six Genotypes

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ABSTRACT

Dormancy in wheat grain has been associated with red pigmentation of the grain-coats. The development from anthesis to harvest-ripeness of two-white-grained and four red-grained genotypes of varying dormancy was investigated. Grain growth was measured as changes in fresh weight and dry matter. Dehydration to harvest-ripeness (17.5% moisture) was calculated. The developmental rates of grain of the six genotypes were similar.

Dormancy-breaking germination tests showed that embryo maturity was attained at similar stages of development of four genotypes. It appeared to be somewhat delayed in two red-grained genotypes, which also had the lowest germination rates in standard germination tests. Dormancy was estimated as the percentage of grains with mature embryos, which did not germinate in the standard germination tests. Grain of all the genotypes had a period of dormancy during development. However, in white-grained genotypes it had disappeared before harvest-ripeness was attained and it lasted only a little longer in one of the red-grained genotypes. In the other three red-grained genotypes, dormancy was prolonged for at least several weeks beyond harvest-ripeness.

The concentrations of flavonoid precursors were similar in grains of all six genotypes throughout their development. Assays of crude extracts of a group of enzymes (phenolases) involved in pigment synthesis did not reveal peaks of activity associated with the appearance of mature grain-coat colour. Successive extractions of the grains showed that the pigment was probably a large flavonoid polymer. The amounts of endogenous abscisic acid in developing grains was analysed by high pressure liquid chromatography. Significant quantities of the 2-trans isomer, as well as of the common 2-cis isomer (abscisic acid) were found. The amounts did not appear to be related to either dormancy or to maturation and dehydration of the grain, as had been suggested.

The mechanisms prolonging dormancy beyond harvest-ripeness in wheat grain were discussed with reference to pigmentation. It was considered that dormancy of the red-grained wheats was probably due to impermeability of the grain-coat to oxygen, possibly resulting from molecular properties of the pigment. These properties were the ability to absorb oxygen, which might prevent it reaching the embryo, and the ability to complex with the large proteins of the immature testa, which might prevent their degradation during grain development. During imbibition the complexed proteins might swell to create a physical barrier to oxygen permeation.

CHAPTER 1

DORMANCY AND SPROUTING DAMAGE IN WHEAT

Sprouting damage in wheat may be a problem when it is grown in cool maritime climates (Mackey, 1976), where wet weather may cause unharvested grain to begin germinating. The embryo of an imbibed grain may start to synthesise proteins and gibberellins, which are translocated to the aleurone layer of the endosperm where they stimulate enzyme activity (Belderok, 1968). The enzymes activated and synthesised during early germination include proteolytic ones, starch phosphorylases and hemicellulases (Ching, 1972; Kruger and Preston, 1976). They are probably the initators of degradation in the starchy endosperm, where starch granules are embedded in a protein matrix (Bradbury *et al*, 1956a; 1956b; Simmonds. 1972; Fincher and Stone. 1974; Gordon, 1977).

Later amylases are synthesised (Kruger. 1976) and hydrolyse the exposed starches. Different amylases are present in the pericarps of developing grain, but usually disappear as the grain matures (Kruger. 1976, Olered. 1976, Daussant and Renard. 1976). Flour milled from grain with high amylase activity, due to germinative processes or to high residual levels in the pericarp. may be much reduced in bread-making quality (Kent-Jones and Amos. 1967; Olered. 1976; Gale. 1976).

Dormant grain, which fails to germinate in optimal conditions of moisture, temperature and light, is resistant to germinative sprouting damage. However, the mechanisms in wheat, which control the duration of the dormant period and the onset of germinability, are unknown (Kruger, 1976). For convenience, grain dormancy can be considered to be imposed primarily by limiting factors in either the embryo, the endosperm or the grain-coat. Coordination of the processes in these tissues ultimately determines whether the embryo actually does sprout.

Embryos are immature during early grain development and are therefore incapable of germinating even in conditions which would release dormancy-imposing mechanisms. If the period of immaturity was sufficiently long, it could result in apparent dormancy at harvest-ripeness. However, in both dormant and non-dormant wheat varieties, embryos reach maturity in similar lengths of time, as shown by dormancy-breaking tests on whole grain (Gordon. 1975) and germination of excised

embryos (Miyamoto *et al*, 1961). Wheat embryos are mature some time before the grain reaches harvest-ripeness (Robertson and Curtis, 1967; King, 1976), although the period of immaturity is relatively longer in cooler environments (Cross, 1977). Embryo growth in cereals begins with the release or synthesis of gibberellins within the embryo (Belderok, 1976). Blockage of this process, which would prevent germination, probably does not occur in wheat. Grain of some dwarf wheats, which do not show the normal aleurone response to gibberellin, were shown to release as much gibberellin as those of normal varieties (Gale and Marshall, 1973; 1975).

The endosperm may be the limiting factor, if it does not respond to stimuli from the embryo, in which case the stored reserves necessary for embryo growth are not made available. Lack of response to gibberellin by the aleurone layer of the endosperm has been found in several different dwarf wheats (Gale and Marshall, 1975; Gale, 1976; Bhatt *et al*, 1976). Their insensitivity to gibberellin is controlled by one gene (Gale *et al*, 1975a), whose effects are synergised by certain other genes, which produce sub-threshold levels of inhibitors but do not inhibit alpha-anylase synthesis (Gale, 1976).

The grain-coat tissues are involved in imposing dormancy in wheat. Rupturing the grain-coat breaks dormancy (Wellington, 1956b; Belderok, 1968; Gordon, 1970; Lancaster and Wright, 1970), but the grain-coat effect is not due to mechanical toughness (Miyamoto *et al*, 1961; Sander, 1965; Belderok, 1968). It may be due to inadequate permeability to oxygen, because dormancy can be broken by facilitating the access of oxygen to the embryo by either removing or damaging the outer layers or by adding hydrogen peroxide to the imbibing solution, (Belderok, 1976). Dry mature seed-coats of many species are completely permeable to gases, but the imbibed seed-coats of dormant seeds may be impermeable to oxygen until some time after harvest-ripeness (Côme, 1973). In wheat, dormancy may persist until the grain-coat has become more permeable, so that sufficient oxygen can reach the embryo to supply the demands of both respiration and growth (Belderok, 1976). However, no direct measurements of the oxygen permeability of the grain-coat of wheat have yet been made (Belderok, 1976).

The colour of the grain-coat is associated with dormancy in wheat (Nilssohn-Ehle, 1914; Gfeller and Svejda, 1960; Belderok, 1968). White-grained varieties are not dormant, while red-grained ones show

varying degrees of dormancy (e.g. Nilssohn-Ehle, 1914; Wellington and Durham, 1958; Gfeller and Svejda, 1960; Belderok, 1968; Derera *et al*, 1976; Freed *et al*, 1976; La Croix *et al*, 1976). Although there are three independent loci for red grain-coat colour in wheat (e.g. Nilssohn-Ehle, 1914; Gfeller and Svejda, 1960; Kimber, 1971; Freed *et al*, 1976), their numbers in a genotype are not directly related to its dormancy.

Causes of the tendency for grain redness and dormancy to be associated are unknown. Activity of the oxidative enzymes in synthesising the pigment or presence of the pigment itself (Belderok, 1976), may result in a shortage of oxygen in the grain. While the deficit persists, metabolic pathways may be blocked or altered, and respiration may occur via the pentose-phosphate-pathway rather than by glycolysis. (Roberts, 1973). Although energy is produced less efficiently through the pentose-phosphate-pathway, the supply may be sufficient for the early stages of germination (Ching, 1972).

Some precursors of the pericarp pigment, the flavon-3-ols, may be inhibitory to germination, (Miyamoto and Everson, 1958; Miyamoto, *et al*, 1961). Germination tests of excised wheat embryos, from both dormant and non-dormant varieties at various stages after harvest-ripeness, showed that the dormant period was lengthened in the presence of flavan-3-ol (Stoy and Sundin, 1976). The effect was nearly eliminated when gibberellin was also present in the germination medium (Stoy and Sundin, 1976). However, both red and white wheat-grains have relatively high levels of flavanols (Gordon, 1975). Also imbibition of aged red and white grains, in which residual dormancy had disappeared, in flavan-3-ol solutions did not interfere with their germination (Gordon, 1975). The relationships among the flavonols, the red pigment and the enzymes synthesising it, and the association with dormancy require further investigation (Gordon, 1975; Kruger, 1976).

The growth processes of the various tissues, during grain growth, development and germination are probably co-ordinated by interactions of hormone effects (Wareing and Saunders, 1971; Mayer and Shain, 1974; Khan, 1975). Abscisic acid is an inhibitor of germination and its effects may be antagonised by cytokinins (Khan, 1979). Gibberellins promote the breaking of dormancy, if abscisic acid is not present or if cytokinins are also present (Khan, 1975). Indole acetic acid may be required for some germinative processes (Palmiano and Juliano, 1973; Laidman *et al*, 1974; Gaspar *et al*, 1977).

The aim of this study was to examine grain development in wheat, with respect to pigmentation, and relate it to the onset of dormancy. Two white- and four red-grained varieties, of differing dormancy, were used. Grain growth was measured, in terms of changes in fresh weight and dry matter, and dehydration to harvest-ripeness was calculated. Embryo maturity and grain germinability were measured, and used to estimate the duration of dormancy. Concentration of the red-pigment precursors, activity of the enzymes involved and the time when the pigment appeared, were also determined. The level of endogenous abscisic acid during grain development was analysed, to see if it was related to dormancy.

CHAPTER 2.

DEVELOPMENT OF THE WHEAT GRAIN

2.1 Morphological changes during development

2.1.1. Introduction

The development of the wheat caryopsis has been described at length (Percival, 1921). Its structure and the composition of its mature tissues have been detailed (Pomeranz, 1971). Typically, it is 8-9% pericarp, 6-9% aleurone, 75-84% starchy endosperm and 2-3.5% embryo (Pomeranz, 1971). Maternal tissues form the pericarp, which develops first followed by successive cycles of growth of embryo and endosperm. The latter receive substrates from maternal tissues (Rijven and Cohen, 1961) via the vascular tissue of the rachilla and the grain's pigment strand (Bradbury *et al*,1956). Grain growth declines at the same time as vascular tissue becomes blocked (Zee and O'Brien, 1970), but may be due to reduced starch synthesising capacity rather than blockage of substrate supplies (Jenner and Rathjen, 1975).

The parental genotype and its environment influence grain development. Removal of various parts of the plant during grain development indicated that growth substances in the ear, which included awns and glumes, may be adequate for normal grain growth (Wheeler, 1976). Auxins and cytokinins produced by the grain itself apparently control its growth. Gibberellins appear to be associated with the number of grains formed and auxins with their weight (Bhardwaj and Dua, 1975). At maturity, the levels of the growth substances are low (Wheeler, 1972).

2.1.2. Embryo development

Soon after anthesis and pollination,the double fertilization occurs to form a diploid zygote and triploid primary endosperm nucleus. A period of rapid cell division of the zygote follows as it grows into a club-shaped embryo. Its distal end begins to differentiate, eventually becoming the scutellum and storing oil and protein. The coleoptile is initiated and the radical begins to differentiate at about the same time. The first foliage leaf develops, the base of the scutellum elongates and the epithelial cell layer is laid down (Percival, 1921). Further development and growth of embryonic tissues results in a mature embryo usually by the time the grain reaches 40% moisture, which is 20-50 days

after anthesis depending mainly on the environment, especially temperature (Cross, 1977). As the grain is not harvested with a moisture content above 20%, the embryo is usually mature at harvest-ripeness.

2.1.3. Endosperm Development

After fertilization, the primary endosperm nucelus immediately begins to divide rapidly forming many free nuclei within the embryo sac. When the embryo comprises 10-15 cells (8-10 days after anthesis), a single continuous layer of endosperm cells line the embryo sac. Then cell walls are formed around the remaining free nuclei in the rest of the cavity, starting from the inner region (Percival, 1921). Starch deposition begins 10-14 days after fertilization when the endosperm is completely cellular. Accumulation of starch continues steadily through grain ripening, until the protoplasm of the starchy endosperm cells becomes disorganised during grain dehydration. (Kapoor and Heiner, 1976). In the mature dehydrated grain, starch and protein completely fill the cells (Percival 1921). The outer-most layer of the endosperm differentiates as the aleurone, which is morphologically mature 1-2 weeks before starch synthesis ceases (King, 1976).

During germination, the aleurone produces enzymes, including alpha-amylase, in response to gibberellins from the embryo (Radley, 1976). These are secreted into the starchy endosperm where the protein matrix, the cell walls and the starch granules are progressively degraded, starting from the outer regions, while the aleurone layer remains adhering to the grain-coat (Fincher and Stone, 1974).

2.1.4. Development of maternal grain tissues

The antipodals and nucellar cells become disorganised and their contents are absorbed; only traces of them remain outside the mature aleurone. The outer integumentary layer begins to degenerate soon after fertilization and the cuticular layer between it and the inner epidermis of the ovary wall degenerates (Morrison, 1975). The inner integument grows until the grain reaches its maximum size (at about 45% moisture).

It becomes crushed during the final stages of ripening, forming the seed testa with its inner and outer cuticles and central "colour" layer containing the red pigment (Percival, 1921). In a white-grained and in a red-grained non dormant variety, scanning electron micrographs clearly showed that the colour layer and outer cuticle of the testa

consisted of dense homogenous material, in the week before harvest ripeness was attained. A week after maturity, the layers were broken down to a loose granular structure (Belderok, 1976). However, in the unripe grains of a red-grained dormant variety, the layers were thick and denser and became fused as harvest-ripeness was reached. It is not known whether this presumably proteinaceous material broke down during after-ripening (Belderok, 1976).

At fertilization, the ovary wall had parenchymatous tissue between an outer and an inner epidermis. As the grain developed, these layers differentiated and eventually became the outer epidermis, hypoderm, remnants of thin-walled cells, intermediate, cross and tube cell layers of the mature pericarp (Percival,1921). The pericarp was the predominant grain tissue during the first 6-8 days and had high levels of activity of some enzymes e.g. glutamyl transferase, proteolytic enzymes and alpha-amylase. (Rijven and Banbury,1960; Rijven and Cohen,1961; Kruger,1976). It began to senesce about 35 days after anthesis, with its cells becoming increasingly disorganised (Wellington,1956a). Its alpha-amylase activity generally fell to low levels during maturation (Kruger, 1976).

After germination, white grains were observed to have wrinkled grain-coats while those of red grains were smooth (Wellington, 1956). During imbibition, swelling in white grains would initially only remove the wrinkles, but the increasing tension in red grain-coats might limit embryo expansion (Wellington, 1956). The rupture of the grain-coats also occurred differently. In white grain-coats, a longitudinal split simultaneously exposed both coleoptile and coleorhiza; whereas the split in red grain-coats occurred at the base of the embryo and the coleorhiza emerged first (Wellington, 1956). However, Miyamoto *et al*, 1961 observed normal longitudinal breaks in red grain-coats even when the embryo with the overlying grain-coat attached, was excised before being germinated.

2.2 Dormancy and Grain-coat Colour

2.2.1. Grain-coat pigments

The grain-coat pigments of wheat have not been fully identified, although they are known to be flavonoid. The colour in stems, glumes, coleoptiles and grain may be due to anthocyanins, which are widely distributed, especially in the outer epidermal cells of the ears (Lewicki, 1929) and in the grain-coat (Bailey, 1944). Two different anthocyanins have been identified in the pericarps of unusual purple-grained wheats (Gale and Flavell 1971, Dedio *et al*, 1972) where they

would be in cross-layer and parenchyma cells (Percival, 1921). The pigment of the red grain-coats was generally considered to be a flavonoid polymer or phlobaphene (Miyamoto and Everson, 1958; Pomeranz, 1971; Freed, 1972; Gordon, 1975; Cross, 1977).

Flavonoid polymers are known as phlobaphenes, condensed tannins, flavolans or procyanidins. They are the condensation products of flavan-3-ols, flavon-3,4-diols and possibly anthocyanins (Ribéreau-Gayon, 1972). Brown and black phlobaphenes were obtained by heating pyrogallol extracted from brown-red wheat glumes, or catechol for white and black glumes, in the presence of sulphuric acid and oxygen, which lead to the suggestion that pigmentation depended on the presence of phenolics and oxidases (Lewicki, 1929). A positive correlation between the levels of flavon-3-ol, in the pericarps of grain at 40% moisture, and pigmentation of the mature grain was used as the basis for suggesting that the pigment was a condensed tannin (Miyamoto and Everson, 1958). In whole grain, the differences in flavanol levels, throughout the development of red- and white-grained genotypes, were not associated with grain-coat colour. Also, the pattern of change in the flavanol levels was the same for both the red- and white-grained genotypes examined (Gordon, 1975; Cross, 1977).

In sorghum, the seed-coat of the grain contained condensed tannins (Haslam, 1977). The grain developed initially in a sheath and the seed-coat contained no flavan-3-ols or related molecules during that etiolated stage. As chlorophyll formed in the seed-coat, flavan-3-ols and higher oligomers were rapidly synthesised. The level of condensed tannins remained high and constant until the distinctive reddish pigmentation appeared during grain-ripening. The major anthocyanin causing the coloration was luteolinidin. At this stage, a decrease in the levels of flavan-3-ol and its associated co-metabolites and an increase in very highly polymerised condensed tannins occurred (Haslam, 1977). In rice, flavan-3-ols and condensed tannins were present in the pericarp and testa of red grain, to a lesser extent in brown grain and not in white grain (Nagao *et al*, 1956).

2.2.2. Phenolic acids and flavonoids

2.2.2.1. Biosynthesis and structures

Anthocyanins, flavan-3-ols and flavan-3-diols all have a

C_6-C_3-C_6 flavonoid structure. The B ring and the 3 carbon atoms of the heterocyclic ring originate from phenolic acids synthesised via the shikimic acid pathway (Fig. 2.1A). The A ring of the flavonoids is derived from acetate or malonate (Fig. 2.1B) (Ribereau-Gayon, 1972; Wong, 1973; Stafford, 1974b; Walker, 1975). The mechanism of synthesis of different phenolic acids may vary and the flavonoids may not be derived directly from one another or by only the pathways shown in figure 2.1B (Ribereau-Gayon, 1972; Wong, 1973; Sutfield and Weirman 1976; Haslam,1977.) The dihydroflavonone may be reduced to a flavon-3-4-diol, then dehydroxylated to a key intermediate, flav-3-en-3-ol, which lacks an oxygen at C4 of the heterocyclic ring and has a double band between C3 and C4. Reduction of it would give a flavan-3-ol and oxidation would yield an anthocyanidin (Haslam, 1977). Secondary modification by hydroxylation methylation and glycosylation is common, but the stage at which such modifications occur is unknown.

2.2.2.2 In wheat grain

Many phenolic acids have been found in wheat grain. In endosperm, they included benzoic acids (vanillic, syringic, P-hydroxy-benzoic and salicylic), cinnamic acids (P-coumaric, ferulic, sinapic and isoferulic) as well as chlorogenic acid, o-coumaric acid and the coumarin, scopoletin (El-Basyouni and Towers, 1964; Lorenz and Maga, 1975). The phenolic acid content of ground endosperm did not vary much among wheat and triticale and was 1-40 ppm in mature grain (Gallus and Jennings, 1971; Lorenz and Maga, 1975). Aleurone cell-walls contained a ferulic acid-carbohydrate complex, which was lost in a specific pattern during germination (Fulcher *et al*, 1972).

Wheat embryos contained ferulic and vanillic acids and two glyoflavones (King, 1962). The pericarps of purple-grained wheats contained the anthocyanins cyanidin and peonidin, their glycosides, rutinosides and two acylated forms (Dedio *et al*, 1972.) The pericarps of red-grained wheats may contain phlobaphenes (Miyamoto and Everson, 1958) as discussed in section 2.2.1. Lignin, a complex type of polymer condensed from various phenolic acids (Harkin, 1973) occurred in pericarp walls of mature grain (Bradbury, McMasters and Cull, 1956). In whole grain, high levels of flavanols were reported, particularly during their early stages of development (Gordon, 1975; Cross, 1977).

2.2.3. Functions of phenolics

2.2.3.1. As inhibitors

In addition to their functions as aromatic amino acids and pigment and lignin precursors, phenolic compounds have been increasingly recognised as modifiers of many plant processes. Generally, they have been inhibitory; e.g. salicylic, p-hydroxybenzoic, p-coumaric, caffeic, ferulic and chlorogenic acids were active inhibitors of some growth processes; salicylic acid inhibited alpha-amylase; scopoletin and ferulic acids inhibited germination and several flavanones inhibited wheat coleoptile growth (Kefeli and Kadyrov, 1971; Gross, 1975). Flavonoids affected bioenergetic reactions by inhibiting the synthesis of Adenosine triphosphate (ATP) and gave a general uncoupling affect (Popovici and Reznik, 1976).

FIGURE 2.1A

BIOSYNTHESIS OF PHENOLICS & FLAVONOIDS.
FLAVONOID "B"-RING FORMATION.

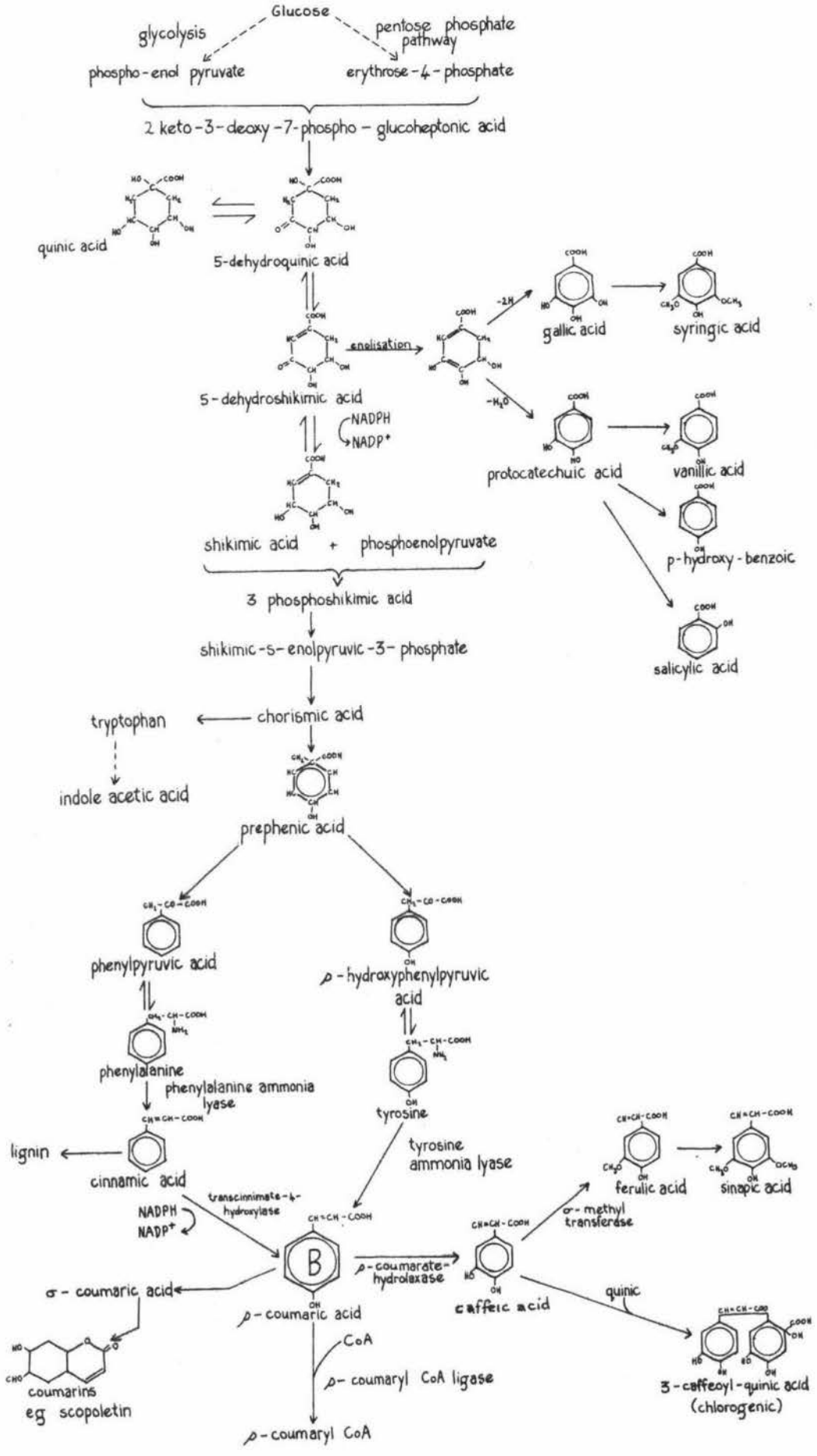
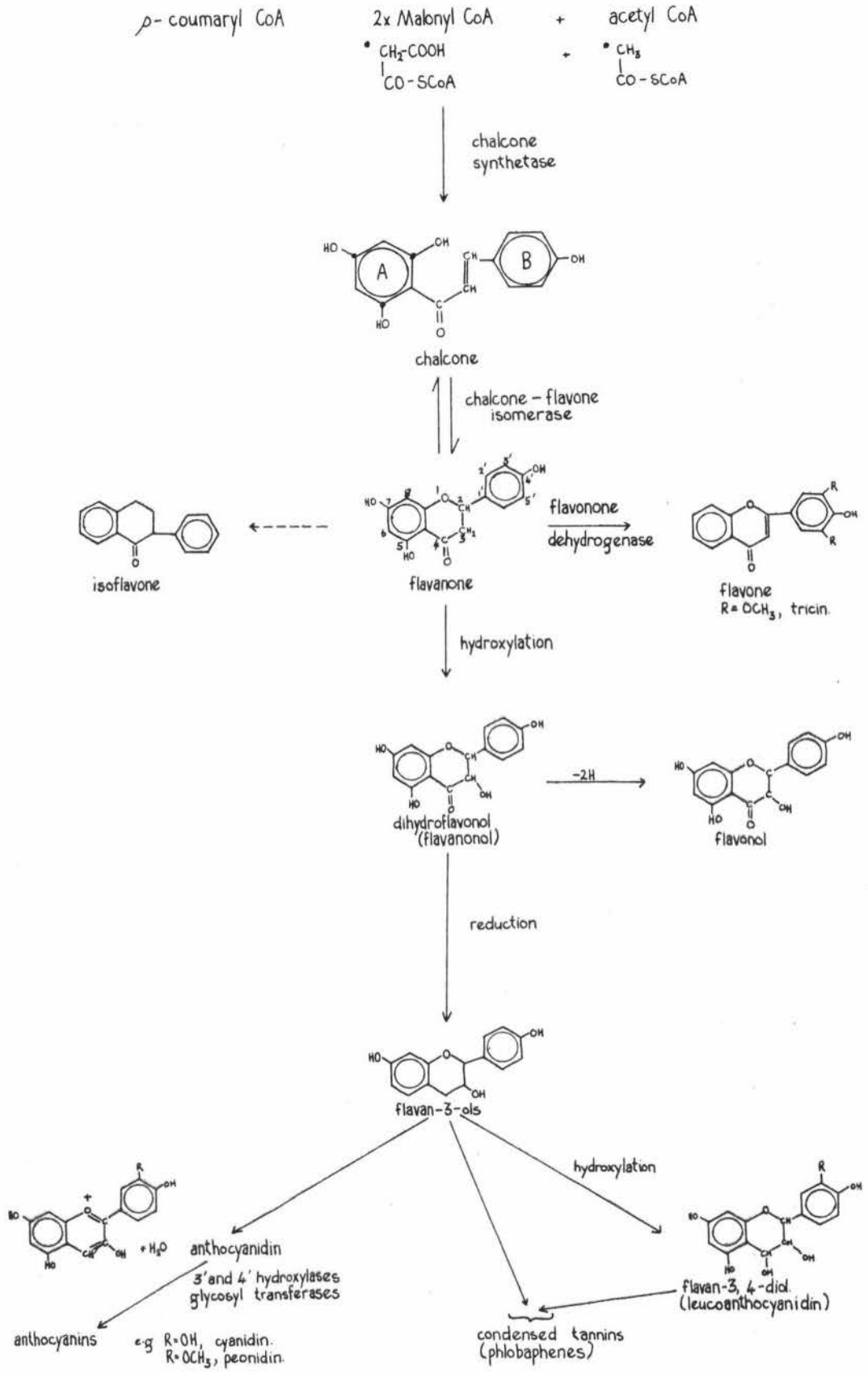


FIGURE 2.18

FLAVONOID "A" - RING FORMATION.



Phenolic acids have a generalised inhibitory effect on active ion uptake, probably due to reversible alterations in membrane permeability (Glass, 1974). The reconstitution of membranes is an important process in germination (Laidman *et al*, 1974; Ching, 1972), because membranes lose their integrity during dehydration of seed and solutes could leak out during early inhibition (Simon and Harum, 1972). The ferulic acid-carbohydrate complexes of aleurone cell-walls might make them relatively resistant to the hydrolases, which digest the starch endosperm, and possibly make them impermeable to water (Fulcher *et al*, 1972). Cellulase activity would release the ferulic acid, which is a germination inhibitor (Taylorson and Hendricks, 1977).

2.2.3.2. Phenolics and respiratory metabolism

Enzymes of pathways for catabolysing sugars may be partially controlled by phenolics. Tests with many phenolics showed that scopoletin and chlorogenic acid and, to a lesser extent, caffeic and ferulic acids might regulate the activity of glucose-6-phosphate dehydrogenase (G6PDH) isozymes (Hoover *et al*, 1977). G6 DPH:$NADP^+$ -1-oxidoreductase (EC 1.1.1.49) is the first enzyme in the pentose phosphate pathway and, together with phosphogluconate dehydrogenase, may control it (Hoover *et al*, 1977). Phospho-enol pyruvate from the glycolytic pathway and erythrose-4-phosphate from the pentose phosphate pathway are the initial substrates for the shikimic acid pathway of phenolic biosynthesis (Fig. 2.1.A). The nicotinamide adenine dinucleotide cofactor $NADPH_2$, which supplies much of the reducing power for the synthesis of phenolic acids, might come from the pentose-phosphate pathway (Pryke and Rees, 1977). Oxidation of $NADPH_2$, which the shikimic acid pathway could do, immediately stimulated activity of the pentose-phosphate pathway (Ishikawa *et al*, 1977). The supply of NADP was a rate-limiting factor for the pentose phosphate pathway and activity of G6PDH was repressed by $NADPH_2$ (Ishikawa *et al*, 1977) and by ATP (Taylorson and Hendricks, 1977).

The glycolytic and pentose-phosphate pathways are regulated by the availability of substrates, and enzymes as well as by the cofactors NAD and NADP. The concentrations of these cofactors are rapidly affected by light, oxygen, low temperature, auxin and kinetin (Yamamoto, 1969). Under anaerobic conditions, the level of NADP is high, which would stimulate

activity of the pentose phosphate pathway. Under aerobic conditions, it is low and respiration would probably be via the glycolytic pathway which is the more efficient energy producer. The levels of NADP may regulate the relative activity of the two pathways as NADP enzymes occur at many branch points (Tekzuka and Yamamoto, 1970). The respiratory Pathways and the shikimic acid pathway are inter-related through their products, substrates and cofactor requirements. It is possible that the regulatory mechanisms for each pathway are coordinated. During germination of wheat embryos, both the respiratory pathways were active (Ishaikawa *et al*, 1976), but it is not known if the shikimic acid pathway is active at that stage.

2.2.3.3. Phenolics and hormones

Phenolic compounds also interact with some of the plant hormones. The hydrolysable tannins, chebulinic acid and tara-tannin, and some phenolic acids including chlorogenic, caffeic, ferulic, cinnamic and coumaric acids were specific antagonists of gibberellin-induced growth in pea and cucumber seedlings (Corcoran *et al*, 1972). The hydrolysable tannins affected only the extra growth induced by exogenously applied gibberelin; they did not affect growth of untreated controls nor growth induced by exogenous applications of auxin (Corcoran *et al*, 1972). They also inhibited the synthesis of gibberellin-induced enzymes in barley grains, but did not block secretion of the enzymes nor inhibit existant enzymes (Jacobsen and Corcoran, 1977). Chebulinic acid and tara-tannin may act on the gibberellin receptor proteins, which were found in wheat aleurone (Jelsema *et al*, 1975), or directly on the gibberellin molecule (Green and Corcoran, 1975). The action of the hydrolysable tannins was specifically in the inhibition of gibberellin-requiring processes (Jacobsen and Corcoran 1977).

The metabolism of the hormone indole acetic acid (IAA) is also affected by phenolics. Mono-phenols, which have a single hydroxyl group, are cofactors of IAA oxidase, while diphenols and polyphenols are inhibitors (Andreae and Collet, 1967; Stafford, 1974a, Wolf *et al*, 1976). The presence of high concentrations of inhibitors, such as ferulic acid, in actively growing tissues, suggested that they may promote growth by preventing the oxidation of IAA (Gelinas, 1973). Phenolic acids cause a lag phase in the oxidation of IAA and they may be oxidised during the delay. It was suggested that phenolics may provide the fine-tuning

control on IAA-induced growth (Wolf *et al*, 1976).

Oxidation of diphenols produces σ-quinones, which may interact with disulphide and thiol groups to regulate the redox potential to a level favourable to growth (Wolf *et al*, 1976). There are three intrachain disulphide groups and no thiol groups in the single polypeptide chain of IAA oxidase (Stafford, 1974). Interaction of these with diphenols or σ-quinones could directly inhibit the enzymes' activity, resulting in decreased oxidation of IAA. The protein-bound thiol and disulphide groups in the inner grain-coat layers of wheat disappear during late maturation (Belderok, 1976).

2.2.4. Enzymes of Pericarp Pigment Biosynthesis

2.2.4.1. Introduction

Oxidative mechanisms are involved in the synthesis of flavonoids and condensed tannins. Phenolases and peroxidases oxidise a large group of substrates including monophenols, diphenols and complex molecules containing phenolic groups. Oxidation products include the phlobaphenes, which may be the dark pigments of seed-coats and outer tissues of higher plants (Stafford, 1974). There may be several phenolase isozymes and several peroxidase isozymes in the same tissue. The isozymes may vary in their substrate affinities and occur during different developmental periods.

Some evidence showed that peroxidase, IAA oxidase and phenolase activities were due to isozymes of one enzyme group (Srinvasta, 1973; Schneider and Wightman, 1974; Srivasta and van Huyslee, 1977a,b). However, it seems more generally accepted that the phenol oxidases are a different group from the IAA oxidases and peroxidases (Stafford, 1974; Shinshi and Noguchi, 1975; Anon, 1976). IAA oxidase activity is apparently due solely to peroxidase (Gelinas, 1973), although not all peroxidase isozymes show such activity (Schneider and Wightman, 1974).

2.2.4.2. Phenolases

1. Classification and Reactions

Enzymes with the major function of catalysing the oxidation of phenols are currently classified as monophenol dihydroxyphenol:oxygen oxidoreductase E.C.1.14.18.1 (Anon, 1976). Synonyms include phenclase, tyrosinase, catechol oxidase, polyphenol oxidase, monophenol oxidase,

laccase, σ- and p-diphenol oxidase, σ-phenolase, cresolase and dihydroxyphenylalanine (DOPA) oxidase. The reactions catalysed (Stafford, 1974) are:-

Monophenol + H_2 donor + O_2 = diphenol + oxidised H_2 donor + H_2O
Diphenol + ½ O_2 = σ-quinone + H_2O
σ-quinone = pigmented polymers

Phenolases were inhibited during the catalysis of diphenols to quinones (Padron *et al*, 1975). The inhibition was irreversible and accompanied by incorporation of substrate into the enzyme. It may be due to the reaction of a nucleophilic group near the active site with the quinone formed there (Gutteridge *et al*, 1977). Chelating agents and thiols were non-competitive inhibitors of phenolase activity (Coombs *et al*, 1974, Gutteridge *et al*, 1977).

The monophenolase activity (Taneja and Sachar, 1974b) and diphenolase activity (Ben Shalom *et al*, 1974) have been found in separate proteins. Some phenolases were located on mitochondrial and plastid membranes, while others were soluble enzymes in some tissues (Parish, 1972; Ben Shalom, 1977). Monophenolase and diphenolase activities reached their maximum levels at different times during development (Taneja *et al*, 1974d) and may have different mechanisms of action (Lerner and Mayer, 1975). As members of this enzyme group do not all catalyse the same reaction on the same substrates, it is possible that their classification may be revised in Enzyme Nomenclature Supplement 2 (Biochemica et Biophysica Acta, 1978).

2. Flavonoid Polymer Synthesis

Many intermediates of flavonoid pigment biosynthesis are substrates for phenolase isozymes. In addition, trans-cinnamate-4-monooxygenase, p-coumarate hydroxylase and flavanone hydroxylase, which all use $NADPH_2$ as the H_2 donor, catalyse similar reactions to phenolases (Stafford, 1974; Fritsh and Grisebach, 1975; Anon, 1976). The possible existance of a complex of many enzymes of the shikimic acid pathway has been discussed (Stafford, 1974). The flavan-3-ols and flavan-3,4-diols may also be phenolase substrates, forming anthocyanins, dimers (biflavans) and polymers.

The mechanism of action of phenolases on their flavonoid substrates is unknown (Ribéreau-Gayon, 1972; Lerner and Mayer, 1975), but the result is the formation of polymers (phlobaphenes, condensed tannins, flavolans or procyanidins). Possibly their quinones or quinone methides are formed initially. These are electrophilic at C4, C2 or C6[1] (see fig 2.1B) and may react with the nycleophilic C8 in the A ring of other flavanols to form condensed tannins (Wong, 1973). The linkages between flavan units are mainly C4-C8 (Haslam, 1977).

Alternatively, these polymers may be derived from carbonium ions produced by reduction of flav-3-en-3-ol (Haslam, 1977) or formed at C2 of flavan-3-ols or C4 of flavan-3,4-diols (Ribéreau-Gayon, 1972; Wong, 1973). Reaction of these with the C6, C8 or alcoholic hydroxyl groups of other flavanols could result in C4-C8, C4-C6, C3-O-C7 and C3-O-C2 bonds, linking them in three dimensions (Ribéreau-Gayon, 1972; Wong, 1973). Owing to their asymmetry, the stereo-chemistry of flavonoid isomers is important in determining the position of the linkages and the restricted rotation around the interflavan bond partly determines the three dimensional structure of the polymer (Haslam, 1977). The complete structures of condensed tannins are probably as complex as those of lignin.

It is not clear whether enzymes actually are involved in the formation of condensed tannins. *In vitro*, the reactions of the appropriate flavan dimers and flavan-3-ol isomers produced a final array of products exactly matching, both quantitatively and qualitatively, those found in plants from which the same precursors had been extracted (Haslam, 1977). The predominant formation of certain products in each reaction was determined solely by purely thermodynamic stability considerations. The reactions could therefore take place in the plant cell without being controlled by enzymes (Haslam, 1977).

If formation of flavonoid polymers in the testa was a spontaneous process during grain maturation, the genes for grain-coat colour would have to control the availability of flavonoid monomers. However, the levels of flavanols in whole red- and white-grained wheats were similar throughout maturation (Gordon, 1975; Cross, 1972). Also, it would be possible to have a red- and a white-grained wheat that differed by only one gene, dominant for redness. It seems unlikely that the colour difference could be due to inhibition of flavonoid synthesis or to

differential localisation of them. It is more probable that the genes for grain-coat colour code for enzymes involved in synthesizing the polymers. Possibly such enzymes activate the flavonoid substrates, which then polymerise according to stereochemical and thermodynamic considerations.

3. Changes during Development

During the development of wheat grains, diphenolase and monophenolase activities appeared at different times. Diphenolase activity reached its peak soon after anthesis (Taneja and Sachar, 1974d). Its activity with different substrates varied during the growth period; with flavan-3-ol, the maximum occurred 30-40 days after anthesis (Taneja and Sachar, 1974d). At that time the grains would be expected to be turning to their mature grain-coat colour. The flavan-3-ol-based activity was three times higher in a red-grained wheat than in a white-grained one (Kruger, 1976b), which may indicate that phenolase is involved in producing the red grain-coat pigments. Up to twelve diphenolase isozymes were present, in developing wheat grains, and only one monophenolase (Kobrehel and Gaulier, 1974; Teneja and Sachar, 1974d). The monophenolase activity appeared when the grain changed colour at the onset of maturation, but its maximum was very low compared with those of diphenolase (Tikoo *et al*, 1973; Taneja and Sachar, 1974d; Kruger, 1976b).

In mature grain there was comparatively little monophenolase or diphenolase activity. Most of the diphenolase activity occurred in the grain-coats but some was also present in mature embryos (Tikoo *et al*, 1973, Kruger, 1976; Taneja and Sachar, 1977 a,b). Monophenolase activity occurred in the mature aleurone (Taneja and Sachar, 1973). During the first few days of germination, the activity of diphenolase increased considerably in the embryos. The increase was due to new diphenolase isozymes, which were synthesised from messages conserved in mature ungerminated embryos (Taneja and Sachar, 1975). Cycloheximide, but not Actinomycin-D, strongly inhibited the appearance of new isozymes which indicated control at the level of translation (Taneja and Sachar, 1977a,b).

Phenolase activity in wheat grains is affected by hormones. Gibberellic acid induced a doubling of monophenolase activity in the endosperm, which was completely counteracted by abscisic acid (Taneja and Sachar, 1974a). Diphenolase activity increased during incubation of embryo-less half-grain and the increase was greater in the presence of gibberellin. Both increases were dependent on protein synthesis and could be inhibited

by cycloheximide and abscisic acid respectively (Jennings and Duffus, 1977b). Gibberellic acid and kinetin did not affect the phenolase activity nor the number or presence of isozymes. Abscisic acid reduced the activity and the number of isozymes by processes independent of transcription (Taneja and Sachar, 1977a,b). While auxin relieved the inhibition of phenolase activity induced by abscisic acid, it had no direct effect on total enzyme activity. However, auxin did affect the number of isozymes revealed by electrophoresis, by association and dissociation among the multiple forms, and the process required new translation (Taneja and Sachar, 1977a,b). It is not known whether similar effects and interactions due to endogenous hormones occur during germination (Taneja and Sachar, 1977a,b).

2.2.4.3. Peroxidase

1. Classification and Reactions

Peroxidase is classified as E.C.1.11.1.7. (Anon, 1976). It catalyses many reactions including oxidation of phenols to complex coloured products, oxidation of IAA and $NADPH_2$, lignification of cell walls and production of ethylene (Shin and Nakamura, 1962; Kamel *et al*, 1977). Also, it may regulate the level of an important precursor of flavanoid biosynthesis, p-coumaric acid (Stafford, 1974a). There are many isozymes of peroxidase, which differ in their affinities for various substrates. With IAA, they vary from the typical peroxidase isozyme which will oxidase IAA slowly in the absence of exogenous H_2O_2 to ones with both high peroxidative and IAA oxidative activities to those which have only peroxidative or only oxidative activity (Schneider and Wightman, 1974). The isozymes change in activity from H_2O_2 activation to O_2 activation to O_2 carrying, which may be associated with progressively fewer acidic groups in the haeme cleft of the peroxidase molecule (Schneider and Wightman, 1974).

Peroxidase is affected by free and bound phenols, organic acids, manganese salts and coumarins, particularly scopoletin which at low levels stimulates IAA oxidase activity and inhibits it at high levels. Peroxidase is located in cell walls and on a variety of membranes including those of endoplasmic reticulum, nucleus, vacuoles, dictyosomes, ribosomes and polysomes (Stafford, 1974). Peroxidases are readily soluble and relatively heat stable (Parish, 1972; Stafford, 1974).

2. Changes during Development

In developing wheat grains, peroxidase activity rose rapidly in the second week after anthesis, reached a maximum around the end of the third week and decreased to low levels at maturity (Kruger and Laberge, 1974a). Most peroxidase activity during early grain growth occurred in the pericarp. Activity in the aleurone, starchy endosperm, embryo was very low at early stages but increased during development. The greatest increase was in the endosperm, where it coincided with the build-up of storage proteins. The presence of up to twelve peroxidase isozymes in immature grains was shown by polyacrylamide slab electrophoresis (Kruger and Laberge, 1974a). Most of the peroxidase activity occurred in the bran (Honold and Stahman, 1968). Changes in the intensity of staining of the various isozymes detected during development reflected quantitative changes in total peroxidase activity in various tissues (Kruger and Laberge, 1974a).

High levels of IAA-oxidase activity were reported in resting wheat grains (Laurema, 1974; Tao and Khan, 1975). In the hulls of rice grains, peroxidase activity decreased progressively during storage or breaking of dormancy (Navasero *et al*, 1975). In germinating barley grains, there was a high negative correlation between alpha-amylase activity and peroxidase activity in the coleoptile (Gasper *et al*, 1969). It was possible to modify the alpha-amylase activity by affecting the supply of IAA (Gasper *et al*, 1969). Wheat embryos may not be able to germinate unless auxin levels are above a threshold level, which could depend on low levels of peroxidase activity (Gasper *et al*, 1977). Incubation of embryo-less half-grain in gibberellin increased peroxidase activity in the aleurone within a day, which was similar to the effect of the embryo in germinating whole grain (Tao, 1975). Gibberellin may regulate IAA oxidase activity (Bolduc *et al*, 1970; Tao, 1975; Gale and Marshall, 1975) although there are contradictory cases (cited in Bolduc *et al*, 1970).

Dihydroxybenzoic and ferulic acids cause a lag in the oxidation of IAA during which they are oxidised, while p-coumaric acid and resorcinol act as cofactors of IAA oxidase from young wheat seedlings (Machackova *et al*, 1975; Machackova and Zmrhal, 1976). The wheat coleoptile has three peroxide isozymes, one of which has IAA oxidase activity, and all are inhibited by application of IAA (Whitmore, 1971). In wheat coleoptile segments, p-coumaric acid and ferulic acid at higher concentrations inhibited IAA-induced growth, while lower concentrations of ferulic acid were stimulatory (Machackova

and Zmrhal, 1976). Cytokinins stimulated the *in vivo* activity of IAA oxidase in barley grain (Gaspar *et al*, 1969).

2.3 Hormones in Developing Wheat Grains

2.3.1. Introduction

Hormones are probably involved in the integration of developmental processes in the various grain tissues. Unsynchronised development could result in apparent dormancy of mature tissues, while the others continued to develop. Different interactions among the hormones in red- and white-grained wheats during development, might result in processes leading to their different responses under germinative conditions. The action of some of the hormones during development might induce dormancy at later stages, but the continuous presence of abscisic acid is required for its inhibitory effect (Millborrow 1964).

2.3.2. Cytokinins

Ovules contained only small amounts of cytokinins, but by the end of anthesis the grains reached their maximum cytokinin content (Wheeler, 1972). The content declined during the period of rapid cell division until there was little after three weeks from anthesis when the endosperm had ceased dividing (Wheeler, 1972). Cytokinins probably originated in the roots and moved up in the transpiration stream. The exudate from cut wheat stems contained most cytokinin before anthesis and little while the grains were growing. Any cytokinin metabolised during grain growth is not replaced, so what was present at anthesis might regulate the early stages of grain growth and possibly final grain size (Wheeler, 1972).

2.3.3. Auxin

Maximum IAA content was found just before the grains reached their maximum fresh weights, when starch and protein were accumulating rapidly and cell expansion had ceased (Wheeler, 1972). Maximum peroxidase activity, which may metabolise auxins, occurred at about the same time (Kruger and Laberge, 1974a). The maximum levels of auxin were much higher than those found in vegetative tissues (up to 100 ng/grain),

but rapidly declined to nothing in mature grain (Wheeler, 1972). IAA was apparently involved in regulating the accumulation of reserves, in the endosperm, thus determining grain weight (Wheeler, 1976). The levels of IAA were not related to those of the precursor tryptophan, although its phenolic precursors were found during the first four weeks after anthesis (Wheeler, 1972). In developing barley grains, instead of IAA there was an unidentified indolic compound, which was also a product of metabolism of exogenous IAA as were catechol and pyrogallol. The system involved was independent of gibberellin action (Minchin and Harmey, 1975).

2.3.4. Gibberellins

At anthesis, levels of gibberellins were low but rose rapidly as starch accumulated in the endosperm. Gibberellin content reached a peak three weeks after anthesis, when endosperm cells had ceased dividing and were expanding, then disappeared during deyhdration (Wheeler, 1972). The smallness of the changes of gibberellin content in stem exudates or husk extracts suggested that the gibberellins were synthesised within the grains (Wheeler, 1972). Embryos from grains at maximum endosperm enlargement contain high concentrations of gibberellins but it is not known how early the synthesising systems are developed (Radley, 1976). Chloroplasts of the inner pericarp may be the site of synthesis as the increase in gibberellins ceased at about the time that chloroplast activity declined (Radley, 1976). Also, isolated endosperm tissue of *Cucurbita* could synthesise gibberellins (Graebe *et al*, 1974).

Gibberellin production may be necessary to coordinate growth of the various grain tissues and may be synthesised at different sites during different stages of development (Radley, 1976). The several different gibberellins present in developing grain were not uniformly distributed. They occurred mainly in the pericarp and decreased as alpha-amylase increased (Radley, 1976). However, there was no evidence that the synthesis of the pericarp alpha-amylases of immature wheat grains was controlled by or responsive to gibberellin (Olered and Jonsson, 1970; Radley, 1976).

2.3.5 Abscisic Acid

There was little abscisic acid in wheat grains during their first stages of growth (King, 1976). Then its level increased rapidly and remained high during the fourth week after anthesis, when the grains were turning colour, followed by an abrupt drop to a low level in mature grain (McWha, 1975; Radley, 1976; King, 1976). A brief increase in the amount of bound abscisic acid coincided with the decrease in the free form during dehdration (King, 1976). The embryo and endosperm of immature grain could synthesise abscisic acid from mevalonic acid, with the embryo being the more active (Milborrow and Robinson, 1973) and containing relatively more abscisic acid (King, 1976). The amount and time of appearance of peak abscisic acid content were similar in several genotypes of differing grain colour (King, 1976).

The role of abscisic acid in grain development is unknown but it did not appear to inhibit growth. The fastest growing grain in the spikelet had the most abscisic acid and differences in grain growth rate up and down the ear were not reflected by differences in abscisic acid content (King, 1976). Application of abscisic acid to ears in the third week after anthesis had no effect on the rate of grain growth (King, 1976). It was considered that its role might be in grain maturation, as rapid dehydration occurred immediately after its peak (Radley, 1976) and application of it at 31 days after anthesis hastened grain water loss (King, 1976). Its action may be due to inhibition of synthesis or activity of enzymes, such as those of starch synthesis. Alternatively it could be involved in the sealing of vascular tissue from the maternal plant so that the supply of sucrose and water to the grain is reduced, or it may increase membrane permeability and contribute to the loss of water through the pericarp (Radley, 1976). As abscisic acid can prevent mature grain from germinating, it is possible that it may prevent premature sprouting of immature grains and the break-down of starch in them (King, 1976).

2.4. Biosynthetic Changes During Germination

The sequence of events in the re-establishment of active metabolism during germination of grain might indicate which metabolic processes might be blocked during dormancy. Presumably the most efficient block

would occur at the earliest possible stage. The first events after the start of imbibition of non-dormant grain occur in the embryo and involve the activation of metabolic systems. The next stage is the release of latent enzymes and the *de novo* synthesis of enzymes, which occurs mainly in the aleurone. These break-down the protein, carbohydrate and lipid reserves of the endosperm to products which are transported to the embryo, where they provide substrates for its growth (Kruger, 1976).

An early change during imbibition in a non-dormant wheat embryo is the synthesis of ATP. Its concentration increased 5 times within 30 mins and 10 times within an hour and then remained constant (Obendorf and Marcus, 1974), which correlated with the activity of the mitochondrial respiratory chain. ATP is required for early organisation of polysomes, functioning of t.RNA and protein synthesis. The maintenance of a high concentration of ATP in imbibed seeds is a necessary but not sufficient cause for germination (Taylorson and Hendricks, 1977). All components of protein synthesis are present in wheat embryos before imbibition. Active protein synthesis occurred within 30 minutes after the start of imbibition in rye, maize and wheat grains and paralleled water uptake (Taylorson and Hendricks, 1977). The mRNA for the polyribosomes may be supplied from pre-existant sources in wheat grains. Although synthesis of RNA apparently started immediately after wetting, it was considered unlikely to serve any mRNA function during the first three hours of germination. DNA synthesis was not appreciable before 9 hours of imbibition (Spiegel *et al*, 1975; Taylorson and Hendricks, 1977).

It has been suggested that a shift in the operative respiratory pathway from glycolysis to the pentose phosphate pathway may break dormancy (Roberts, 1973). The pentose phosphate pathway does not produce ATP from glucose-6-phosphate as efficiently as glycolysis, but may be active when oxygen concentrations are too low for the glycolytic pathway to be active. In non-dormant isolated embryos of wheat, the glycolytic pathway operated at a constant rate during the first five days of germination, while activity of the pentose phosphate pathway increased during the first 3 days of germination before decreasing (Ishikawa *et al*, 1977).

In non-dormant oat embryos, the activity of the pentose phosphate pathway was high, relative to that of the glycolytic pathway, during the early stages of germination. In dormant embryos, its activity was stimulated when gibberellic acid was used to break dormancy (Simmonds and Simpson, 1972). G-6-P dehydrogenase, the first enzyme of the pentose-

phosphate pathway, is present in dry seeds. The activity of G-6-P dehydrogenase increased sharply in germinating oat grains but declined in dormant imbibed ones (Kovacs and Simpson, 1976).

The early use of oxygen by imbibing seeds or embryos raises the question of reductants. $NADPH_2$ and $NADH_2$ are the immediate sources of substrates which are readily oxidised by dehydrogenases. The level of NADP, dependent in part on NAD:NADP kinase and phosphatase activities, is a point of metabolic control for many biosynthetic reactions. Oxidative respiration and activity of G-6-P dehydrogenase. In isolated wheat embryos, the contents of NAD and of $NADH_2$ rose during the first day of germination, then fell to low levels. The increases in NADP and $NADPH_2$ were less and their peaks occurred a day later (Ishikawa *et al*, 1977).

2.5 Hormones and Germinative Response

Hormones are involved in imposing and breaking dormancy in seeds (Wareing and Saunders, 1971; Mayer and Shain, 1974). Gibberellins have consistently enhanced seed germination and are implicated in many seed processes (Khan, 1971). In barley aleurone cells, they controlled the *de novo* synthesis of hydrolytic enzymes which mobilised stored substrates (Chrispeels and Varner, 1967b). Abscisic acid is a potent inhibitor of germination in many species when their seeds are treated with solutions of it (Milborrow, 1974). This type of observation, together with its presence as the major growth inhibitor in dormant seed of many species, have implicated it in the maintenance of seed dormancy (Milborrow, 1974). Cytokinins allow gibberellin-induced germinative or enzymatic processes to be completed when they are inhibited by abscisic acid or coumarin (Khan, 1975). Cytokinin-inhibitor antagonism is known to occur in alpha-amylase synthesis, peroxidase synthesis, RNA synthesis and polysome formation (Khan, 1975).

It has been suggested that gibberellins have the primary role in controlling seed dormancy, while cytokinins and inhibitors interact with one another and with the gibberellins, rather than directly on the control systems (Khan, 1975). Much evidence has supported this hypothesis (Khan, 1975), which provides an explanation for otherwise anomalous situations such as dormancy without inhibitors or germination when their levels are high. The former case could be caused by inability to synthesis gibberellins or lack of response to them. In the latter, high levels of cytokinins may counteract the inhibition. In mature cereal grains, cytokinin-inhibitor antagonism was not considered to be the

cause of dormancy (Khan, 1975; Belderok, 1976). However, both abscisic acid and flavanols have been implicated as germination inhibitors (King 1976, Stoy and Sundin, 1976, Varner and Ho, 1976) and the effect of cytokinins in breaking dormancy in wheat have not been investigated.

In barley grains, application of gibberellin broke dormancy, while applied cytokinins were ineffective. However, when grains were pre-treated with abscisic acid, cytokinin was then required to permit gibberellin to break dormancy (Khan and Waters, 1969). The gibberellin effect was delayed if grains were imbibed in IAA, which was metabolised during the lag (Minchin and Harmey, 1975). Abscisic acid prevents the gibberellin-induced alpha-amylase and protease syntheses, membrane-bound polysome formation, incorporation of ^{32}P into membrane phospholipids, poly-A RNA synthesis and activation of phosphorylcholine transferases, in barley aleurone (Varner and Ho, 1976). Although barley grains were capable of response to all three types of hormone, abscisic acid and cytokinins were possibly not essential for the control of dormancy or germination (Khan, 1975).

The germinative response to hormones of mature wheat embryos differed among dormant and non-dormant cultivars (Stoy and Sundin, 1976). Germination of non-dormant ones was prevented by abscisic acid only in samples harvested in the first few weeks after embryo maturity was reached. The effect was completely suppressed by the addition of gibberellic acid. By contrast, in dormant cultivars abscisic acid completely suppressed germination in embryos from grains harvested over several months after embryo maturity had been reached. Also, its effects were only partially overcome by gibberellic acid, particularly in earlier samples (Stoy and Sundin, 1976). DNA-RNA hybridisation experiments showed that no new mRNA or r RNA was associated with the ribosomes of germinating wheat embryos. The indication was that the hormones regulated the use of mRNA, already stored as ribonucleo-protein in the unimbibed embryo, rather than controlling the transcription of new mRNA (Varner and Ho, 1976). Actinomycin-D had no effect on germination although protein synthesis was initiated immediately after imbibition, again indicating post-translocational control by the hormones (Varner and Ho, 1976).

The embryo-less half-grains of dormant and non-dormant wheat cultivars also had different alpha-amylase responses after incubation in gibberellic acid for 24 hours (Stoy and Sundin, 1976). Non-dormant cultivars gave a high response and had a high germination percentage shortly after maturity. Dormant cultivars had very low responses and low germination

percentages even in samples taken after several months of after-ripening (Stoy and Sundin, 1976). However, the alpha-amylase response to gibberellin was low in the first 30 hours of treatment (McMaster, 1976; Gordon, 1977), so the results of Stoy and Sundin (1976) may have reflected a response to endogenous gibberellin rather than that of the incubation medium. Also, the responses were closely related to the water content of the samples when they were harvested, indicating that germinative process might have already begun in the non-dormant cultivars which would result in high responses. Grain of another cultivar germinated readily after a short period of after-ripening, but its response to gibberellin within the 24 hours was low for a considerably longer period (Stoy and Sundin, 1976).

Abscisic acid prevented the response to gibberellin, although no direct effects of it in aleurone cells have been observed. The inhibition of response induced by abscisic acid was not due to simple competition, because a high concentration of gibberellin could not completely overcome it (Varner and Ho, 1976). Abscisic acid enhanced the uptake and metabolism of tritiated GA_1 in barley aleurone layers (Nadeau *et al*, 1972). It may inhibit alpha-amylase synthesis by stimulating the conversion of GA_1 to inactive products (Nadeau *et al*, 1972; Stolp *et al*, 1977). After 12 hours of gibberellin treatment the alpha-amylase response of barley aleurone was no longer inhibited by 3' deoxyadenisine which inhibited transcription. At that stage, abscisic acid still effectively inhibited the response, except when 3'deoxyadenosine was added after it, which suggested that the inhibition depended on RNA synthesis (Ho and Varner, 1976). Gibberellin also directly affected coleoptile growth of isolated wheat embryos (Kefford and Rijven, 1966).

In wheat aleurone, 20% of the triglyceride reserves were mobilized in the first 24 hours of germination by cytokinins or extracts of starchy endosperm, and 10% by auxin, which was also required for continued mobilization (Laidman *et al* , 1974). Gibberellin was not effective in mobilizing the triglycerides, but induced beta-oxidation of fatty acids and the glyoxalate cycle (Laidman *et al*, 1974). Oxidation of the fatty acids of triglyceride may provide substrates for the synthesis of phosphotidyl polar lipids which are essential components of membranes. They increased in the embryo of germinating seeds of several species which probably paralleled the re-establishment of membrane integrity (Taylorson and Hendricks, 1977). Gibberellin from the embryo and cytokinin from the endosperm act sequentially in the aleurone of germinating

wheat to control the mobilization of potassium, calcium, magnesium, and phosphates from phytin (Laidman *et al*, 1974).

The aleurone layer of two dwarf wheats of different origin (Minister Dwarf, Tom Thumb and a white-grained derivative of it, Tordo) did not respond to gibberellin and the grain had low germination rates (Gale and Marshall, 1975; McMaster, 1976; Bhatt *et al*, 1976). During development, their grains had more gibberellin and less auxin than those of tall varieties (Gale and Marshall, 1973; 1975; Gale *et al*, 1975). The lack of response may be due to a defect in the gibberellin receptor proteins of the aleurone (Jelsema *et al*, 1975), which would prevent the detection of gibberellin. Alternatively, there may be an inhibitor, which competes with gibberellin for the active sites (Gale and Marshall, 1973).

The pericarps of grain from these dwarf wheats had higher levels of diphenolase activity than those of tall varieties, but it was not clear whether the activity was due to new synthesis or to modification of the existing enzymes (Tikoo *et al*, 1973). The increased activity may indicate that more phenolic acids are being synthesised. Higher levels of phenolics may inhibit the gibberellin-induced activities in the aleurone or affect the rate of oxidation of IAA. Low peroxidase activity in wheat embryos, was associated with a high percent germination and high alpha-amylase activity (Gasper *et al* , 1977). Also, embryo-less half-grain of rice and wheat were apparently unable to secrete essential enzymes in the absence of IAA (Palmiano and Juliano, 1973). Interference with auxin metabolism in Tom Thumb and Tordo may contribute to the gibberellin insensitivity. However, increased peroxidase activity during germination was not related to dormancy-breaking (Thevenot, 1977, Tao and Khan, 1975).

In wheat, a sufficiently low peroxidase activity and a minimum auxin level of the embryo were considered to be responsible for the onset of germination, but not for the breaking of dormancy in wheat (Gasper *et al*, 1977). High IAA-oxidising activity occurred in resting wheat grains (Laurema, 1974; Tao and Khan, 1975). Coleoptile growth and alpha-amylase activity in barley grains were parallel processes and hormonal modification of coleoptile growth induced modifications of alpha-amylase synthesis (Gasper *et al*, 1977). Removal of the coleoptile was followed by decreasing synthesis of alpha-amylase and insensitivity to hormone treatments. Kinetin strongly inhibited root and coleoptile growth of germinating barley in the dark, greatly increased the activity of peroxidase and specifically reduced the level of polyphenolics

(Gasper *et al*, 1969). Kinetin also reduced alpha-amylase synthesis in the grain (Gasper *et al*, 1977). Peroxidase-mediated auxin metabolism in the coleoptile was considered to participate in alpha-amylase synthesis in the grain (Gasper *et al*, 1977).

CHAPTER 3

GRAIN GROWTH, EMBRYO MATURITY AND GRAIN DORMANCY

3.1 Introduction

Basic tests on the developing grain were made to determine the differences in grain maturation and dormancy of various red- and white-grained genotypes. Dehydration of the grain was used as a general indication of approach to harvest-ripeness, so that development of the genotypes could be compared at similar stages. Germination tests were used to show embryo maturity and dormancy. The information provided by these tests will form the basis to which other information can be related.

3.1.1. Cultivation and sampling of parental populations

Grain development and maturation was investigated in five genotypes of diverse genetic origin, which represented a range of grain colour and dormancy and had been used in previous studies (Gordon, 1975; Cross, 1977). The genotypes were:-

Gamut, a white-grained non-dormant wheat of Australian origin.

Karamu, a red-grained New Zealand cultivar with one gene for redness.

Pembina, a red-grained dormant wheat of Canadian origin and an unknown number of red genes.

Sonora-64A, a red-grained wheat of Mexican origin with little or no dormancy and one gene for redness.

Timgalen, a white-grained non-dormant wheat of Australian origin.

In addition, the F_2 from the cross Timgalen x Pembina (Tim x Pem) was included, making a total of six genotypes.

Prior to sowing, any possible residual grain dormancy was broken by peeling back the grain-coat overlying the embryo with a scalpel. The grains were germinated on 0.2% w/v KNO_3-soaked filter paper in petri dishes in the light at 21°C for 3-4 days. Eighteen plants were sown in each plot, which was 2.5 m long, with inter-plot spacing of 0.4 m. The plots were set out as six randomised complete blocks in a glasshouse in late January, 1976. The glasshouse soil was Manawatu silt-loam, which had been fumigated with methyl bromide to kill soil-

borne pathogens and weeds. It was fertilized with super-phosphate, dug and stones removed. Irrigation was from soak-hoses laid between alternate plots. The plants were sprayed weekly against aphids and fungi. Rat-poison and mouse-traps were laid as the grain ripened, but rodents still caused considerable losses.

Each individual head of grain was labelled with a 22H jeweller's tag above the lowest spikelet, on the day it reached anthesis, which was when the anthers first extrude from the florets. The number of days from planting until the first heads reached anthesis were:- Timgalen, 53; Sonora, 56; Tim x Pem, 63; Karamu, 64; Pembina, 67; and Gamut, 74. The tagged heads formed the populations available for sampling during grain development. Owing to the limited plot size, most heads had to be tagged despite their reaching anthesis at different times. Consequently, grain development among samples did not occur in a constant environment. Photoperiod was kept at 14 hours per day with supplementary lighting. An attempt was made to prevent night temperatures from dropping below $4^{o}C$, with kerosene heating as the glasshouse was not connected to the main heating system at that time. Automatic vents and fans came into operation when the temperature reached $20^{o}C$.

Samples of tagged heads were taken at nine-day intervals, starting 5 days after anthesis and continuing until about 80 days. Further sampling would have been desirable for some genotypes, as developmental processes were not complete. However, it was not possible due to the limited number of heads, which resulted from the small plot size and rodent damage. Grains were hand-threshed from the lower 2 florets of spikelets in the middle two-thirds of the spike, giving samples in which the ages of the grains did not vary more than a day (Rawson and Evans, 1970; Evans *et al*, 1972). Grains not involved in germination tests were oven-dried at $60^{o}C$ for 24 hours before being stored at $1^{o}C$.

3.2 Statistical Analysis

3.2.1. Fitting Regression Equations

Each set of measured data from each genotype was used, in turn, as the dependent variable (Y) in a regression analysis. Time, expressed as days from anthesis, was the independent variable (X). The equations of regression analysis provided a single function describing the trend in the measured variable with time. The regression statistics of the

equations for each genotype could be compared for significant differences. Regression equations also allowed interpolation and limited extrapolation from the data points, so that the timing or magnitude of events of biological interest could be estimated, along with the accuracy of those estimates.

Plots of the data points of each dependent variable against time were used to suggest likely types of equations for the regression curves. Biological principles and expectations from previous studies were also considered. Some variables were expected to follow a sigmoidal curve, accelerating from an initial asymptote through an exponential phase before decelerating to a final asymptote (e.g. grain dehydration, percent embryo maturity and dormancy). In these cases, asymptotic curves were generally preferable to the straight line, quadratic, cubic or exponential curves appropriate to data sets lacking asymptotes. Logistic and quadratic logistic curves were fitted, where it was possible to estimate the upper asymptote. In all cases, the usual method of least squares fitting was used (Draper and Smith, 1966).

The criteria for selecting the best-fitting equation included its appropriateness to biological expectations and the accuracy of the regression. The preference was for curves with higher coefficients of determination (R^2), where $R^2 = \Sigma(\hat{Y}_i - \overline{Y})^2 / \Sigma(Y_i - \overline{Y})^2$, in which $\overline{Y}$ is the mean of the observations of Y, Y_i is the i^{th} observation of $\hat{Y}$ and $\hat{Y}_i$ is the value of Y_i estimated by the regression equation; $R^2 \rightarrow 1$ as $\Sigma(Y_i - \hat{Y}_i)^2$ tends to zero (Draper and Smith, 1966). The curve with the highest R^2 was not necessarily accepted, depending on the other criteria. The actual shape of the fitted equation was considered with respect to the observed data by examining a plot of it and the residual variation of the data points $(Y_i - \hat{Y}_i)$. In general, the simpler equation was preferred if alternatives existed.

3.2.1.1. Logistic Equations

The following description was made by Gordon (1975) based on Bliss (1970). The logistic function is one where the rate of change in Y is proportional to change in X according to the relationship:-

$dy/dx = \beta_1 Y\left((y_0 - Y)/ y_0\right)$, where β_1 is the initial rate of change and y_0 is the upper asymptote in Y. On integration this becomes $Y = y_0 / \left(1 + e^{-(\beta_0 + \beta_1 X)}\right)$ where β_0 is a constant. Rearrangement gives $(y_0 - Y) /Y = e^{-(\beta_0 + \beta_1 X)}$, of which the reciprocal is $Y/(y_0 - Y) = e^{(\beta_0 + \beta_1 X)}$. Therefore, in $\left[Y/(y_0 - Y)\right] = \beta_0 + \beta_1 X$, which is the linear form of the logistic equation and can be fitted by linear regression (Steel and Torrie, 1960; Bliss, 1970). By the original definition, $\ln \left(Y/(y_0 - Y)\right)$ = logit Y. So the linear logistic equation can be expressed as logit $Y = \beta_0 + \beta_1 X$. The quadratic logistic equation introduces an additional term to reduce curvature remaining in a logistic equation, so that logit $Y = \beta_0 + \beta_1 X + \beta_2 X^2$.

Some data sets have theoretical upper asymptotes which can be used in fitting curves. For those in which Y_0 is unknown, it is initially estimated from either the original data plot or from previous studies. Improved estimates are obtained by using an auxillary maximum likelihood variate in the regression (Bliss, 1970). The auxiliary variate is $X^* = y_0 /(y_0 - Y)$ and the linear equation becomes logit $Y = \beta_0 + \beta_1 X + \beta_2 X^*$. This is used to estimate the next value of y_0 ($\hat{y}_{02}$) as $\hat{y}_{02} = \hat{y}_{01} (1 + \beta_2)$ (Bliss, 1970). The regression is repeated using the new value of y_0. After the first two iterations, $y_{0(i + 1)}$ may be estimated using the equation:-

$$\hat{y}_{0i} = \left(\hat{y}_{0\ (o - 1)}\ \hat{d}_{(i - 2)} - \hat{y}_{0(i-2)}\ \hat{d}_{(i - 1)}\right) / \left(\hat{d}_{(i-2)} - d_{(i-1)}\right)$$

where $\hat{d}_{(i - 2)} = \hat{b}_{2(i - 2)}.\ \hat{y}_{0(i - 2)}$; $\hat{d}_{(i - 1)} = \hat{b}_{(i - 1)}$. $\hat{y}_{0(i - 1)}$ and i is the index of the interaction. Iteration is terminated when $\hat{y}$ is stabilised. as indicated by one of two methods: (1) $\hat{\beta}_2$ is not significantly different to zero for at least two successive iterations. as indicated by its t-test for the $H_0 : \beta_2 = 0$. $H_1 : \beta_2 \neq 0$; (2) y_{0i} was different to $Y_{0_{(i - 1)}}$ by less than an arbitary fraction (e.g. 0.01) of the previous value for at least three successive iterations. The mean of the last few estimates may be used as the final $\hat{y}_0$ (Bliss, 1970; Gordon, 1975).

The equations, logit Y = $\beta_0 + \beta_1 X$ and logit Y = $\beta_0 + \beta_1 X + \beta_2 X^2$, were used in the REGRESSION program of Statistical Packages for the Social Sciences (SPSS/V6), using observed values of Y at the various X's and estimating y_0. Values of Y lying on the upper asymptote were adjusted downwrds by 0.01 and zero values were adjusted upwards to 0.01. The regressed line is in logits and cannot be empirically transformed to the original Y units. Antilogit tables were used to perform the transformation. The SPSS/REGRESSION program scales the data by the reciprocal of the upper asymptote. Antilogits of estimated Y values must be multiplied by the appropriate y_0 to align them with the original data.

3.2.2 Comparison of Equations among Genotypes

Differences among the fitted equations were tested for significance by comparing the Y-intercepts (β_0) and the regression coefficients (β_1 and β_2). A significant difference in any of the regression statistics was taken to indicate a difference in the pattern of development. As the variances of the statistics were heterogeneous, all possible pair-wise t-tests were estimating by using the test:- $t = (\hat{X}_2 - \hat{X}_1)/(\hat{\sigma}_2^2 + \hat{\sigma}_1^2)^{\frac{1}{2}}$, where X_1 and X_2 are the appropriate regression statistics and $\hat{\sigma}_1^2$ and $\hat{\sigma}_2^2$ are their respective variances. The absolute value of t was compared with the theoretical t at P = 0.05 and the sum of degree of freedom for variance about regression $(n_1 + n_2 - 4)$. Where the variability of the data was high and R^2 was low or where the estimate of a point of biological interest had a high variance, the significance level was raised to P = 0.10. This increased the probability of detecting real differences, although there was also an increased risk that the observed differences were due to chance.

The variance of β_0 is not given by SPSS/REGRESSION. For linear equations, $y = \beta_0 + \beta_1 X$, the variance of β_0 was estimated using the equation:-

$$\hat{\sigma}^2_{\beta_0} = \sigma^2_{y.x} \left(X_i^2 / (n\Sigma (x_i - \bar{X})^2) \right) \text{ . (Draper and Smith, 1966).}$$

SPSS/REGRESSION output includes n, $\bar{X}$, $\bar{Y}$, s_x and $\hat{\sigma}_{y.x}$, so $\hat{\sigma}^2_{\beta_0}$ was expressed in terms of them by the following method :

$\Sigma(X_i - \bar{X})^2 = (n-1)S_x^2$, as $S_x^2 + \left[\Sigma(X_i - \bar{X})^2\right] / (n-1)$ by definition

and $\Sigma(X_i - \bar{X})^2 = \Sigma X_i^2 - (\Sigma X_i^2) /n$

$= \Sigma X_i^2 - n\bar{X}^2$ as $\Sigma X_i^2 / n = (n\bar{X})^2 /n$ which simplifies to $n\bar{X}^2$

Rearrangement gives: that $\Sigma X_i^2 = \Sigma(X_i - \bar{X})^2 + n\bar{X}^2$ and dividing

$\Sigma(X_i - \bar{X})^2 + n\bar{X}^2 + n\bar{X}$ by (n-1) gives $(n-1)S_x^2 + n\bar{X}^2/ (n-1)$,

resulting in the identity $\Sigma X_i^2 = (n-1)S_x^2 + n\bar{X}^2/ (n-1)$

Substituting the identities for $\Sigma(S_i - \bar{X})^2$ and ΣX_i^2 into equation (3)

results in the expression:-

$$\hat{\sigma}^2_{\beta_0} = \sigma^2_{y.x} \left[(n-1\ S_x^2) + \frac{n\bar{X}^2}{n-1} \right] / \left[n(n-1)S_x^2 \right]$$

which can be simplified to:-

$$\hat{\sigma}^2_{\beta_0} = \sigma^2_{y.x} \left[\frac{1}{n} + \frac{\bar{X}^2}{(n-1)^2 S_x^2} \right]$$

For the quadratic equations, the program REGSPS (Gordon, unpublished 1977) was used to obtain the variance-covariance matrix of the β's ($\mathbf{V}(\beta)$).

$\mathbf{V}(\beta) = \sigma^2_{y.x} (\mathbf{X}'\mathbf{X})^{-1}$ where $\sigma^2_{y.x}$ is the variance about regression, $\mathbf{X}$ is the 2xn matrix of the independent X variables in which all values in the first column are unity, $\mathbf{X}'$ is the nx2 transpose matrix of $\mathbf{X}$ and $(\mathbf{X}'\mathbf{X})^{-1}$ is the nxn inverted matrix (Draper and Smith, 1966). The variances of the β's lie on the diagonal of $\mathbf{V}(\beta)$.

3.2.3 Estimation of Points of Biological Interest

3.2.3.1 Timing Differences

An estimate of X, $\hat{x}$, for a particular value of y, y_k, may be obtained, where appropriate, from the reverse regression,

$x = \beta_0' + \beta_1' y + E$ (Steel and Torrie, 1960; Draper and Smith, 1966). However, it is not a valid regression if the X variable of the forward regression ($y = \beta_0 + \beta_1 X + E$) is invariate with no intrinsic E's e.g. time in this study. For such a case, $\hat{x}$ can be obtained from a backward solution of the forward regression:- at y_k, $\hat{x} = (y_k - \beta_0)/\beta_1$. Although X is invariate, the $\hat{x}$ has a variance due to variance in $\hat{\beta}_1$ and $\hat{y}_k$. An estimate of the variance of $\hat{x}$ is required for the estimation of t-statistics for the differences among the $\hat{x}$'s of the various genotypes at y_k.

The forward regression can also be written as $(y_k - y) = \beta_1 (x - \bar{x})$ with the solution for x being $\hat{x} = (y_k - \bar{y})/\beta_1 + \bar{x}$, where the mean of x, $\bar{x}$, is a constant which does not contribute to the variance. The variance of $\hat{x}$, $\sigma^2_{\hat{x}}$, is therefore the variance of the ratio

$(y_k - \bar{y})/\beta_1$ i.e.

$$\sigma^2_{\hat{x}} = \left| (y_k - \bar{y})^2 \sigma^2_{\beta_1} + \beta_1 \sigma^2_{(y-y)}, -2 (y_k - \bar{y}) \beta_1 \right. \left. .\mathrm{cov}\ (y_k - \bar{y}, \beta_1) \right| / \beta_1^4$$

(Gordon unpublished, 1977)

As the covariance of y_k and β_1 is zero and the covariance of $\bar{y}$ and β_1 is zero, (Draper and Smith, 1966) it is assumed that the covariance of $(y_k - \bar{y})$ and β_1 is also zero. Therefore,

$$\sigma^2_{\hat{x}} = \left[(y_k - \bar{y})^2 \sigma^2_{\beta_1} + \beta_1^2 \sigma^2_{(y-\bar{y})} \right] / \beta_1^4 .$$ As $\sigma^2_{\beta_1} = \sigma^2_{y.x} / \Sigma (x - \bar{x})^2$,

where $\sigma^2_{y.x}$ is the variance about regression. The equation can be simplified to:-

$$\sigma^2_{\hat{x}} = \sigma^2_{y.x} \left[(y_k - \bar{Y})^2 / \sum_i (X_i - \bar{X})^2 + \beta_1^2 (1 + \tfrac{1}{n}) \right] / \beta_1^4 ,$$

Where all terms have their usual meanings or have been defined previously. All possible pair-wise t-tests were performed using the test given in 3.3.2 and comparing the calculated values with the theoretical values at P = 0.05 or P = 0.10 and $(n_1 + n_2 - 2)$ degrees of freedom.

3.2.3.2 Response Differences

Estimates of y for particular values of x, $\hat{y}_k$, were obtained from forward solutions of the forward regressions, $y = \beta_0 + \beta_1 X$. For equations with one X variable, the variance of the estimates was estimated using:- $\sigma^2_{y_k} = \sigma^2_{y.x} \left(1/n + (X_k - X)^2 / \Sigma(X_i - \overline{X})^2\right)$ (Draper and Smith, 1966). For quadratic equations, the estimated variance of $\hat{Y}_k$ was obtained as

$\hat{\sigma}^2_{y_k} = \mathbf{X}_k' (\mathbf{X}'\mathbf{X})^{-1} \mathbf{X}_k \sigma^2_{y.x}$ (Draper and Smith, 1966), where

$\mathbf{X}_k'$ is the vector $(1, X_k, X_k^2)$ and $(\mathbf{X}'\mathbf{X})^{-1}\sigma^2_{y.x}$ is the variance-covariance matrix of the β's obtained from REGSPS. All possible pair-wise t-tests were performed as before, to find significant differences among the $\hat{y}_k$ for the various genotypes.

3.3 Grain Moisture Content

3.3.1 Methods

One of the agricultural criteria of grain maturity is its moisture content, which is usually expressed as a percentage of its fresh weight (FW). FW was measured by immediately weighing the first 10 grains threshed from a sample. Then the grains were dried at 60°C for 24 hours, cooled in a desiccator over silica gel and reweighed to give the dry weight (DW). The percentage moisture content of the grains was calculated as 100 (FW-DW)/FW.

Logistic and quadratic logistic equations were fitted, with the first estimate of the upper asymptote ($\hat{y}_{01}$) for each genotype being based on the final estimates of Gordon (1975) and Cross (1977). Improved estimates were obtained using the auxiliary variate, as described in Statistical Methods 3.2.1.1. Logistic and quadratic logistic curves were fitted because they were compatible with biological expectations for dehydration of developing grain, and previous studies had found them suitable (Gordon 1975; Cross, 1977). However, under the environmental conditions of the present experiment, dehydration was slow. Plots of the data did not show an upper asymptote and the lower one had not been reached by the final sampling date. A straight

line, with y as % moisture, gave a better fit (higher R^2) to the data than the logistic curves, even after their fourth iteration. Although the $\hat{y}_0$'s were not stabilized by then, it was decided that further iteration was not worthwhile. The straight line, Y (% moisture) = $\beta_0 + \beta_1$ X (days from anthesis), was used as the simplest statistical description of the trends in the data over the time it was collected.

3.3.2 Results

The linear regression equations and data points for each genotype are shown in figure 3.1. Estimated statistics of these equations are given in table 3.1.1 Estimated t-tests for differences among the Y-intercepts (β_0's) and regression coefficients (β_1's) of the genotypes are given in table 3.1.2. The β_0's of Gamut and Pembina, which averaged 86.9%, were significantly different at P = 0.05 from all the others. The differences among the β_0's of the other genotypes divided them into two groups of over-lapping significance. These were Karamu and Tim x Pem with an average of 80.2%, and Tim x Pem, Timgalen and Sonora with an average of 78.2% moisture at anthesis. Comparison of the β_1's showed that Pembina was significantly different at P = 0.05 from Gamut and Sonora, while all the other differences were non-significant. Considering both regression statistics, Gamut, Pembina and Sonora were all different from one another and from the other genotypes, which formed a single significance grouping.

Despite the equations being non-asymptotic, the y-intercept (i.e. the maximum positive value) was similar to the upper asymptotes of logistic curves fitted in previous studies (Gordon, 1975; Cross, 1977). In these, the upper asymptote of all genotypes in every environment was 78.2% moisture. Previous studies also showed that the differences, in % moisture equations among the genotypes, were not consistent in different environments. The differences tended to be less when grain development was faster in warmer environments (Gordon, 1975; Cross, 1977).

The moisture content of grain, along with colour and hardness, usually determines when it is ready for harvesting (Andersen, 1965). The normal range is 10-20% moisture content, so harvest-ripeness (HR) was arbitrarily defined as 17.5% for the purpose of comparing development among genotypes. The level was chosen at the higher end of the scale as development was relatively slow in the environment of the study. It also concurred with agricultural practice in New Zealand.

The number of days from anthesis to harvest-ripeness is shown in table 3.2.1.Estimated t-statistics for the differences are given in table 3.2.2. No difference was significant at P = 0.10. Previous studies have shown that significance groupings for days to harvest-ripeness among the genotypes Gamut, Timgalen, Pembina, Sonora and Karamu were not consistent in different environments (Gordon, 1975; Cross, 1977). The groupings tended to form over-lapping series in cool environments (12-18^{o}C) and the differences disappeared in a warm one (20-30^{o}C) (Cross, 1977). However, the genotypes were compared over all the investigations by ranking them in order of increasing number of days from anthesis to harvest-ripeness in each environment. There was a general tendency for Gamut to have a longer period of grain development to harvest-ripeness and for Pembina and Timgalen to take fewer days (Gordon, 1975; Cross, 1977). The trend was the same in the present study.

3.4 Embryo Maturity

3.4.1 Methods

A dormancy-breaking germination test was used to determine embryo maturity. Twenty fresh grains per sample were placed on Whatman no. 1. filter paper in a 9 cm petri dish with an imbibing solution of 0.2% w/v aqueous potassium nitrate. The tests were carried out in a germination cabinet where conditions were 20^{o}C in the light for 16 hours alternating with 15^{o}C in the dark for 8 hours. This incorporated several of the International Seed Testing Association's recommendations for breaking dormancy (Anon, 1966, 1976) and was used by Gordon (1975). The grains were counted as germinated if any embryo tissue was visible at five days.

As the test is designed to break dormancy, lack of germination would have been due either to embryonic immaturity or dead seeds. However, previous extensive testing of grains which failed to germinate in the dormancy-breaking tests, with tetrazolium, showed that embryo inviability was extremely rare (Gordon, 1975). Therefore, the results of the present dormancy breaking tests were assumed to measure only embryo maturity (EM), which can be expressed as:-

$$EM\% = \frac{D}{T} \times 100$$ where D is the number of germinated grains and T is the total number of grains.

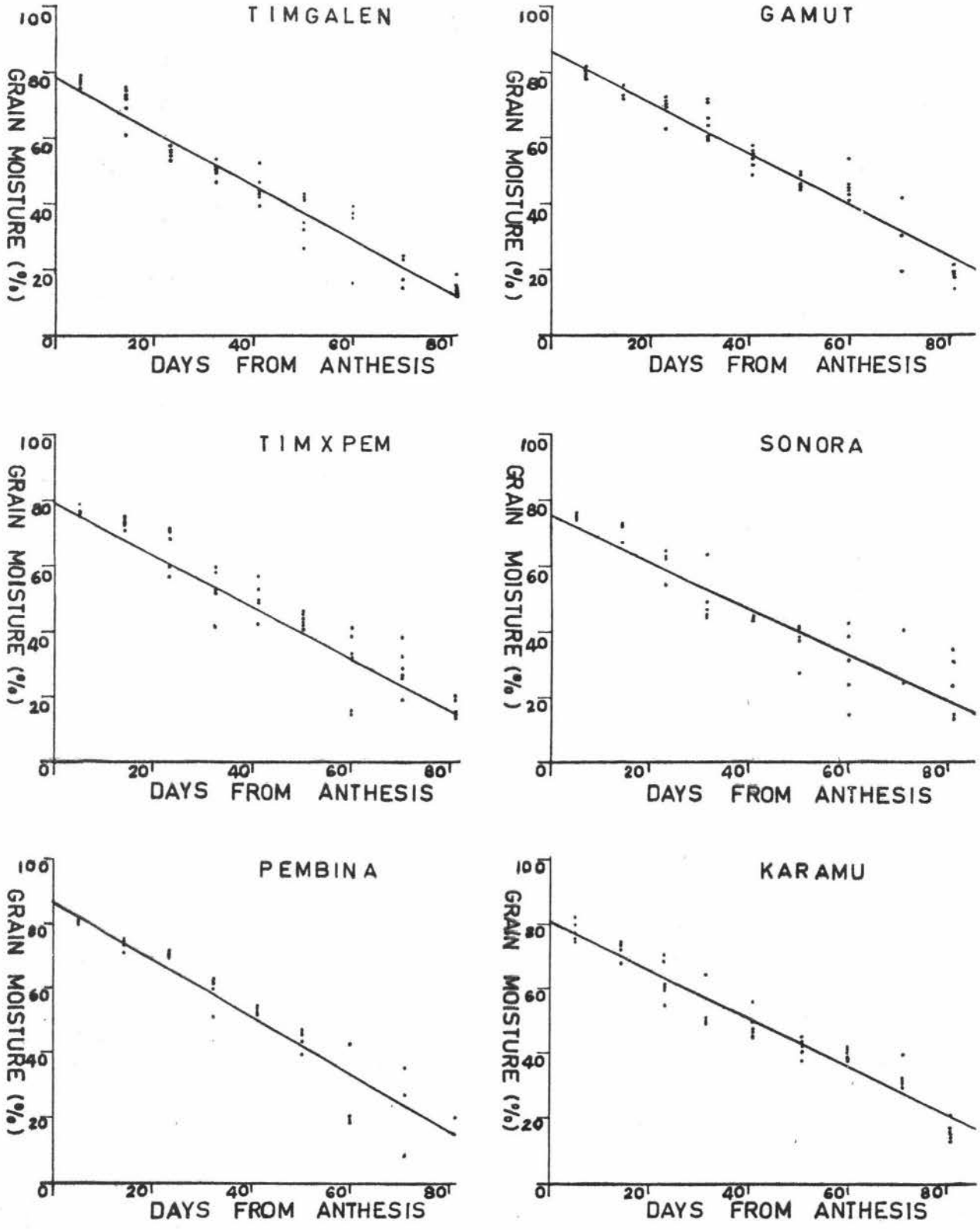


FIGURE 3.1

CHANGES IN PERCENTAGE MOISTURE

DURING GRAIN DEVELOPMENT IN

SIX WHEAT GENOTYPES

TABLE 3.1.1 ESTIMATED STATISTICS FOR EQUATIONS ($Y = \beta_0 + \beta_1 X$) DESCRIBING CHANGES IN % GRAIN MOISTURE DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM X PEM	PEMBINA	SONORA	KARAMU
β_0	86.2412	78.7520	79.1158	87.1429	76.5993	81.0268
s.e of β_0	0.7496	0.7522	1.2346	1.0503	1.2655	0.7167
β_1	-0.7780	-0.8168	-0.7821	-0.8983	-0.7117	-0.7538
s.e of β_1	0.0308	0.0299	0.0477	0.0469	0.0495	0.0283
R^2	0.9341	0.9408	0.8512	0.9105	0.8516	0.9391
$F_{regressn}$	638.21	746.65	268.95	366.35	206.61	709.29
	*[2]	*	*	*	(NS)[1]	*
$\sigma_{y.x}$	4.9799	5.1225	8.3960	6.2344	7.5266	4.8114

[1] (NS) non-significant at the 5% level

[2] * 0.05 >P

TABLE 3.1.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF REGRESSION STATISTICS OF EQUATIONS FOR % GRAIN MOISTURE

β_1 \ β_0	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	-----	7.0524 **[4]	4.9333 **	0.66987 NS	6.5553 **	5.0279 **
TIMGALEN	0.9040 NS[1]	------	0.2768 NS	6.4951 **	1.4623 NS	2.1895 *
TIM x PEM	0.0722 NS	0.1954 NS	-----	0.1845 NS	1.7628 (*)	1.3387 NS
PEMBINA	2.1438 *[3]	1.4654 NS	0.2504 NS	----	6.4111 **	2.1994 *
SONORA	1.1372 NS	1.8177 (*)[3]	1.2693 NS	2.7364 **	----	3.0443 **
KARAMU	0.5785 NS	1.5307 NS	0.5102 NS	0.3841 NS	0.2384 NS	----

SIGNIFICANCE SYMBOLS

[1] NS - non significant at the 10% level

[2] (*) - 0.10 >P >0.05

[3] * - 0.05 >P >0.01

[4] ** - 0.01 >P

TABLE 3.2.1 ESTIMATED NUMBER OF DAYS FROM ANTHESIS TO HARVEST-RIPENESS (17.5% MOISTURE) OF WHEAT GRAIN OF SIX GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
X	88.4	75.0	78.8	77.5	83.0	84.3
s.e. X	6.7	6.5	11.1	7.3	11.1	6.3

TABLE 3.2.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "DAYS TO HARVEST-RIPENESS"

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	1.4319	0.7396	1.0863	0.4092	0.4316
	NS	NS	NS	NS	NS
TIMGALEN	------	0.2994	0.2593	0.6264	1.0027
		NS	NS	NS	NS
TIM x PEM		------	0.3355	0.3299	0.4257
			NS	NS	NS
PEMBINA			-----	0.4139	0.7065
				NS	NS
SONORA				-----	0.0955
					NS

SIGNIFICANCE SYMBOLS

NS - non-significant at 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P 0.01

** - 0.01 >P

3.4.2 Results

Previous studies (Gordon, 1975; Cross, 1977) suggested that logistic or quadratic logistic curves were appropriate. These were fitted with days from anthesis as the X variate and logit of adjusted percent embryo maturity as the Y variable. The upper asymptote was 100%. The fitted curves for the logistic equations and the data points for each genotype are shown in figure 3.2. The curves are shown superimposed in figure 3.3. Estimated statistics of these equations in their linear form and the estimated t-statistics for differences among them are given in tables 3.3.1 and 3.3.2.

The regression coefficient (β's) divided the curves for percent embryo maturity into two groups of overlapping significance at P = 0.05. One group comprised Timgalen, Sonora, Gamut and Tim x Pem. The second group included Gamut and Tim x Pem as well as Pembina and Karamu. However, data were insufficient for Pembina and particularly for Karamu, especially over the period when a rapid increase in embryo maturity would be expected. The $\hat{\beta}_1$'s for these two genotypes are therefore unreliable. The $\hat{\beta}_0$ for Sonora was significantly different to those of Tim x Pem, Timgalen and Karamu at P = 0.05. Although no other significant differences were found, embryo maturity did appear to be delayed in Pembina and Karamu.

The numbers of days to reach 50% embryo maturity for each genotype were estimated and appear in table 3.4.1. Estimated t-statistics of differences among genotypes are shown in table 3.4.2 Karamu was significantly different from all but Pembina at P = 0.05 and all other differences were non significant.

Estimates of % embryo maturity at harvest-ripeness and the estimates of t-statistics for their differences are shown in tables 3.5.1 and 3.5.2.

At P = 0.10 there were two significantly different groups. The higher probability level was used because the variability in the data might obscure real differences. For the group comprising Timgalen, Sonora, Pembina and Tim x Pem, % embryo maturity at harvest ripeness was 84.7. For Karamu and Pembina embryo maturity averaged 33.5% at harvest-ripeness. Gordon (1975) found that embryo maturity in Pembina was significantly lower (P = 0.05) than that of Timgalen, Gamut and Sonora. Cross (1977) found that Pembina embryos did not reach maturity before harvest-ripeness in 12-18^{o}C environments and percent embryo maturity was significantly lower at P = 0.05 in an 20-30^{o}C environment.

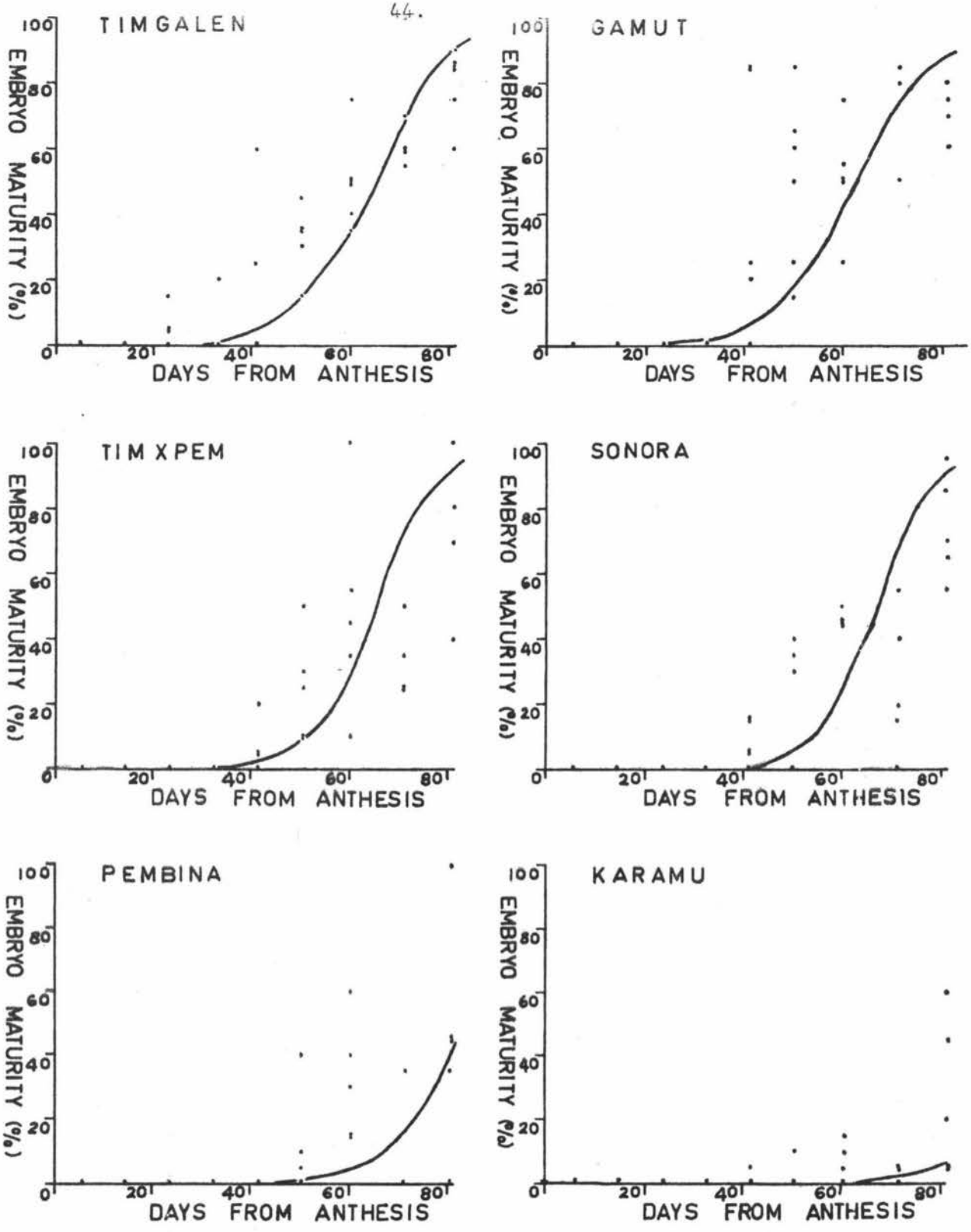


FIGURE 3.2

CHANGES IN % EMBRYO MATURITY

DURING GRAIN DEVELOPMENT

IN SIX WHEAT GENOTYPES

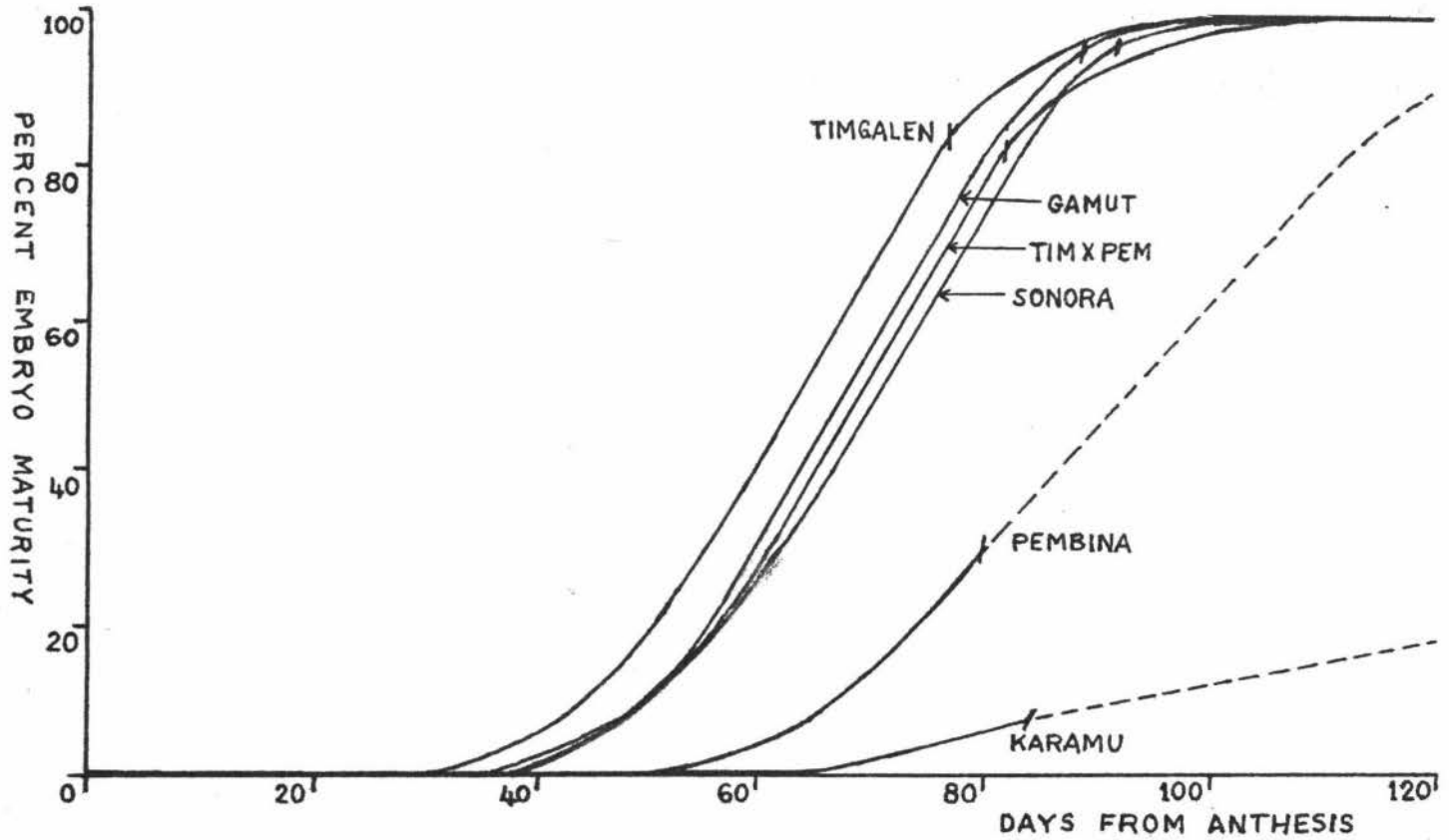


FIGURE 3.3

LOGISTIC CURVES DESCRIBING PERCENT EMBRYO MATURITY DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

(/ - HARVEST RIPENESS)

TABLE 3.3.1 ESTIMATED STATISTICS FOR EQUATIONS (LOGIT $Y=\beta_0 + \beta_1 X$) DESCRIBING CHANGES IN % EMBRYO MATURITY DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
β_0	-8.2813	-8.1171	-8.1447	-8.5248	-9.1870	-8.0080
s.e. of β_0	0.3420	0.2895	0.3795	0.3407	0.3244	0.2371
β_1	0.1236	0.1281	0.1196	0.0905	0.1321	0.0533
s.e. of β_1	0.0140	0.0170	0.0148	0.0136	0.0131	0.0096
R	0.6456	0.7570	0.6141	0.5314	0.6939	0.3770
$F_{regression}$	78.333 (NS)	143.23 (NS)	65.239 (NS)	44.230 (NS)	102.02 (NS)	30.851 (NS)
$\sigma_{y.x}$	2.2298	1.8858	2.4233	2.1119	2.2170	1.6798

[1](NS) - non-significant at the 5% level

TABLE 3.3.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF REGRESSION STATISTICS OF EQUATIONS FOR % EMBRYO MATURITY

β_1 β_0	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	----	0.3365 NS	0.2667 NS	0.5044 NS	1.8996 (*)	0.6567 NS
TIMGALEN	0.2556 NS	----	0.0546 NS	0.9119 NS	2.4280 **	0.2916 NS
TIM x PEM	0.1988 NS	0.0904 NS	----	1.4885	2.3293 *	0.3055 NS
PEMBINA	1.6988 (*)	2.1744 *	1.4478 NS	-----	1.3916 NS	0.8805 NS
SONORA	0.4405 NS	0.2365 NS	0.7088 NS	2.2017 *	----	2.8888 **
KARAMU	4.1453 **	5.2114 **	3.7596 **	0.1922 NS	4.8498 **	----

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level
(*) - 0.10 > P > 0.05
* - 0.05 > P > 0.01
** - 0.01 > P

TABLE 3.4.1 ESTIMATED NUMBER OF DAYS TO 50% EMBRYO MATURITY DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
X	66.97	63.59	68.12	94.30	69.50	150.22
s.e X	18.5	15.0	20.8	25.0	17.20	37.38

TABLE 3.4.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "DAYS TO 50% EMBRYO MATURITY"

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	0.1417	0.0580	0.8752	0.1020	1.9956
	NS	NS	NS	NS	(*)
TIMGALEN	------	0.2750	1.0500	0.2607	2.1509
		NS	NS	NS	*
TIM x PEM		-----	0.1922	0.0376	2.1578
			NS	NS	*
SONORA			-----	0.8120	1.2634
				NS	NS
KARAMU				----	1.9601
					(*)

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level
(*) - 0.10 >P >0.05
* - 0.05 >P >0.01
** - 0.01 >P

TABLE 3.5.1 ESTIMATED % EMBRYO MATURITY AT HARVEST-RIPENESS (HR) OF GRAIN OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
Days to HR	88.4	75.0	78.3	77.5	83.0	84.3
Y_{HR} (Logits)	2.640	1.489	1.277	-0.823	1.783	-0.561
Y_{HR} (%)	93.3	81.6	78.3	30.6	85.6	36.4
se Y_{HR} (Logits)	0.843	0.543	0.800	0.612	0.7321	0.4741

TABLE 3.5.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "% EMBRYO MATURITY AT HARVEST-RIPENESS"

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	1.1478	1.1723	3.3429	0.7676	3.3097
	NS	NS	**	NS	**
TIMGALEN	-----	1.1977	2.8258	0.3220	2.8488
		NS	**	NS	**
TIM x PEM		-----	2.1559	0.5413	1.9765
			*	NS	(*)
PEMBINA			-----	2.7311	0.4511
				**	NS
SONORA				-----	2.6874
					**

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level
(*) - 0.10 >P >0.05
* - 0.05 >P >0.01
** - 0.01 >P

Other genotypes, including Karamu, formed different overlapping groups in the different environments (Cross 1977).

3.5 Grain Dormancy

3.5.1 Methods

As noted in 3.5.1, non-germination can be due to embryo immaturity, dormancy or inviability. Assuming inviability to be trivial in the present case (as found by Gordon, 1975), lack of germination in a standard germination test could be due to either or both the other causes. The standard germination test was as follows:- Twenty fresh grains per sample were germinated on Whatman no. 1 filter paper in a 9 cm petri dish kept at a constant $20^{o}C$ in the dark. Distilled water was the imbibition medium and germinated grains were counted at five days. The test gives directly the germination percentage (GM%), which is the percentage of embryos which are both mature and non-dormant. GM% = 100G/T, where G is the number of germinated grains and T is the total number of grains in the sample.

Embryo dormancy, the proportion of mature embryos which do not germinate under normal germinative conditions, can be estimated by combining the results of the standard germination test with those of the dormancy-breaking one. The dormancy-breaking germination test gave an estimate of the percentage of embryos capable of germinating ("mature"), regardless of grain dormancy. Dormancy (D) is the proportion of grain which are not germinable under standard conditions (1-GM) excluding those with immature embryos (1-EM), out of the total number of grain with mature embryos (EM).

$$D = \left[(1 - GM) - (1 - EM)\right] / EM$$
$$= (EM - GM) / EM$$
$$= 1 - (GM/EM)$$

For curve-fitting purposes, dormancy was expressed as a percentage, and for samples taken soon after anthesis, when all embryos were immature (GM = 0 and EM = 0), it was set at 100%. However, it does not have real meaning until some embryos have reached maturity (EM >0). Subject to that limitation, if percent dormancy is greater than zero, then some actual dormancy-imposing mechanism does exist.

However, the estimates of both embryo maturity and dormancy are based on results of the dormancy-breaking test. The values obtained in that test depend on the efficiency of the method used

to break dormancy. A more efficient method of breaking dormancy (some possibilities for which include puncturing the grain-coat or excising the embryos) might result in higher estimates of embryo maturity. As the same standard germination test would be used, estimates of dormancy (1 - (GM/EM)) would also be increased, as (GM/EM) would be reduced.

3.5.2. Results

Logistic or quadratic logistic equations were considered appropriate (Gordon, 1975; Cross, 1977). They were fitted with days from anthesis as the X variable, logit of adjusted % dormancy as the Y-variable and 100% as the upper asymptote. The fitted curves of the logistic equations and the data points for each genotype appear in figure 3.4 and are shown superimposed in figure 3.5. Estimated statistics for the equations in their linear form appear in table 3.5.1. The estimated t-statistics for the differences among β_0's and β_1's are given in table 3.5.2. At P = 0.10, the only significant differences in the regression coefficients (β_1) were between Gamut and each of TimxPem, Pembina and Karamu. The y-intercept (β_0) of Timgalen was significantly different from all others at P = 0.05. The other β_0's formed an over-lapping series of differences at P = 0.05, in which the only significant ones were between Karamu and Sonora, Karamu and Tim x Pem, and Pembina with Tim x Pem.

Considering both regression statistics, four distinct patterns of decreasing dormancy were apparent. Timgalen was different from all the others; Gamut and Sonora were similar; Tim x Pem was different from all the others; while Karamu and Pembina were similar. Gordon (1975) found different patterns of decreasing dormancy (P = 0.05) for each of Timgalen, Gamut, Sonora and Pembina, although the differences were less between genotypes of the same grain-coat colour. In 12-18°C environments, for genotypes in which some embryos did reach maturity, the pattern of decrease in dormancy of each genotype was significantly different (Cross, 1977). In the 20-30°C environment, only Karamu was different (Cross, 1977).

The number of days taken from anthesis to median dormancy is shown in table 3.7.1. Estimated t-statistics for the differences among genotypes are given in table 3.7.2. At P = 0.10, the differences separated the genotypes into two groups. The white-grained wheats,

Timgalen and Gamut, formed a group which reached 50% dormancy in significantly fewer days. The red-grained wheats, Pembina and Karamu, as well as Tim x Pem comprised the other group. In Tim x Pem, most of the grain-coats were red, as this population would show an F_2 segregation ratio for grain-coat colour and red is dominant to white (Freed *et al*, 1976). The grouping of Tim x Pem with the dormant red wheats showed there was an association between dormancy and the red gene(s) of Pembina. Sonora was not significantly different at P = 0.10 from Gamut nor from Tim x Pem. Previous observations have also been that, although red-grained, Sonora showed not much more dormancy than white-grained wheats (Gordon, 1975), indicating that the association of dormancy with redness is not invariable.

Estimated percent dormancy at harvest-ripeness and t-statistics for differences among genotypes are presented in tables 3.5.2. and 3.8.2. At P = 0.05, the genotypes were divided into two groups, again correlating with grain-coat colour. The first was Gamut and Timgalen with dormancy at 0 and 6.1 respectively. The other was Tim x Pem, Pembina and Karamu, where the average percent dormancy at harvest ripeness was 82.6. Sonora, with 22.3% dormancy was not significantly different from either Timgalen or Tim x Pem. These results are comparable with those of Gordon (1975) and Cross (1977). Gordon (1975) found that Gamut and Timgalen were not different at P = 0.05, but Sonora and Pembina were different. Cross (1977) found the only separation into distinct groups at P = 0.05 occurred in his 20-30°C environment, where Timgalen, Gamut and Sonora formed one group, and Karamu with Pembina another.

As grain may not be harvested until some days after it first attains harvest-ripeness, the duration of dormancy relative to harvest-ripeness was of interest. Dormancy was considered to be virtually finished when it dropped to 10% (D10). Estimates of the number of days to D10 are given in table 3.9.1 and the t-statistics of their differences in table 3.9.2. Due to the high variances of these estimates, there were no significant differences at P = 0.10. However, comparison of days to D10 with days to harvest-ripeness could give an indication of the relative duration of useful dormancy in each genotype.

Both white-grained wheats had less than 10% dormancy by the time they reached harvest-ripeness. Gamut reached 10% dormancy 16 days before harvest-ripeness and Timgalen reached it 4 days before. Sonora had a 2-day period after arvest-ripeness before dormancy fell to 10%, which is

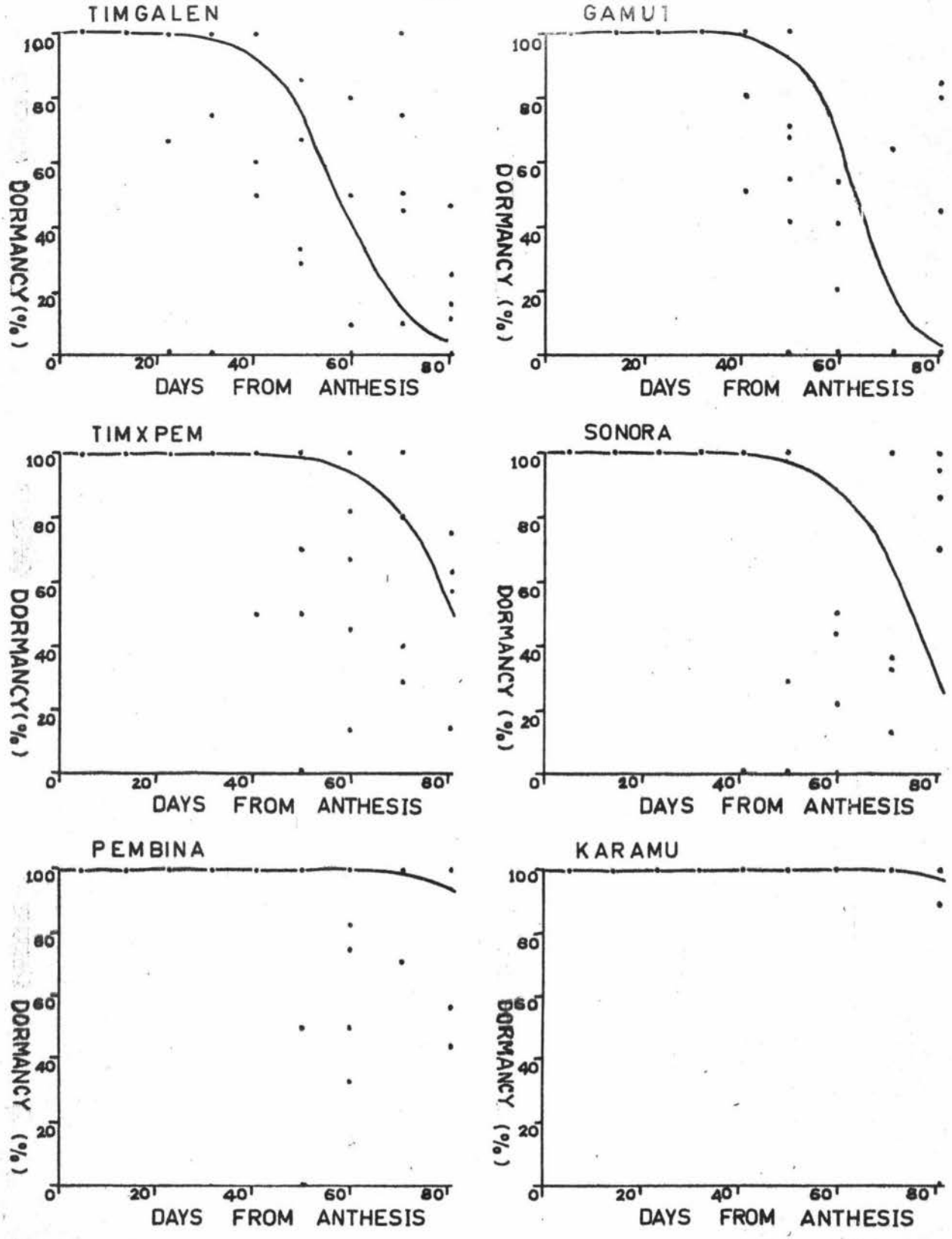


FIGURE 3.4

CHANGES IN PERCENT DORMANCY

DURING GRAIN DEVELOPMENT

IN SIX WHEAT GENOTYPES

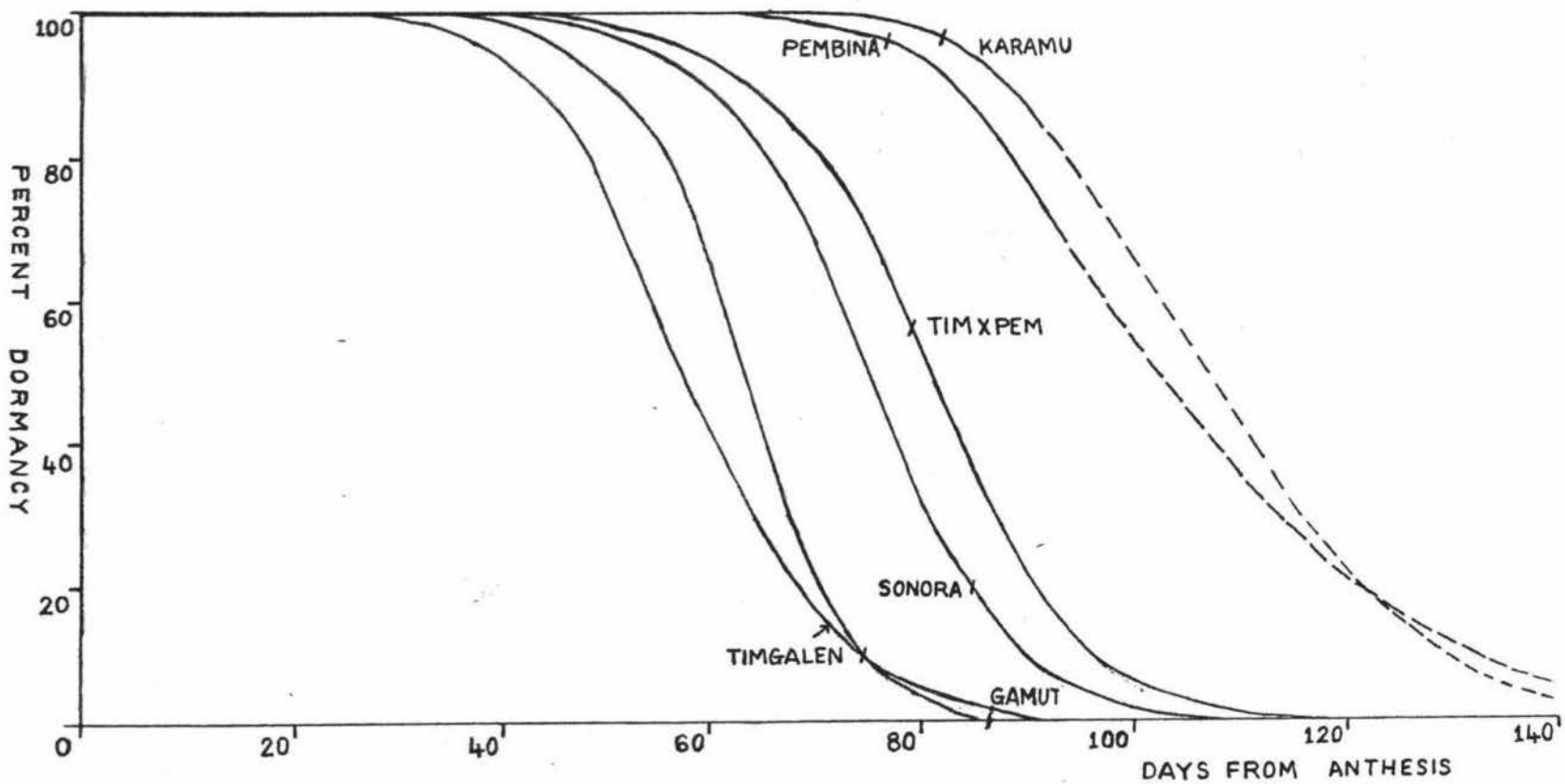


FIGURE 3.5

LOGISTIC CURVES DESCRIBING PERCENT DORMANCY DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

(/ - HARVEST RIPENESS)

TABLE 3.6.1 ESTIMATED STATISTICS FOR EQUATIONS (LOGIT Y $=\beta_0 + \beta_1 X$) DESCRIBING CHANGES IN % DORMANCY DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
β_0	11.9734	8.0438	10.5140	10.6082	10.5969	12.6560
s.e β_0	0.6471	0.8383	0.6024	0.6349	0.7628	0.6626
β_1	-0.1962	-0.1436	-0.1311	-0.0935	-0.1427	-0.1111
s.e. β_1	0.0275	0.0321	0.0235	0.0260	0.0298	0.0313
R^2	.5538	.3037	.4316	.2544	.3420	.2189
$F_{Regression}$	50.8785 (NS)	20.0600 (NS)	31.1331 (NS)	12.9678 (NS)	22.8720 (NS)	12.6100 (NS)
$\sigma_{y.x}$	4.1198	5.6499	3.8461	3.8840	3.0327	4.6934

[1](NS) non-significant at the 5% level

TABLE 3.6.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF REGRESSION STATISTICS OF EQUATIONS FOR % DORMANCY

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	----	3.7107 **	1.6507 NS	1.5058 NS	1.3761 NS	0.7370 NS
TIMGALEN	1.2446 NS	-----	2.0843 *	2.4386 **	2.2526 **	4.3163 **
TIM x PEM	1.8000 (*)	0.1196 NS	-----	4.5260 **	0.0926 NS	2.3920 *
PEMBINA	2.7139 **	1.2130 NS	1.0730 NS	-----	0.0114 NS	1.8650 (*)
SONORA	1.3195 NS	0.0206 NS	0.2965 NS	1.2441 NS	----	2.0379 *
KARAMU	2.0437 *	0.7253 NS	0.5113 NS	0.1268 NS	0.7315 NS	----

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level
(*) - 0.10 >P >0.05
* - 0.05 >P >0.01
** - 0.01 >P

TABLE 3.7.1 ESTIMATED NUMBER OF DAYS TO 50% DORMANCY DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
X	61.0	56.0	80.2	113.5	74.3	113.9
s.e X	21.5	37.9	30.6	47.0	36.4	44.2

TABLE 3.7.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "DAYS TO 50% DORMANCY"

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	1.9677	2.3012	2.4929	1.6536	2.7707
	NS	*	**	NS	**
TIMGALEN	----	3.6617	2.7362	2.3090	3.0397
		**	**	*	**
PEMBINA		------	0.2766	0.2931	1.6655
			NS	NS	NS
SONORA			----	1.7840	0.0175
				(*)	NS
KARAMU				----	1.9715
					(*)

SIGNIFICANCE SYMBOLS

NS - non-significant at 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P >0.01

** - 0.01 >P

TABLE 3.8.1 ESTIMATED % DORMANCY AT HARVEST-RIPENESS (HR) OF GRAIN OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
Days to HR	88.4	75.0	78.8	77.5	88.0	84.3
Y_{HR} (logits)	-5.363	-2.726	0.186	3.359	-1.253	3.295
Y_{HR} (%)	0	6.1	54.7	96.6	22.3	96.4
s.e. Y_{HR} (logits)	1.680	1.574	1.270	1.330	0.985	1.512

TABLE 3.8.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "% DORMANCY AT HARVEST-RIPENESS

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	1.1454	2.6349	4.0705	2.1105	3.8311
	NS	**	**	**	**
TIMGALEN	----	2.6822	2.9528	0.7933	2.7589
		**	**	NS	**
TIM x PEM		----	0.8218	0.7289	1.5750
			NS	NS	NS
PEMBINA			----	**	NS
SONORA				----	**

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P >0.01

** - 0.01 >P

TABLE 3.9.1 ESTIMATED NUMBER OF DAYS TO 10% DORMANCY (D10) DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
Days to HR	88.4	75.0	78.8	77.5	88.0	84.3
X	72.2	71.3	97.0	137.0	89.7	133.7
s.e X	21.8	40.3	31.5	50.3	35.8	49.2
D10 - HR	-16.2	-3.7	19.2	50.5	1.7	49.4

TABLE 3.9.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "DAYS TO 10% DORMANCY"

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	0.0198	0.6450	1.1812	0.4160	1.1436
	NS	NS	NS	NS	NS
TIMGALEN	----	0.5063	1.0187	0.3403	0.9817
		NS	NS	NS	NS
TIM x PEM		----	0.1325	0.1250	0.6296
			NS	NS	NS
PEMBINA			----	0.7666	0.0459
				NS	NS
SONORA				----	0.7249
					NS

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P >0.01

** - 0.01 >P

too short to be of practical value in field situations. Tim x Pem had a 20 day period of useful dormancy after harvest-ripeness. Pembina and Karamu both had relatively long periods of dormancy after harvest-ripeness:- 50 and 49 days respectively. These may be over-estimated, as the data on which they are based was insufficient to give a reliable estimate of β_1. Gordon (1975) found that Pembina had a 33 day period of dormancy after harvest-ripeness. Cross (1977) found that in the warmer (20-30°C) where dormancy relative to harvest-ripeness was more marked than at 12-18°C, Pembina and Karamu both had a 7 day period of dormancy and Sonora did not reach harvest-ripeness until after D10.

3.6. Discussion

The data described by the curves for embryo maturity and dormancy were extremely variable. The major cause of the variability was probably changes in the environment during grain development. An attempt was made to reduce the variability by analysing a subset of the data, which was all recorded within a short period (14-29 May). The estimated statistics of logistic curves in their linear form for embryo maturity and dormancy are given in tables A6 and A8. The estimated t-statistics for differences among the regression statistics are given in tables A7 and A9 respectively. In general, the variance about regression, $\sigma^2_{y.x}$, was higher for these curves than for the ones fitted to all the data. Consideration of both regression statistics for the curves, at P = 0.10, showed that the genotypes formed overlapping series of groups for both embryo maturity and dormancy.

The variability among genotypes in an experiment of this sort could be reduced either by growing the plants in a controlled environment or by having a very much bigger population in which a large number of heads would reach anthesis on the same day. It would be preferable to have both options, so that all the sampled heads would have developed under the same environment. Under non-ideal conditions, extensive recording of sampling dates for every test and weather data might allow some of the variability to be accounted for during the statistical analysis. In addition, examination of larger samples and a greater number of replicates would help to reduce the variability.

The germinability of each genotype at harvest-ripeness is shown

in figure 3.7. Lack of germinability is due to embryonic immaturity as well as to grain dormancy. In this environment, the white-grained wheats Gamut and Timgalen were highly germinable and so was the red-grained Sonora. Pembina and Karamu had a lower germinability than Tim x Pem, mainly due to a higher proportion of embryo immaturity. As discussed previously, the dormancy-breaking method may affect the estimates of embryo maturity, which might alter the proportions of germinable and non-germinable grains.

There appeared to be some grain dormancy, after the embryos became mature, in each of the wheat varieties that have been investigated. With the present type of test, complete lack of dormancy would have shown up as a straight line at 0% dormancy. Such a line did not appear in the present study, nor was it found for any of these genotypes in other environments (Gordon, 1975; Cross, 1977). Several studies have tested the germination of embryos excised from grain of white, red-dormant and red-non-dormant genotypes and compared the results with the germination of intact grains of the genotypes, during development from anthesis to well after harvest-ripeness. In all cases the germination of intact grains occurred later than the germination of excised embryos, although the delay was much greater in the case of dormant wheats (King, 1976; Stoy and Sundin, 1976).

The general observation that white-grains are not dormant is because germination tests have usually been made close to and after harvest-ripeness, by which time their dormancy has long since disappeared. Consequently, the dormancy-imposing mechanism of intact white grains were not of agricultural interest. The dormancy may simply be an oxygen permeability effect, which is reduced as the pericarp tissues become senescent. The dormancy in intact red grains is more prolonged and may persist for some time after harvest-ripeness has been attained. It may be due to a greater duration of the same mechanism which imposes dormancy on white grains during the early stages of their development. However, it is possible that some additional mechanism accounts for the persistence of dormancy in some of the red-grained wheats. Such a mechanism could come into operation in conjunction with the activities associated with the formation of grain-coat pigments.

These tests gave the basic information about the development of each genotype with respect to dehydration to harvest-ripeness, embryo maturity and dormancy. They were necessary to provide a background for the interpretation of information about the other processes investigated in the genotypes during grain development.

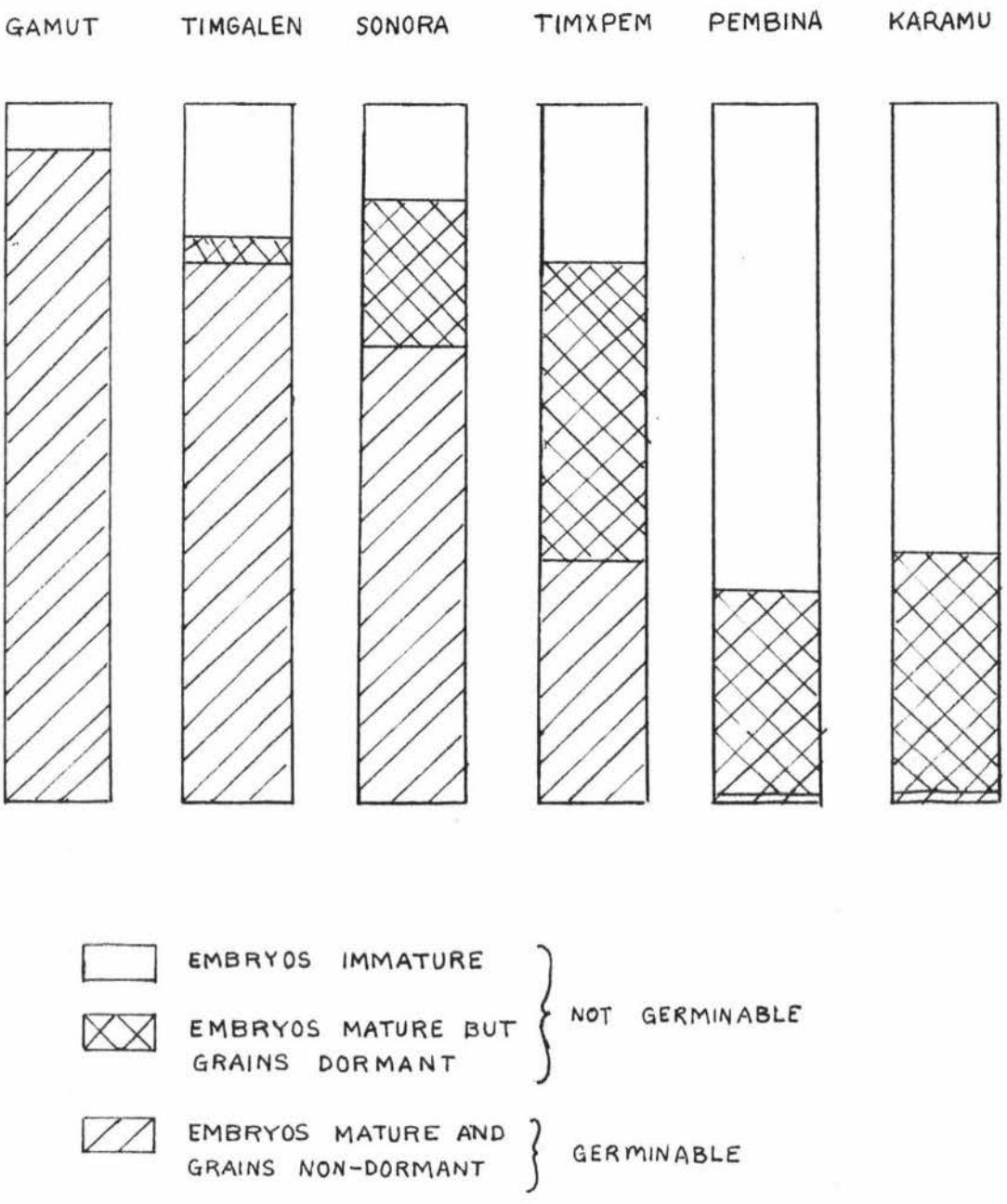


FIGURE 3.7

GERMINABILITY AT HARVEST-RIPENESS IN GRAIN OF SIX WHEAT GENOTYPES

CHAPTER 4.

THE DEVELOPMENT OF MATURE GRAIN-COAT COLOUR IN WHEAT

4.1 Introduction

The development of grain-coat colour was investigated in the four red genotypes of the study and compared to that of the two white genotypes. The changes in concentration of flavonoid precursors of grain-coat pigments (which were discussed in sections 2.2.1 and 2.2.4) were measured during grain maturation. The differences in pigmentation of mature red and white grains was investigated. The role of the phenolases in catalysing production of grain-coat pigments was discussed in section 2.2.4. Their activity during grain development was assayed by measuring the coloured products of their action on various substrate solutions. Phenol was used as a substrate for mono-phenolase activity. The diphenol, catechol, was used as a substrate for diphenolase activity. In addition, one of the flavonoid precursors of phlobaphene, flavan-3-ol, was used as a substrate. It also has two phenolic hydroxyl groups.

4.2 Precursors of the grain-coat pigments

4.2.1 Methods

Concentration of the flavonoid precursors of condensed tannins were determined by the vanillin-sulphuric acid method (Swain and Hillis, 1959). The test is specific for flavonoids with a single bond at the C2-C3 position of the "A" ring and free meta-oriented hydroxyl groups on the "B" ring (see figure 2.1) i.e. for flavan-3-ols, flavan-3,4-diols, dihydrochalcones and anthocyanins (Sarkar and Howarth, 1976). Where the C4 of the heterocyclic ring is involved in bonds to oxygen or to other flavonoids, i.e. in flavanones, dihydroflavanols and polymers, the vanillin reaction does not occur (Swain and Goldstein, 1964). Ten grains per sample were weighed, broken up in a grinder and ground with a mortar and pestle in 10 cm^3 of 10% (v/v) aqueous methanol. They were extracted for 24 hours in the dark with occasional shaking, then centrifuged at approximately 2000XG (3000RPM, 4 way swing-out rotor-head, BTL bench centrifuge) for 10 minutes. One cm^3 aliquots of supernatant were put in each of 2 glass-stoppered vials and diluted with 1.0 cm^3 of water. Four cm^3 of freshly prepared 1% (w/v) vanillin in 70% (v/v) aqueous

sulphuric acid (vanillin reagent) was added during 10-15 seconds from a burette to vial A, and 4.0 cm^3 of 70% sulphuric acid to vial B. The vials were shaken well in iced water and left for exactly 15 minutes at room temperature for the colour to develop. Vial C contained 4.0 cm vanillin reagent and 2.0 cm^3 water. The absorbances were read in a Spectronic-20 at 500 nm against a blank containing 4.0 cm^3 70% H_2SO_4 and 2.0 cm^3 water. The absorbance due to reaction of flavonoids with the vanillin reagent was calculated as A-(B + C).

A standard curve was constructed using standard solutions of flavan-3-ol ((+)-catechin, Flüka, Germany) at 2,4,6,8,10,12,14,17 and 18 $\mu g/cm^3$. Assays of all concentrations were done in a randomised complete block design with three replications. The absorbance of vanillin solutions is known to change with time (Swain and Hillis, 1959, Gordon 1975), but the ageing effect was not detectable within the 3-hour period during which the solution was used. Vanillin reagent was prepared freshly twice a day for the samples. The predictive equation, $Y = \beta_0+\beta_1 X$, was estimated as

$$Y = 46.9439 \quad (A-(B + C)) \quad - 0.0183,$$

Where Y is the equivalent concentration of flavan-3-ol in $\mu g/cm^3$ and (A-(B + C)) is the absorbance due to reaction with the vanillin solution.

The coefficient of determination, R^2, was 0.9978 and F for regression was 11569.97 (significant at P = 0.01). The estimated standard error of the estimate was 0.2492 and estimated standard errors of β_0 and β_1 were 0.1048 and 0.4364 respectively. Final concentration in grain samples was expressed as mg/gDW, which was calculated by multiplying the amount per 1.0 cm^3 aliquot of extractant in $\mu g/cm^3$ by 10/w where 10 is the volume of the extractant in cm^3 and w is the oven dry weight of the sample in g.

4.2.2 Results and Discussion

The regression equations fitted to the data were quadratic ($Y = \beta_0 + \beta_1 X + \beta_2 X^2$) and cubic ($Y = \beta_0+ \beta_1 X + \beta_2 X^2 + \beta_3 X^3$), as well as exponential ($LnY = \beta_0+ \beta_1 X$) and quadratic exponential ($LnY = \beta_0+ \beta_1 X + \beta_2 X^2$). In every case, Y was the concentration of flavanols (i.e. flavan-3-ols and flavan-3,4-diols) in $\mu g/g$ dry weight and X was days from anthesis. The exponential equations gave very poor fits (R^2 was less than 0.15). The R^2's for the quadratic and

cubic equations for each genotype were very similar, so the cubic was discarded. The quadratic equations and data points for each genotype are shown in figure 4.1. Estimated statistics of these equations are given in table 4.1.1.The estimated t-statistics among the estimated regression statistics are given in tables 4.1.2 and 4.1.3.

Differences among the β_0's at P = 0.05 grouped the genotypes into an overlapping series, in which Timgalen was significantly different from each of Karamu, Sonora and Tim x Pem. For the β_1's, Timgalen was different at P = 0.05 from all but Gamut; Gamut was different from Karamu. For the $\hat{\beta}_2$'s at P = 0.05, Timgalen differed from all the others, as did Karamu; Gamut was different from Tim x Pem. Gamut, Sonora and Pembina were not significantly different for any of the regression statistics. But Timgalen, Karamu and Tim x Pem were each significantly different at P = 0.05 to all the others in at least one of their regression statistics. There was no apparent connection between the concentration of flavanols at any stage of development and grain colour.

Previously, it had been found that the best fit to data on flavanol concentrations in developing wheat grain was provided by Makeham equations (Gordon, 1975; Cross, 1977). The linear form of this type of equation is

$\ln Y = \beta_0 + \beta_1 X + \beta_2 B^X$, where X = days/7, Y = concentration of flavanols and B is a slope parameter estimated initially from an eye-fit curve (Gordon, 1975). The equations for the genotypes were all significantly different at P = 0.05 for the $\hat{\beta}_0$'s but there was no consistent segregation for either regression coefficient in the various environments (Gordon, 1975; Cross, 1977). The present study confirms the lack of association between concentration of the pigment precursors in whole grain during development and grain-coat colour at maturity. The implication of all these observations was considered to be that the difference in grain-coat colour was due to the presence of particular enzymes in the red wheats. These would act specifically in the polymerisation of flavonoids in the grain-coat to form the red-coloured phlobaphenes.

There was a common pattern of change during grain development for all genotypes. There was a rapid decline in flavanol concentration for about thirty days after anthesis, then a gradual decline to a minimum at around fifty days. The concentration rose gradually as harvest-ripeness was approached. A similar pattern has been found previously (Gordon, 1975; Cross, 1977) but the concentrations were

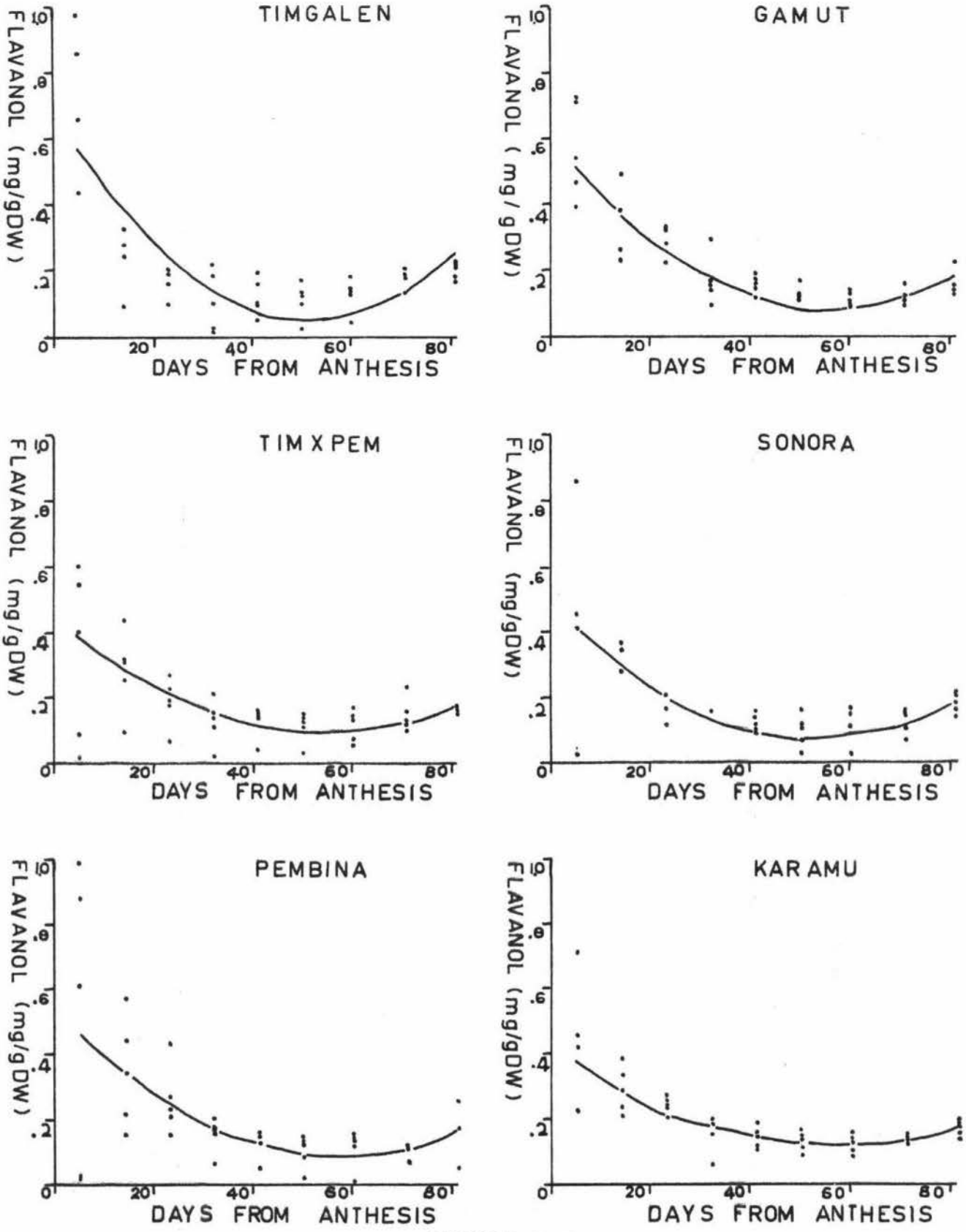


FIGURE 4.1
CHANGES IN FLAVANOL CONCENTRATION IN
DEVELOPING WHEAT GRAINS
OF SIX GENOTYPES

TABLE 4.1.1 ESTIMATED STATISTICS FOR EQUATIONS ($Y = \beta_0 + \beta_1 X + \beta_2 X^2$) DESCRIBING CHANGES IN CONCENTRATION OF FLAVANOLS ($\mu g/gDW$) IN GRAIN OF SIX WHEAT GENOTYPES DURING DEVELOPMENT

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
β_0	605.559	700.901	442.633	539.244	495.771	421.772
s.e β_0	37.5232	55.4003	42.5179	67.5491	59.0611	42.9864
β_1	-18.736	-25.505	-12.704	-15.641	-16.458	-10.493
s.e.β_1	2.0355	3.0053	2.3065	3.6648	3.2006	2.3318
β_2	0.1669	0.2499	0.1175	0.1360	0.1569	0.0901
s.e. β_2	0.0230	0.0339	0.0260	0.0414	0.0362	0.0263
R^2	0.7651	0.6836	0.5160	0.4626	0 5106	0.4656
$F_{Regression}$	65.1555 (NS)	42.1410 (NS)	20.7884 (NS)	15.0618 (NS)	16.6907 (NS)	17.4251 (NS)
$\sigma_{y.x}$	79.8496	116.4832	89.3966	134.9394	112.9415	91.4749

[1] (NS) non-significant at the 5% level

TABLE 4.1.2 ESTIMATED t-STATISTICS OF DIFFERENCES AMONG PAIRS OF Y-INTERCEPTS OF EQUATIONS FOR FLAVANOL CONCENTRATION

β_1 \ β_0	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	1.4243	2.8738	0.8588	1.5696	3.2217
	NS	**	NS	NS	**
TIMGALEN	----	3.6041	1.8505	2.5332	3.9807
		**	(*)	**	**
TIM x PEM		----	0.6755	0.8789	0.3450
			NS	NS	NS
PEMBINA				0.4845	2.4540
				NS	NS
SONORA				----	1.0130
					NS

TABLE 4.1.3 ESTIMATED t-STATISTICS OF DIFFERENCES AMONG PAIRS OF REGRESSION COEFFICIENTS OF EQUATIONS FOR FLAVANOL CONCENTRATION

β_1 \ β_0	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	----	1.9669	1.8407	0.6501	0.4793	2.5434
		(*)	(*)	NS	NS	**
TIMGALEN	2.026	----	2,4568	2.0814	2.0403	3.9466
	*		**	*	*	**
TIM x PEM	1.4231	0.3614	----	0.0259	1.1721	0.6739
	NS	NS		NS	NS	NS
PEMBINA	0.6520	2.1286	0.3795	----	0.1864	1.0504
	NS	*	NS		NS	NS
SONORA	0.2332	1.8752	1.0653	0.3800	----	1.5289
	NS	(*)	NS	NS		NS
KARAMU	2.1952	3.7244	0.7408	0.2089	1.4929	----
	*	**	NS	NS	NS	

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P >0.01

** - 0.01 >P

TABLE 4.2.1 ESTIMATED CONCENTRATION OF FLAVANOLS (µg/gDW) AT HARVEST-RIPENESS (HR) OF GRAINS OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
Days to HR	88.4	75.0	78.8	77.5	83.0	84.3
Y_{HR} (µg/gDW)	253.49	193.68	171.21	143.95	210.66	177.48
s.e. Y_{HR}	13.27	53.71	42.60	42.09	48.09	44.78

TABLE 4.2.2 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF ESTIMATES OF "FLAVANOL CONCENTRATION AT HARVEST-RIPENESS"

	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	1.0800	1.8439	2.4818	0.8585	1.6273
	NS	(*)	**	NS	NS
TIMGALEN	----	0.3270	0.7287	0.2355	0.2316
		NS	NS	NS	NS
TIM x PEM		----	0.6104	0.6282	0.1014
			NS	NS	NS
PEMBINA			----	1.0438	0.5480
				NS	NS
SONORA				----	0.5049
					NS
KARAMU					-----

SIGNIFICANT SYMBOLS

NS - non-significant at the 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P >0.01

** - 0.01 >P

much higher. The differences were probably due to the treatment of the grains between harvesting and assaying. Grains should be used fresh (e.g. Gordon, 1975), because the concentrations in the dried, stored grains in this study were a hundred times lower. If they must be stored, deep-freezing in sealed containers is probably the most efficient method.

At harvest-ripeness, the only significant differences at P = 0.10 of flavanol concentration among the genotypes were between Gamut and Tim x Pem or between Gamut and Pembina. However, these differences were probably due to the longer period to harvest-ripeness in Gamut, which meant that the estimate of its flavanol concentration at harvest-ripeness lay in the rising part of the quadratic curve. The estimates of flavanol concentration at harvest-ripeness and the estimated t-statistics of the differences among genotypes are given in tables 4.2.1 and 4.2.2.

As well as being a precursor of the pericarp pigment, flavan-3-ol has been implicated as a germination inhibitor (Miyamoto and Everson, 1958; Miyamoto *et al*, 1961; Stoy and Sundin, 1976). However, no inhibition of germination or alpha-amylase response has been found in grains imbibed in solutions of flavan-3-ol (Gordon, 1975). There was apparently no association between the concentrations of endogenous flavanols in whole grains and dormancy. Nor was there any association between them and grain colour at maturity.

4.3 Grain Coat Pigments

4.3.1 Qualitative Identification

The evidence that the pigment in mature grain-coats of wheat is phlobaphene is not conclusive. (see discussion in 2.2.1 based on the works of Lewicki, 1929 and Miyamoto and Everson, 1958). Miyamoto and Everson (1958) based their suggestion on a correlation between levels of flavan-3-ol at one early stage of grain development with grain-coat colour at maturity. That finding has been contradicted by the similar patterns of change in levels of flavanols over the whole period of grain development found by Gordon (1975) and Cross (1977) as well as in the present study. Consequently, an attempt was made to investigate the flavonoid pigments of the grain-coats.

The method was based on those outlined by Walker (1972) and Ribéreau-Gayon (1972). A 100 grains of mature colour of Timgalen, Pembina, Sonora and Karamu were each ground and twice extracted in

10 cm^3 of 1% v/v HCl in absolute methanol in the dark at 40°C for 12 hours. The extracts were filtered through whatman no. 1 paper under partial vacuum. The filtrates were partitioned 3 times with equal volumes of petroleum ether to remove carotenoid endosperm pigments. Small aliquots of the methanolic fractions were tested with 10% (w/v) vanillin in 50% v/v conc. Hcl. All genotypes gave a strong positive reaction, indicating the presence of flavanols and/or anthocyanins.

Each methanolic fraction was condensed under vacuum at 30°C and and repeatedly spotted onto dry whatman no. 1 chromatography paper. The paper had previously been washed overnight in descending 3% v/v aqueous conc. HCl to remove phenolic contaminants. The spotted extracts were partitioned with descending n-Butanol, acetic acid, water (40:10:22) (Walker, 1972) for 12 hours. The solvent front was marked and the chromatograms were air dried. No coloured spots due to anthocyanins were visible and examination under ultra-violet light, before and after fuming with ammonia, failed to reveal any spots with Rf's comparable to those listed for phenolics or flavanoids (Ribéreau-Gayon, 1972). As the vanillin test was positive, it is probable that there was insufficient material on the chromatograms to be visible. There was not enough grain left to repeat the investigation.

The residue of the grain after the 2x10 absolute methanol extractants had been filtered off was re-extracted in absolute methanol until the filtrates gave a negative reaction with vanillin/HCl. This indicated that all the phenolic acids, flavonoids, lower molecular weight polymers had been removed. Then the residue was extracted in 50% aqueous methanol in the dark at 4°C. The flavonoids removed by this treatment are more highly polymerised and would be immobile in paper chromatography. They are not necessarily insoluble in absolute methanol, but may be linked to polysaccharides in the cell walls, or to proteins, by hydrogen bonds which can only be broken when there is water in the extraction medium (Ribéreau-Gayon, 1972). Vanillin/HCl gave a positive test for all four genotypes.

The remaining residue of the grains was tested directly for tannins which were not extractable in either absolute or 50% methanol. These are probably high molecular weight polymers which would be firmly bound to other cell constituents. The vanillin/HCl test was positive for the three red-grained genotypes, Sonora, Pembina and Karamu, and negative for Timgalen. The results of these successive

extractions provide indirect evidence that the grain-coat pigment of wheat is high molecular weight with flavonoid components i.e. probably a phlobaphene. It would be interesting to perform the successive extractions at different times over the period in which the grain turned from green to the mature colour. A coincidence of a positive test in the final residue with first appearance of mature grain-coat colour would provide further evidence.

4.3.2 Development During Maturation

Noticeable differences in mature grain-coat colour have been observed not only between red- and white-grained genotypes, but also among red-grained ones (Cross, 1977). A comparative scoring system based on those differences was devised by Gordon (pers. comm, 1975) and used by Cross (1977). The system was based on a set of carefully chosen genotypes which were ranked according to the intensity of colour from 1 to 10 and used as standards for comparing other genotypes. The time when the grains turn from green to their mature colour can be determined by sampling during their development and testing them against the colour standards.

Comparative colour tests are improved by immersing the grain in 5% w/v aqueous sodium hydroxide solution for half an hour before recording the grain colour. Green pericarps remain bright green; mature pericarps of white-grained wheats turn a light straw-yellow colour; and mature pericarps of red-grained wheats turn to a red-brown colour. The red-brown colour is probably due to phlorogenic acid, which is formed from phlobaphene in the presence of strong alkali (Miyamoto and Everson, 1958).

However, it was found that, with ageing of the stored colour-standard grains, the response to 5% sodium hydroxide had changed. Differences among the colour standards were difficult to detect and scores given to experimental genotypes were not comparable with those given by other workers previously (Cross, 1977). Genotypes at the lower end of the red part of the scale were comparatively lighter than previously, which could be a possible indication of changes in the grain-coat during after-ripening. When grains of plants grown from the original colour-standard grains were tested, it was found that they had to be ranked in a completely different order. The indication is that the environment affected the intensity of grain redness.

All the red genotypes in this experiment were as dark as darkest colour standard, as was also observed by Cross (1977). The indication was that the presence of the dominant allele at one of the loci for redness is sufficient for the grain to show maximal pigmentation. So there was no detectable difference in intensity of colour among the red-grained genotypes at harvest ripeness. The white-grained genotypes were distinctly different from the red-grained ones. Samples were not taken frequently enough to detect any slight differences in the occurrence of the change from green to the mature colour. In each genotype it occurred between 32 and 41 days.

The embryos of Tim x Pem had F_3 genotypes, but the grain-coats were maternal tissue. Therefore, the population should show the F_2 segregation ratio for grain-coat colour. The F_2 segregation, observed after testing with sodium hydroxide, was 328 red and 94 white grains, which is a reasonable fit to the 3:1 expectation ($X^2 = 1.6715$; $0.20 < P < 0.50$) for a single dominant gene. Pembina, like Sonora and Karamu, apparently has only one gene for redness, although it is not necessarily the same gene in each of the genotypes.

4.4 Activity of the Enzymes of Pigment Formation

4.4.1 Monophenolase Activity with Phenol as Substrate

4.4.1.1. Methods

Phenolases produce a dark pigment in the presence of 1% w/v aqueous phenol (Fraser and Gfeller, 1936). The reaction has been used as a seed-testing procedure (Anon, 1966). The development or otherwise of colour when imbibed seeds were soaked in 1% phenol was used to indicate phenolase activity in the grain-coat. Mature wheat grains of various genotypes have been reported to show a colour range from black through brown to white (Joshi and Banerjee, 1969). They have also shown an increase in the intensity of the reaction with increasing dry matter content (Fraser and Gfeller, 1936).

A set of scores based on intensity of colour and relative area of stained pericarp was devised. It is given in table A4.1. Two grains per sample were soaked in distilled water for 16 hours, drained and let dry for an hour, then immersed in 3 cm^3 of 1% w/v phenol solution for 4 hours. They were drained and scored for phenol colour reaction.

4.4.1.2. Results and Discussion

Logistic and quadratic logistic equations were fitted, with days from anthesis as X, phenol colour score as Y and 10 (the maximum score) as the upper asymptote. The fitted curves and the R 's for the equations for each genotype were similar, so the quadratic logistic was discarded. Data points and the logistic curves fitted to them are shown in figure 4.2. Estimated statistics of the equations are shown in table 4.3.1. and the estimated t-test for differences among them are given in table 4.3.2. The differences at P = 0.05 among the y-intercepts formed the genotypes into a series of overlapping groups. Among these, Sonora was different from all but Timgalen; Pembina was different from all but Karamu; and Karamu was different to Timgalen and Gamut. There were no significant differences at P = 0.05 among the regression coefficients.

By 70 days after anthesis, grain from all the genotypes tested reacted strongly with the phenol solution over extensive areas of the grain-coat. The pattern of phenolase activity during development was similar in both the red-grained and white-grained genotypes investigated. The only significant differences (P = 0.05) at harvest-ripeness were between Pembina and either Sonora or Tim x Pem (see tables 4.7 and 4.8). However, the crudeness of the technique and the 16-hour pre-incubation in water may have obscured real differences in phenolase activity of unimbibed grains.

Somewhat more sophisticated techniques have been used to extract phenolases from wheat grain at various stages of development. The monophenolase activity (with L-tryosine not phenol as substrate) was very low in comparison with diphenolase activity (Taneja *et al*, 1974; Taneja and Sachar, 1974; Kruger, 1976). It was not detectable until the grains were turning colour, when there was an increase in activity for up to 5 days before it disappeared again (Taneja *et al*, 1974). Although its appearance coincided with the colour-change, it is not known whether this enzymic activity would be involved in the formation of pigments. It is unlikely to be involved in the actual polymerisation. However, it might have an effect through alteration of substrates of the polymerising enzyme, which might change their reactivity.

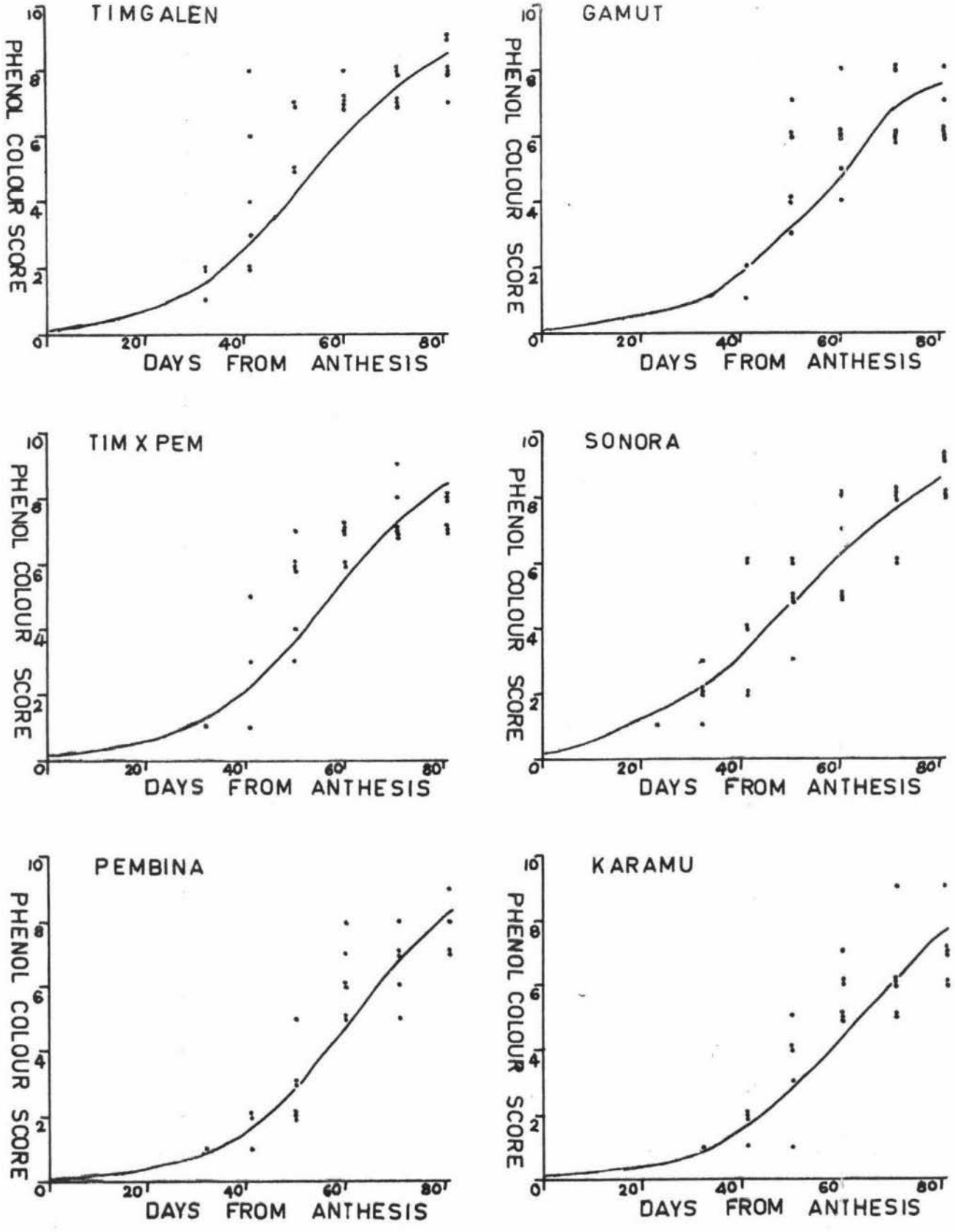


FIGURE 4.2

CHANGES IN PHENOL COLOUR SCORE

DURING GRAIN DEVELOPMENT

OF SIX WHEAT GENOTYPES

TABLE 4.3.1 ESTIMATED STATISTICS FOR EQUATIONS (LOGIT Y = $\beta_0+\beta_1 X$) DESCRIBING CHANGES IN PHENOL COLOUR SCORE DURING GRAIN DEVELOPMENT OF SIX WHEAT GENOTYPES

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
β_0	-3.9959	-3.7296	-4.0940	-4.8379	-3.3014	-4.4330
s.e. β_0	0.1330	0.2555	0.1316	0.1108	0.1110	0.1260
β_1	0.0644	0.0683	0.0717	0.0793	0.0634	0.0700
s.e. β_1	0.0069	0.0132	0.0068	0.0059	0.0058	0.0065
R^2	0.7205	0.4397	0.7649	0.8527	0.7815	0.7784
$F_{Regression}$	87.626 (NS)	26.678 (NS)	110.599 (NS)	179.491 (NS)	121.585 (NS)	115.927 (NS)
$\sigma_{y.x}$	0.6960	0.3376	0.6889	0.5484	0.5809	0.6498

(NS) - non-significant at the 5% level

TABLE 4.3.2 ESTIMATED t-STATISTICS OF DIFFERENCES AMONG PAIRS OF REGRESSION STATISTICS (β_0's and β_1's) OF EQUATIONS FOR PHENOL COLOUR SCORE

β_1 \ β_0	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	-----	0.9243 NS	0.5245 NS	4.8644 **	4.0086 **	2.3861 *
TIMGALEN	0.2617 NS	----	0.6864 NS	3.9797 **	1.5371 NS	2.4692 **
TIM x PEM	0.7447 NS	0.0596 NS	----	9.7384 **	4.3501 **	1.8608 (*)
PEMBINA	1.6367 NS	0.7536 NS	0.8393 NS	----	9.7967	0.6153 NS
SONORA	0.1208 NS	0.3453 NS	0.8798 NS	1.9218 (*)	----	6.7389 **
KARAMU	0.5915 NS	0.1140 NS	0.1738 NS	1.0594 NS	0.7576 NS	----

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - 0.10 >P >0.05

* - 0.05 >P >0.01

** - 0.01 >P

4.4.2 Diphenolase Activity with Catechol as Substrate

4.4.2.1 Methods

The method was based on those of Taneja *et al*,(1974) and Kruger (1976). Twenty-five grains per sample were weighed, broken up, then ground with a mortar and pestle in 10.0 cm^3 of 0.05 M phosphate buffer at pH 6.6 (Appendix A4.2). The extracts were immediately centrifuged at 20,000 x G for 15 min at 4^oC in a Sorvall-RC5 centrifuge (13,000 r.p.m. with SS3 5 rotor head). The supernatants were decanted and refrigerated until the assays were performed a few minutes later. The substrate solutions of 0.01 M catechol (dihydroxyphenol) in 0.05 M phosphate buffer at pH 6.6 were made up in 10.0 cm^3 batches, which were preapred freshly for each assay. Substrate solutions and glassware used in the assay were kept at 25^oC for 2 min prior to the addition of enzyme extracts.

The Spectronic-20 Spectrophotometer was set at 430 nm and zeroed with a blank containing 6.0 cm phosphate buffer at pH 6.6. The absorbance due solely to the enzyme extract (B) was read from a blank containing 5.6 cm phosphate buffer and 0.4 cm of the extract. Each assay tube contained 1.6 cm of phosphate buffer and 4.0 cm of catechol substrate solution. Its absorbance was read (A), then 0.4 cm of enzyme extract was added and a stop-clock was set going simultaneously. The tube was shaken well and its absorbance was read after 60 seconds (C). The activity per 0.4 cm sample was expressed as $\left(C - (A + B)\right)$ absorbance units per minute. This is equivalent to the activity per grain as 0.04 of the extract was assayed and the extract contained 25 grains.

4.2.2.2. Results and Discussion

For each genotype at each sampling time, the mean phenolase activity, in absorbance units per grain per minute, and its standard error are shown in figure 4.3. There were no consistent differences between the red- and white-grained genotypes. Nor did any peak of activity consistently coincide with the time when the grains were turning colour (31-40 days after anthesis).

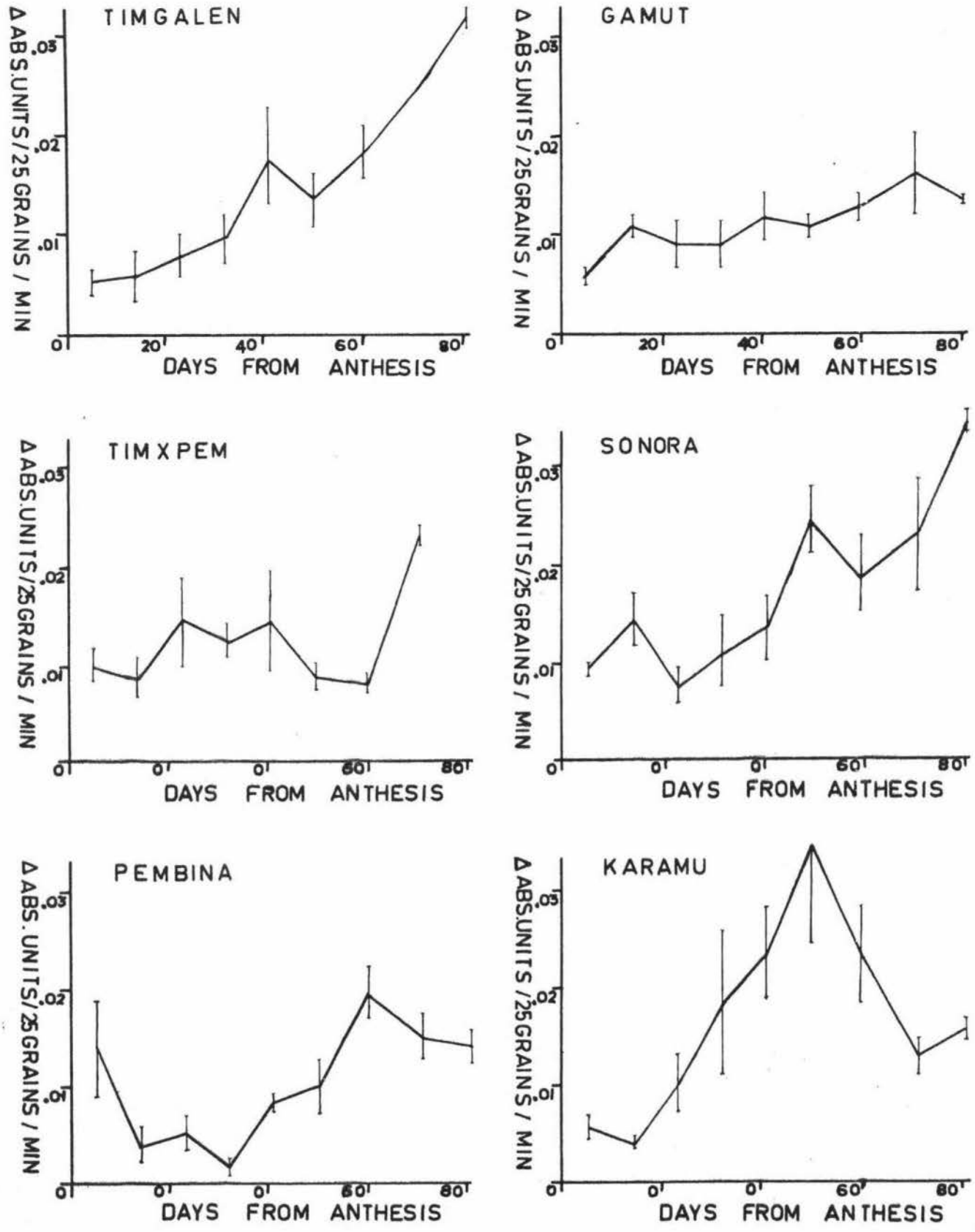


FIGURE 4.3

CHANGES IN ACTIVITY OF PHENOLASES FROM DEVELOPING WHEAT GRAINS WITH CATECHOL FOR SUBSTRATE

The time of peak diphenolase activity during the development of wheat grains has been found to differ considerably among various diphenolic substrates, including L-dihydrozyphenylalanine (L-DOPA), caffeic acid and catechol (Kruger, 1976). Also, the magnitude of peak maxima varied among the substrates. With catechol, phenolase activity was ten times greater than with caffeic acid and a hundred times greater than with L-DOPA (Kruger, 1976). The decrease in phenolase activity corresponded with increasing complexity of the substrate molecule, which would imply that phenolase has a lower affinity for the larger phenolic acids. Also, the differences in reactivity may be a reflection of different levels of activity of phenolase isozymes, which may differ in their affinities for the various substrates. Up to 12 phenolase isozymes are present in developing wheat grains (Kruger, 1976).

In the present study, the reactivity with catechol was of the same order of magnitude as L-DOPA. The reduced activity could be due to denaturation of the enzyme between the time when the grains were harvested and when the assays were done. It would be preferable to store the samples of grain in sealed containers below $0^{o}C$.

The precursors of the grain-coat pigment are more complex molecules than the diphenolic acids used as substrates in these assays. Activity of phenolases on the simpler molecules may give no indication of the activity of the phenolases which are expected to be involved in forming the red pigment. Because the concentration of the flavonoid precursors is similar in red and white grains (Gordon, 1975; Cross, 1977; Section 4.2.2), the phenolase involved in the polymerisation is probably quite specific in its substrate affinities. However, it may have several isozymes, as there are three genes for redness. It would not be expected to occur in the pericarps of white-grained genotypes.

4.4.3 Diphenolase Activity with Flavon-3-ol as Substrate

4.4.3.1 Methods

Twenty wheat grains were weighed and ground in 8.0 cm of 0.05 M chilled phosphate buffer at pH 6.6. The suspension was centrifuged at 20,000 x G for 15 min at $4^{o}C$ (Taneja *et al*, 1974). The supernatant was stored briefly under refrigeration. Flavan-3-ol ((+)-catechin, Fluka, Germany) was quickly dissolved in 25 mls of warm phosphate buffer, to give a 0.05 M substrate solution. The solution was used within 30 minutes of preparation because its reactivity increases with age, probably due to the formation of quinones, which

are more readily oxidised by phenolases (Kruger, 1976).

The reaction rate of an enzyme extract with flavan-3-ol was found to decrease rapidly with time (Kruger, 1976). Initial velocities of reaction were estimated by the use of a double blank system. The changes in absorbance were measured at 350 nm in a Hitachi spectrophotometer. Blank (A), containing 5.6 cm^3 of phosphate buffer, was used to set the zero. The absorbance due solely to the extract was found by adding 0.4 cm of the enzyme extract and reading the change in absorbance (A^1). The second value was used as the zero time reading for the assay. Blank B, containing 3.0 cm^3 of substrate solution and 2.6 cm^3 of phosphate buffer, was used to re-set the absorbance reading to zero. It allowed for any differences among the spectrophotometer tubes and automatically subtracted the absorbance of the substrate. For the assay, 0.4 cm^3 of the enzyme extract was added to B and a stop-clock set going simultaneously. The assay tube was mixed and aerated carefully, by inverting it several times against a clean glass stopper. Shaking may produce bubbles which interfere with absorbance readings. The absorbance was read after 60 seconds (C).

The activity of the 0.4 cm^3 aliquot of the enzyme extract was calculated as ($C-A^1$) absorbance units per minute. The result was multiplied by 8/(0.4 x 20), where 8 was the volume of the extraction medium in cm^3, 0.4 was the volume of the aliquot in cm^3 and 20 was the number of trains in the sample. The latter result is the phenolase activity, with a flavan-3-ol substrate, in absorbance units per grain.

4.4.3.2 Results and Discussion

The mean phenolase activities, in absorbance units per grain per minute with flavan-3-ol as substrate, are shown in figure 4.4 for each genotype. The general pattern of change during development was a two-fold increase in activity during the first 40 days after anthesis, i.e. until the grains were turning colour. The subsequent changes in activity were not consistent among all the genotypes, nor between genotypes of with the same grain-coat colour. The peak maxima were as high for the white grains as for the red ones.

The peak phenolase activities with flavan-3-ol were of the same order of magnitude as those which have been found previously for flavan-3-ol and L-DOPA (Kruger, 1976). However, the changes in activity occurred at different times during development. A two-fold increase in activity from anthesis to 30 days (probably when the grains were turning colour) was also found previously, in a red-grained, a

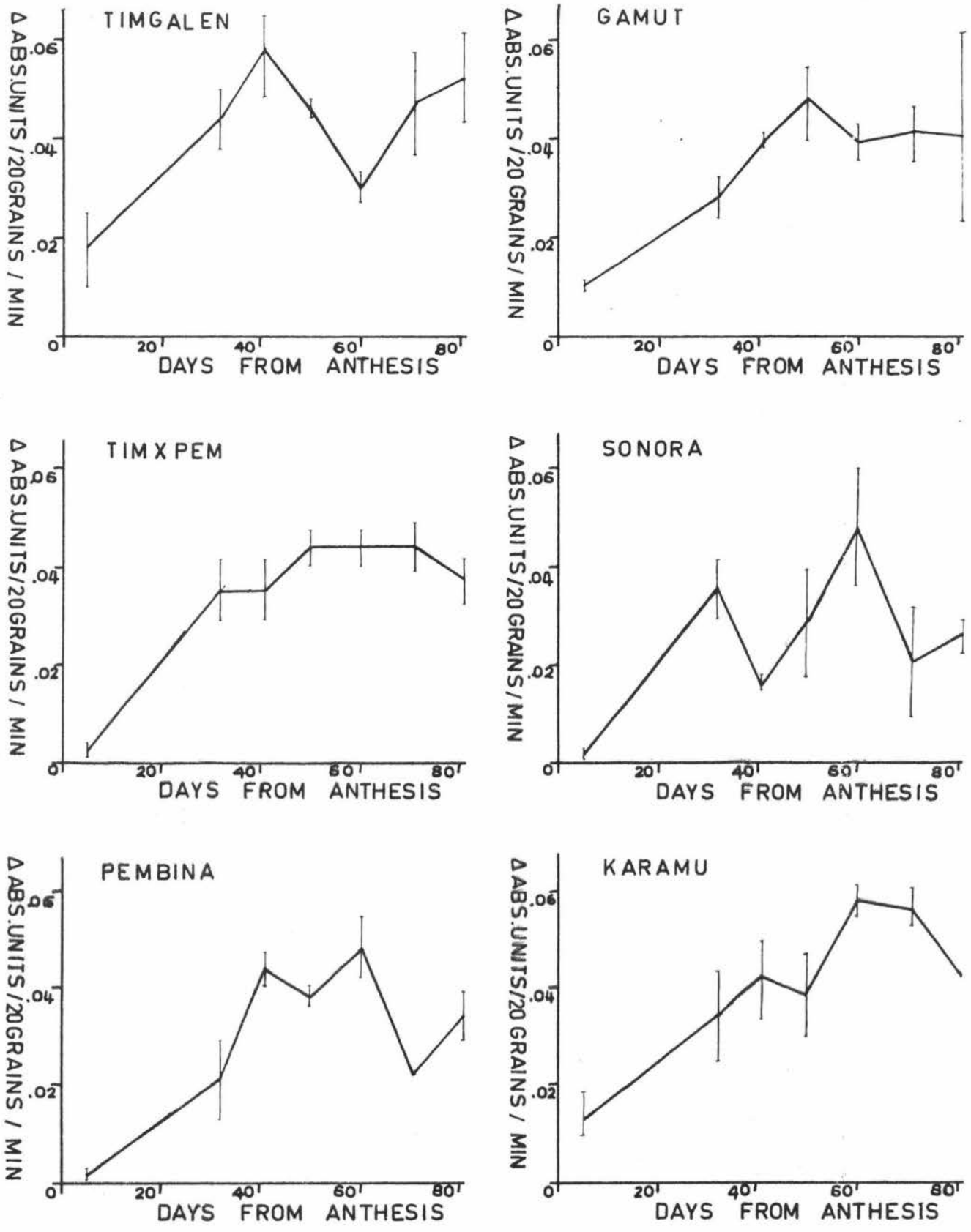


FIGURE 4.4

CHANGES IN ACTIVITY OF PHENOLASES FROM DEVELOPING WHEAT GRAINS WITH CATECHIN FOR SUBSTRATE

white-grained and a durum wheat (Kruger, 1976). In contrast to the present results, the activity subsequently decreased to very low levels in the mature grain (Kruger, 1976). It was possible that the moisture contents of grain, even in the final sample, were higher than those of mature grain in the previous study and phenolases were not yet inactivated.

It was possible that the methods used to extract and assay phenolase activity in all these studies were not sensitive enough to detect differences which may exist between red and white grains. Also, whole grains were used and their total phenolase activity may have obscured that of phenolases acting specifically in the testa to produce the pigment. The possible role of peroxidase in the formation of these pigments has not yet been considered. An investigation of the particular activity of phenolase (and possibly peroxidase) isozymes from the grain-coats of various wheat genotypes is required. A variety of flavanoid substrates should be used and the timing of peaks of phenolase activity during grain development should be related to the appearance of the grain-coat pigments.

CHAPTER 5.

ANALYSIS OF ABSCISIC ACID FROM DEVELOPING GRAINS

5.1 Introduction

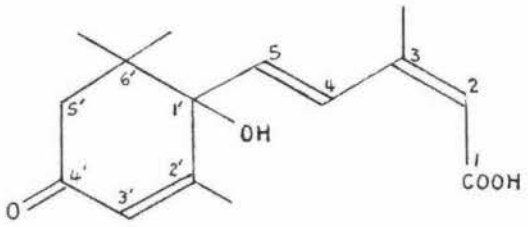
Abscisic acid is an asymmetric molecule with several types of isomers. Their structures and relative physiological activites have been reviewed (Milborrow, 1974). The configuration around a double bond in the side-chain differs in the two geometric isomers, which are 2-cis-4-trans-abscisic acid (ABA) and 2-trans-4-trans-absicic acid (t-ABA) (see figure 5.1). A solution of either t-ABA or ABA readily isomerises in the light to give a 1:1 mixture of the isomers. The double bond at the C2 position, in the biological precursors of abscisic acid, is synthesised in the trans configuration. It is probably isomerised at an early stage of the biosynthesis as tABA has not been found to be enzymatically isomerised to ABA (Milborrow, 1974). ABA is the active naturally occurring isomer in plant tissues and t-ABA is almost inactive in comparison (Milborrow, 1974). The activity of t-ABA was only 6% that of ABA in bean embryonic axes (Milborrow, 1974).

Owing to asymmetry at the C1 position, each geometric isomer has two optical isomers. The naturally occurring structure has (+) stereo-chemistry while synthetic abscisic acid has equal amounts of both (±). The unnatural (-) racemer is almost as active as the (+) one in many bioassay systems. including a dissected wheat embryo assay (Milborrow, 1974). The absolute configuration of the most active, naturally occurring form of abscisic acid is given in figure 5.1.

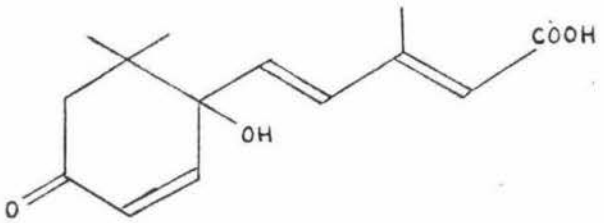
5.2. Methods

In the present study, abscisic acid was extracted from wheat grains at various stages of development. The extracts were analysed using the High Pressure Liquid Chromatography (HPLC) system suggested by Sweetser and Vatvars (1976). The system was sensitive to less than 10 ng of abscisic acid in a sample and the geometric isomers were separated by their different retention times. Initial identification of t-ABA and ABA in the samples was based on retention times as shown by peaks on the HPLC recorder printout, compared to those recorded for solutions of synthetic

I. GEOMETRIC ISOMERS.

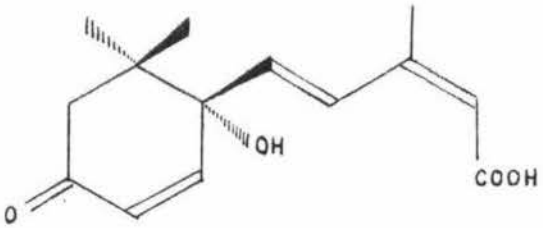


2-cis-4-trans-abscisic acid (ABA)



2-trans-4-trans-abscisic acid (t-ABA)

II. ABSOLUTE STEREOCHEMISTRY OF THE MOST BIOLOGICALLY ACTIVE ISOMER OF ABA.



(+) - (S) - ABA

FIGURE 5.1

THE STRUCTURE OF ABSCISIC ACID

mixed isomers (1:1 tABA:ABA)

Further confirmation of peak identity was obtained by collecting the fraction of the eluate which caused a particular peak and subjecting it to further tests. The "t-ABA" and "ABA" fractions were each co-injected with 1:1 mixed isomers (tABA:ABA) to ensure that peak maxima of the samples and the synthetic isomers coincided. The fractions containing the higher levels of "ABA" were bioassayed, to check that the peak was due to a biologically active compound, not merely one with the same retention time as ABA. Mass spectrometry provided the final identification of "tABA". The quantity of ABA and tABA in the samples was estimated from standard curves relating the peak area to the amount in the sample. The standard curves were estimated from the results of analysing solutions of various concentrations of (±) 1:1 tABA:ABA mixed isomers. The bioassay of ABA provided a quantitative check.

5.2.1 Extraction of Abscisic Acid

The extraction and purification procedure is summarised in figure 5.2. Solutions of abscisic acid should be shielded from direct light throughout the procedure to minimise photo-isomerisation. A determined weight and number of wheat grains (0.5 - 1.5 g) was ground in a modified Phillip's coffee grinder. The flour was transferred to a conical flask with the volume of 80% v/v aqueous Analar grade methanol required to give a 1:10 w/v suspension of it. The suspension was left, for extraction to occur, for 24 hours in the dark at $1^{o}C$. It was filtered through Whatman no. 1 paper into a Buchner flask under suction. The residue was resuspended in a volume of 80% methanol equal to that used before, and extracted for a further 24 hours at $1^{o}C$. It was refiltered and the two filtrates were bulked. The filtered 80% methanolic extracts were reduced to the aqueous phase in 100 ml, pear-shaped flasks, in a rotary evaporator at $35^{o}C$.

An equal volume of 25% concentrated ammonia solution was added to the aqueous extract, to form the more soluble ammonium salt of abscisic acid and reduce losses during purification. The excess ammonia was evaporated under vacuum at $35^{o}C$ and the total volume of the extract was brought down to 0.5 - 1.0 cm^3. This was transferred to a 10 cm^3 centrifuge tube, along with 2 x 0.5 cm^3 distilled water washings of the rotary evaporator flask, and refrigerated overnight. It was centrifuged at 20,000xG (13,000 rpm, SS35 rotor head) for 15 min. at $4^{o}C$ in a Sorvall-RC5 centrifuge. The supernatant was decanted and acidified to pH 3.0 (tested with narrow-

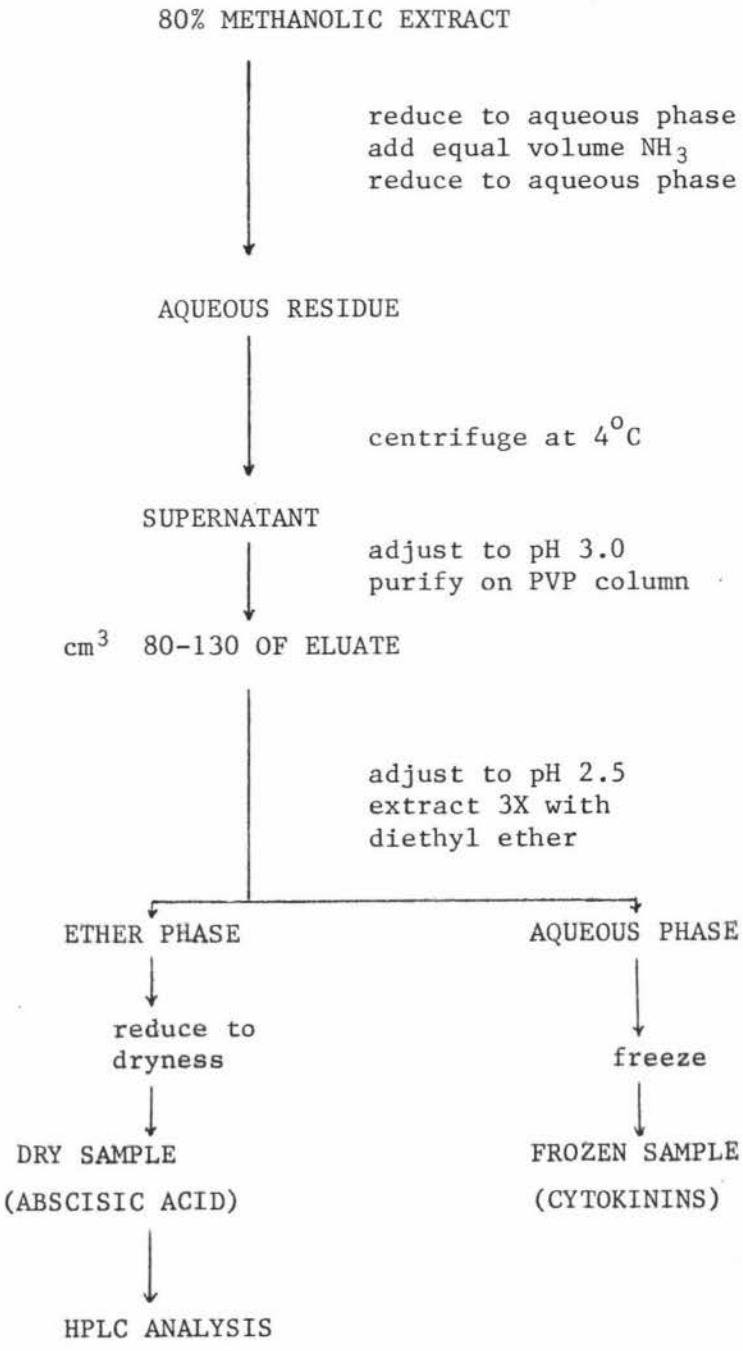


FIGURE 5.2
SUMMARY OF PROCEDURE FOR EXTRACTION OF
ABSCISIC ACID

range pH paper) with 10% v/v HCl. It was carefully layered onto the top of a polyvinyl pyrrolidine (PVP) column and eluted with 0.013 M phosphate buffer at pH 3.0 (Appendix 5.2). Details of the preparation and use of these columns are given in Appendix 5.1. PVP removed phenolic and other organic acid impurities (Glenn *et al*, 1972; Biddington and Thomas, 1976). The 50-130 ml fraction of the eluate, which contained abscisic acid and cytokinins (figure A5.1), was collected.

It was adjusted to pH 2.5 with 50% v/v HCl and shaken vigorously for 5 minutes each time with three equal separate volumes of freshly distilled diethyl ether (Appendix 5.) in 250 cm^3 separating funnels with glass stoppers. The pressure of vaporised ether was released at intervals. The aqueous phase, which contained cytokinins, was deep frozen for possible future use. The ether phase was reduced, half at a time, in 250 cm^3 round bottomed flasks at 35°C in a rotary evaporator. A partial vacuum was applied, then the tubing to the vacuum pump was closed to maximise recovery of the ether for redistillation and reuse. Clips to hold the sample flasks to the rotary evaporator were essential. The last cm^3 of the extract in ether was transferred to a Kimax screw-top test-tube, which had been altered to hold 1 cm^3 only. It was put in a vacuum-oven at 40°C and evaporated to dryness under a vacuum applied very gradually by water-tap suction. The dry extract was stored at 1°C in the dark, ready for HPLC analysis.

5.2.2 Analysis of Extracted Abscisic Acid

5.2.2.1 Separation by HPLC

The High Pressure Liquid Chromatograph used was made by Instrumentation Specialities Co. (ISCO). It comprised a model 1440 liquid chromatograph with a high pressure non-pulsating pump operated by a model 384 Dialagrad, a "Type 6" Optical Unit, a model UA-5 Absorbance Monitor and a model 568 Fraction Collector. In addition, a Rikadenki recorder was connected to the system and set with twice the absorbance units full scale (AUFS) of the ISCO recorder.

The chromatography column was 1.5 m long with an internal diameter of 2 mm. It was manually packed with Zipax-SCX strong cation exchange pellicules (Du Pont Instruments), which have a sulphonated fluoropolymer shell bonded to glass beads. Details of the preparation of this column, its design parameters and efficiency are given in Appendix 5.2. The operating parameters selected were distilled water acidified to pH 1.7 with conc. HNO_3 , a flow-rate of 20 cm^3/hr (560 psi) and 27°C. These gave good selectivity for abscisic acid (Sweetser and Vatvars, 1975),

and good peak separation, as discussed in Appendix 5.2.

The dry abscisic acid samples were prepared for introduction into the pH 1.7 HNO_3 flow at the top of the column. A minimum volume of 0.006 cm^3 (60µl) was required to ensure complete filling of the 0.005 cm^3 sample loop above the column. A volume of 0.012 cm^3 of pH 1.7 HNO_3 was added from a micropipette to the dry abscisic acid sample, which was kept in the dark at room temperature for 1-2 hours while it dissolved. Two HPLC analyses per sample were required to allow for any necessary changes of scale on the recorders, and to give duplicates for the bioassay. The sample was introduced onto the column by the action of a valve, which diverted the elution stream through the 0.005 cm^3 sample loop. Simultaneously the Fraction Collector was advanced a place, and the peak separator of the Absorbance Monitor was switched on. The identity of the sample was immediately recorded on the printouts and the first vial for the sample in the Fraction Collector was marked.

The absorbance of the eluate from the HPLC column was continuously recorded at 254 nm, as it passed through the flow-cell of the Optical Unit. Any substance that is mobile in a particular column has a typical retention time (or retention distance on the printout, if recorded at constant charg speed) from injection until appearance of a peak maximum can be used for initial identification of a sample peak. Synthetic (±) t-ABA and (±) ABA had retention times of 11.3 and 18.2 minutes respectively in the present standard conditions. The corresponding retention distances were 2.9 cm and 4.8 cm at a chart speed of 15 cm/hr. These figures varied slightly (±1mm) in extracts from wheat. The effect was probably due to over-loading of the column's packing, which was of low capacity (3.5 µequivalents/g), as differences due to temperature fluctuations were minimised by the water-jacket around the column. The relative position of the various peaks among sequential samples of a genotype were also used in preliminary identification. Typical recorded printouts for extracts from developing wheat grains are shown in Appendix 5.3.

Fractions of the eluate causing particular peaks were collected in separate scintillation vials, using the automatic peak separator control of the Absorbance Monitor. The recorders made a downward mark each time the Fraction Collector advanced a place to a new vial. The peaks caused by "tABA" and "ABA" were identified on the basis of their retention times. The vials corresponding to those peaks were selected, labelled and deep-frozen as soon as each sample had been run.

5.2.2.2. Quantitative Estimation

2.00 mg of (±) t-ABA and (±) ABA 1:1 mixed isomers were weighed, dissolved in 25% conc NH_3 and transferred to a small rotary evaporator flask with 5 washings of distilled water. The excess ammonia was evaporated off under vacuum, the solution was transferred to a 5 cm^3 volumetric flask and made up with distilled water. This stock abscisic acid solution was diluted to give a series of standard solutions containing 1, 2.5, 5, 10, 25, 50 and 100 ng/0.005 cm^3 of each geometric isomer.

After separation of the geometric isomers in the standard solutions, the peaks recorded on the Rikadenki recorder at 0.10 and 0.04 AUFS (absorbance units full scale) were photocopied onto Xerox paper. A straight base-line, through the minima on either side of the peak, was drawn. The peak area was cut out and weighed on a Sauter 5-figure balance. The cut-and-weigh method is the most accurate manual way of determining peak areas (Nadden *et al*, 1971). Predictive equations were estimated as $Y = \beta_0 + \beta_1 X$, where Y is the concentration of ABA or t-ABA in ng/0.005cm^3 and X is the weight of the peak area on Xerox paper in mg. The data and estimated statistics for these curves are given in tables 5.1 and 5.2.

The peaks from the samples identified as "tABA" or "ABA" were similarly photocopies, cut and weighed. The concentrations in ng/0.005 cm^3 were estimated from the equations and multiplied by 120/50 to give ng/sample. Sample values were divided by the number of grains in the sample to give the amount in ng per grain. They were divided by the weight of the grains to give the concentration in ng per gram dry weight.

5.2.2.3. Efficiency of Extraction Procedure

The recovery of the extracted ABA after purification was determined. A 500 ng/0.005 cm^3 sample of synthetic 1:1 t-ABA to ABA mixed isomers was taken through the entire extraction procedure, then the amount of the isomers remaining was measured. Standard curves for the ISCO recorder printouts at 0.10 AUFS were constructed, over the range 0-250 ng t-ABA or ABA per 0.005 cm^3, as given for the Rikadenki. Data and estimated statistics are given in table 5.3.

TABLE 5.1 DATA FOR STANDARD SOLUTIONS FOR t-ABA AND ABA FROM HPLC CHROMATOGRAMS RECORDED ON RIKADENKI PRINTOUTS

AMOUNT ABA OR tABA UNDER CURVE (ng)	WEIGHT OF XEROX PAPER UNDER CURVE (mg) AT TWO VALUES OF ABSORBANCE UNITS FULL SCALE			
	0.10		0.04	
	t ABA	ABA	tABA	ABA
1			0.83	0.33
2.5			1.64	1.09
			2.09	
5			3.43	
			2.76	1.64
10	2.21	1.32	5.93	3.55
			4.98	3.38
25	4.79	2.29	13.08	7.11
			11.38	8.07
50	7.83	7.63	21.24	14.32
	8.81	6.38	22.35	15.39
100	18.57	12.60	41.04	
	17.83	13.14	44.30	32.39

TABLE 5.2 ESTIMATED STATISTICS OF EQUATIONS FOR HPLC ANALYSIS OF STANDARD SOLUTIONS OF t-ABA AND ABA

	0.10 AUFS		0.04 AUFS	
	tABA	ABA	tABA	ABA
β_0	0.6335	-0.2575	-2.4728	-1.0739
s.e β_0	3.0453	3.7866	0.8688	1.7736
β_1	5.5163	7.6539	2.3887	3.2016
s.e β_1	0.2590	0.4419	0.0444	0.1384
R^2	0.9913	0.9868	0.9962	0.9853
$F_{regression}$	453.6228	299.9511	2898.7641	535.3035
	*[1]	*	*	*
$\sigma_{y.x}$	3.9169	4.8061	2.2744	4.0791

[1] * - significant at the 5% level

TABLE 5.3 DATA AND ESTIMATED STATISTICS OF EQUATIONS FOR HPLC ANALYSIS OF STANDARD SOLUTIONS OF t-ABA AND ABA RECORDED ON ISCO PRINTOUTS AT 0.10 AUES

NG ABSCISIC ACID ISOMER	WEIGHT OF XEROX PAPER UNDER CURVE	
	t-ABA	ABA
250	13.34	9.41
100	5.66	4.22
50	2.84	2.07

STATISTIC	ABSCISIC ACID ISOMER	
	t-ABA	ABA
β_0	-6.0750	-10.8626
s.e. β_0	3.2503	7.7565
β_1	19.1495	27.5534
s.e. β_1	0.3812	1.2772
R^2	0.9996	0.9978
$F_{regression}$	2522.9996 *[1]	465.3830 *
$\sigma_{y.x}$	2.9299	6.8159

[1]* significant at the 5% level

TABLE 5.4 ESTIMATED RECOVERY OF tABA AND ABA AFTER EXTRACTION PROCEDURE AND HPLC ANALYSIS

ORIGINAL CONTENT OF SAMPLE ASSAYED	ESTIMATED RECOVERY AFTER EXTRACTION PROCEDURE AND HPLC ANALYSIS		
	ng t-ABA/sample	ng ABA/sample	total (ng/sample)
A. no plant material 250 ng t-ABA 250 ng ABA	100.3	106.2	206.5
B. Half the extract from 10.0g wheat	227.2	96.9	313.1
C. Half the extract from 10.0 g wheat 250 ng tABA 250 ng ABA	428.0	145.2	573.2

From the equations, the remainder of the 500 ng of mixed isomers (A in table 5.4) was estimated to contain 100.3 ng t-ABA and 106.1 ng ABA. Therefore, the recovery rate of synthetic abscisic acid isomers in this procedure was 41%. The percentage recovery of abscisic acid extracted from plant material was also determined. The methanolic extract of 10 g of wheat grains was divided into two equal volumes. One half (C) spiked with 500 ng of 1:1 mixed synthetic abscisic acid isomers. Each half (B and C) was taken through the entire purification procedure and the amount of abscisic acid remaining was estimated from the HPLC analysis. The difference (C-B) gives an estimate of the percent recovery of synthetic ABA and t-ABA in the presence of other substances also extracted from wheat grains. The total remainder of the 500 ng of synthetic isomers was estimated at 260 ng, which gives a percent recovery of 52%. Previously, recovery of ^{14}C-ABA has been estimated at 40-50% (Sweetser and Vatvars, 1976).

The estimates of percent recovery after the extraction and purification procedure do not take into account that all the abscisic acid present in the plant tissue may not have been extracted.

5.2.3. Identification of Extracted "Abscisic Acid"

5.2.3.1. Co-injection

The results of the procedure, after HPLC analysis are shown in figure 5.3. The first peak is due to contaminants in the sample, The second is "t-ABA" and the third peak is "ABA". The "t-ABA" and "ABA" samples were taken from the appropriate fractions of the eluate of a sample of Tim x Pem grain at 23 days after anthesis. An 0.006 cm^3 aliquot of the "t-ABA" or "ABA" samples was diluted to 0.012 cm^3 with pH 1.7 HNO_3 and reanaylsed by HPLC (1B and 2B).

Standard solutions of 1:1 t-ABA:ABA mixed isomers of about the same concentration as each sample were analysed separately by HPLC (1A and 2A). They contained 50 ng t-ABA/0.005 cm^3 and 100 ng ABA/0.005 cm^3 respectively. A second 0.006 cm^3 aliquot of the Tim x Pem "t-ABA" or "ABA" was taken and added to a standard solution of the synthetic mixed isomers, which was double the concentration of those used previously. These mixtures of the synthetic isomers with either of the tentatively identified ones were analysed by HPLC (1C and 2C). The maxima of peaks from both sources coincided in these co-injections, which confirmed the identity of the sample peaks.

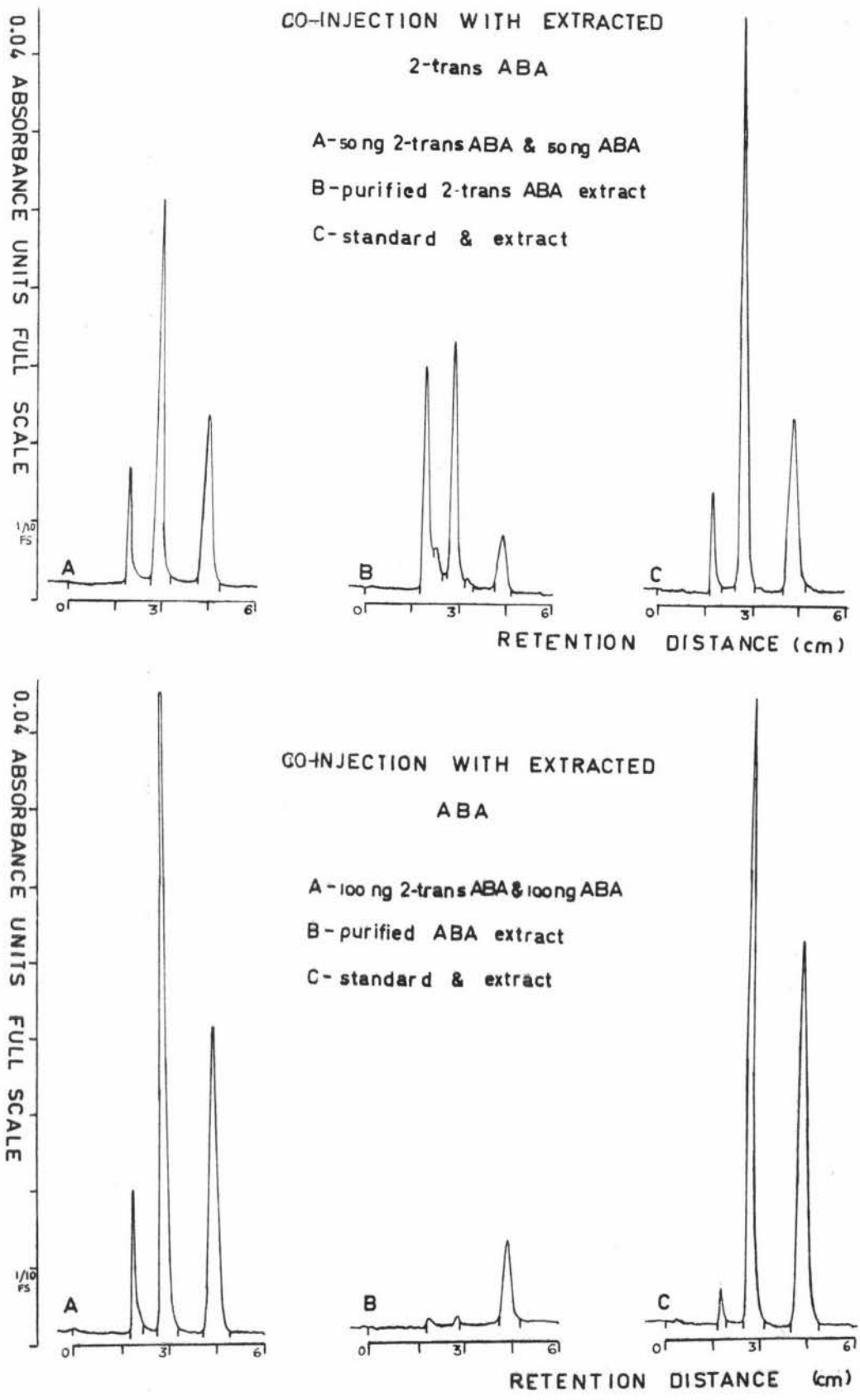


FIGURE 5.3

COINJECTION OF ABA OR 2-trans ABA EXTRACTED FROM DEVELOPING WHEAT GRAINS AND SYNTHETIC ABA AND 2-trans ABA MIXED ISOMERS STANDARDS

(Rikadenki Recorder Printouts X 1/2)

(Peak at 3cm is 2-trans-ABA; Peak at 4.5cm is ABA)

In addition, the peak areas of the co-injected samples (1C and 2C) were approximately equal to the sum of the areas the appropriate peaks in the separate injections, as expected. The rapidity of isomerisation of the geometric isomers was illustrated by the appearance, in the B figures, of the opposite isomer from that collected in the fraction from the initial separation. The photo-isomerisation, as well as the coincidence of peak maxima, confirms that the sample peaks were correctly identified as t-ABA and ABA.

5.2.3.2. Mass Spectrometry

A 5 g sample of Tim x Pem grain 23 days old was extracted, purified and separated by HPLC. The "t-ABA" and "ABA" fractions were collected, neutralised and dried. These samples were analysed by mass spectrometry. The "ABA" sample was lost during the process, but the "t-ABA" sample produced a fragmentation pattern similar to that of authentic abscisic acid. The masses (molecular weights) of the fragments produced were 208, 190, 162, 135, 134, 111 and 91, which respectively corresponded to the compositions C_{11} H_{12} O_4, C_{11} H_{10} O_3, C_{10} H_{10} O_2, C_9 H_{11} O, C_9 H_{10} O, C_9 H_7 O_2 and C_7 H_7 . The fragmentation pattern is consistent with the presence of abscisic acid. (Hodges, pers. com., 1977).

5.2.4 *Triticum aestivum* Coleoptile Bioassay

Grain of the cultivar 'Aotea' was soaked in tap water for two hours, then spread on moist filter paper in plastic trays and covered with glass sheets. It was germinated in the dark at 25°C. After 3 days the coleoptiles were 2-3 cm long and ready for use. Segments were cut from 3 mm below the tip in a device with razorblades fixed 10 mm apart, under a green safelight, and kept in distilled water until used. The deep-frozen vials, which had appeared from the HPLC analysis to contain more than 100 ng ABA, were selected, thawed and neutralised with an equal volume of 10% v/v aqueous ammonia. They were taken to dryness in a vacuum oven at 30°C.

A volume of 2 cm^3 of phosphate-citrate buffer (Appendix 5.4) at pH 5.3, was added to each vial. 10 coleoptile segments were put in under a green safelight and the vial was closed with a plastic cap with

a central needle-hole. The vials were inserted into a polystyrene wheel, which rotated at 1 rpm in the dark at 25°C. After 20 hours, 1 cm^3 of 10% v/v aqueous methanol was added to stop growth. The coleoptile segments were measured under a photographic enlarger at 3X magnification (Ivey, 1974).

A series of ABA standard aqueous solutions was included in each bioassay run. The 1:1 tABA:ABA mixed isomer solutions were made up in the range 4-40,000 ng/cm^3 of water, as described previously, in a log dilution series. For the bioassay of coleoptile growth, 1.0 cm^3 of a standard plus 1.0 cm^3 of double strength buffer was added to each vial in the series. This gave a final concentration range of 1-10,000 ng/cm^3 of ABA, because t-ABA is inactive in the bioassay. The means over 5 replicates and their standard errors are given in table 5.5 and illustrated in figure 5.4. The most useful range of the test is 10-1000 ng ABA.

The bioassay showed that the fractions identified as "ABA" in the HPLC analysis were inhibitory to wheat coleoptile growth. The content of ABA in the samples was estimated from the response curve of the particular set of standards included in that run. The estimates are given in table 5.6, which also shows the amount as estimated from the HPLC standard curves. In general, the estimates from the bioassay were 2.5 times greater than anticipated. A partial explanation might be in the relative activity of the stereo-isomers in the bioassay. The HPLC analysis did not differentiate between (+) and (-) isomers and, in any case, there was presumably none of the unnatural (-) form in the extracts. However, the synthetic ABA, used to construct the standard response curve in the bioassay, was equally (+) and (-). The (-) isomer might have been metabolised more slowly than the (+) form and have been less inhibitory to coleoptile growth. If so, the amount of ABA would have been overestimated by up to double the actual amount.

5.3 Results of Analysis by HPLC

The results of HPLC analyses of abscisic acid are shown in figure 5.5 as the amount in ng of t-ABA or ABA per grain, and in figure 5.6 as the concentration of tABA or ABA in ng per gram dry weight. The values shown were not corrected for losses during extraction. There was no apparent common pattern of change in abscisic acid levels among

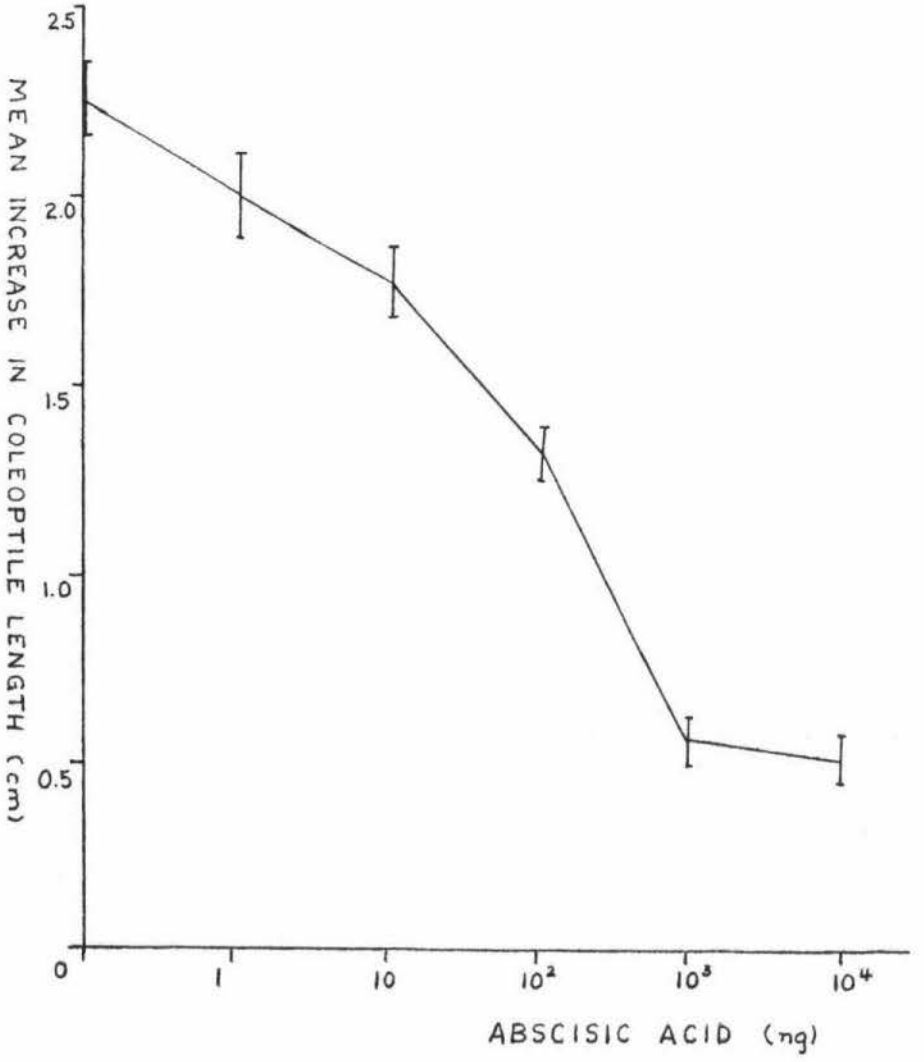


FIGURE 5.4

RESPONSE OF WHEAT COLEOPTILE BIOASSAY TO STANDARD AMOUNTS OF ABA.

(Means of five replicates of ten coleoptiles and standard errors)

TABLE 5.5 BIOASSAY RESPONSE OF WHEAT COLEOPTILES TO STANDARD SOLUTIONS OF ABSCISIC ACID

MEAN INCREASE IN LENGTH OF TEN COLEOPTILES (mm)	AMOUNT ABA (ng) 0	1	10	100	1000	10,000
Rep 1	1.96	1.67	1.67	1.38	.50	.38
2	1.82	1.73	1.66	.93	.37	.30
3	2.56	2.37	1.29	1.10	.42	.37
4	2.53	2.07	2.14	1.48	.74	.59
5	2.30	2.11	2.10	1.73	.79	.94
OVERALL MEAN	2.23	1.99	1.77	1.32	0.56	0.52
S.E. of MEAN	.10	.10	.10	.07	.07	.07

TABLE 5.6 ESTIMATED ABSCISIC ACID CONTENT, OF SAMPLE FRACTIONS FROM HPLC ANALYSIS, BY BIOASSAY RESPONSE OF WHEAT COLEOPTILES

GENOTYPE	GRAIN AGE (DAYS)	ESTIMATED ABA CONTENT (ng) BY BIOASSAY RESPONSE	IN HPLC ANALYSIS
GAMUT	14	112	49
	14	160	
	23	640	185
	32	178	77
	32	177	
SONORA	14	567	375
	41	123	67
	41	151	
TIMGALEN	14	202	53
	14	161	
TIM x PEM	23	243	113
	23	312	
KARAMU	74	290	97

the genotypes. The general tendency was for peaks of ABA content to occur during the second or third week after anthesis, around the time of the colour change or at about harvest-ripeness. However, the peaks did not appear consistently, even among the related genotypes (Tim x Pem and Timgalen or Pembina). Nor was there any obvious relationship between any of these peaks and embryo maturity or dormancy.

In some samples, there were large amounts of t-ABA, which were probably representative of the *in vivo* situation. Although photo-isomerisation of ABA to t-ABA could have occurred during extraction, it could not have resulted in a ratio greater than 1:1 t-ABA to ABA. In some samples (e.g. Pembina and Timgalen at later stages), the ratio was more than 5:1. However, it is possible that the "t-ABA" peaks measured were not entirely caused by t-ABA, because their baselines were not horizontal (see printouts in Appendix 5.3) and other substances may have contributed to their peak areas. While mass spectrometry did show that the "t-ABA" peak was correctly identified, it was not a quantitative test. The occurrence of t-ABA in developing wheat grains has not been reported previously. The role of t-ABA is not known but, in dormant citrus buds where high levels have been found, a suggestion was that it acted as a continuous source of ABA precursor for the maintenance of dormancy (Jones, Coggins and Embleton, 1976). However, in wheat grains during late development, there was no apparent relationship between the concentration of t-ABA and dormancy (figures 5.5 and 5.6).

Generally, the levels of ABA in developing wheat grains have been measured only from the end of the second week after anthesis (McWha, 1975; Radley, 1976). Where levels were measured in the first week after anthesis, they were found to be very low (King, 1976). The largest amount was found in grain harvested a few days before maximum dry weight would have been reached (McWha, 1975; Radley, 1976; King, 1976). Subsequently, the content declined to virtually nil in mature grain (McWha 1975; Radley, 1976; King, 1976). The inconsistency with the present results is probably due to the effects of the environment on grain development, which occurred faster at warmer temperatures. With plants of the same genotype grown in various controlled environments, the major peak appeared 14 days earlier in, grain of the plants grown continuously at 25°C than for those moved to 15°C three weeks after anthesis. For plants grown at 15°C throughout grain development, there were no major peaks and no consistent trend in ABA levels, which were still relatively high at 60 days after anthesis (Radley, 1976). The decreasing temperatures during grain development in the present study may have

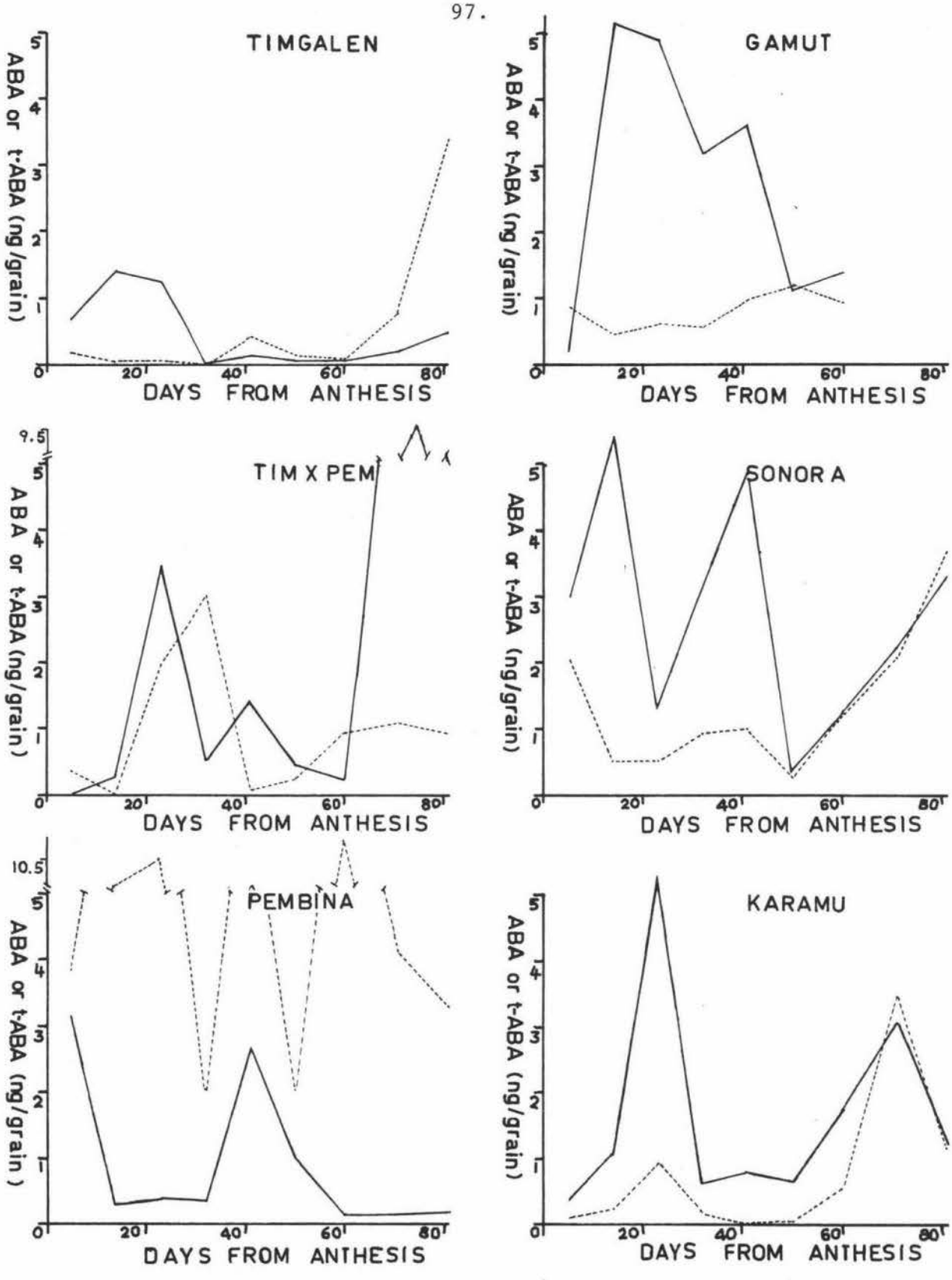


FIGURE 5.5

CHANGES IN AMOUNT OF ABSCISIC ACID AND 2-trans ABSCISIC ACID PER GRAIN IN DEVELOPING WHEAT GRAINS

(—— ABA ; ----- 2-trans ABA)

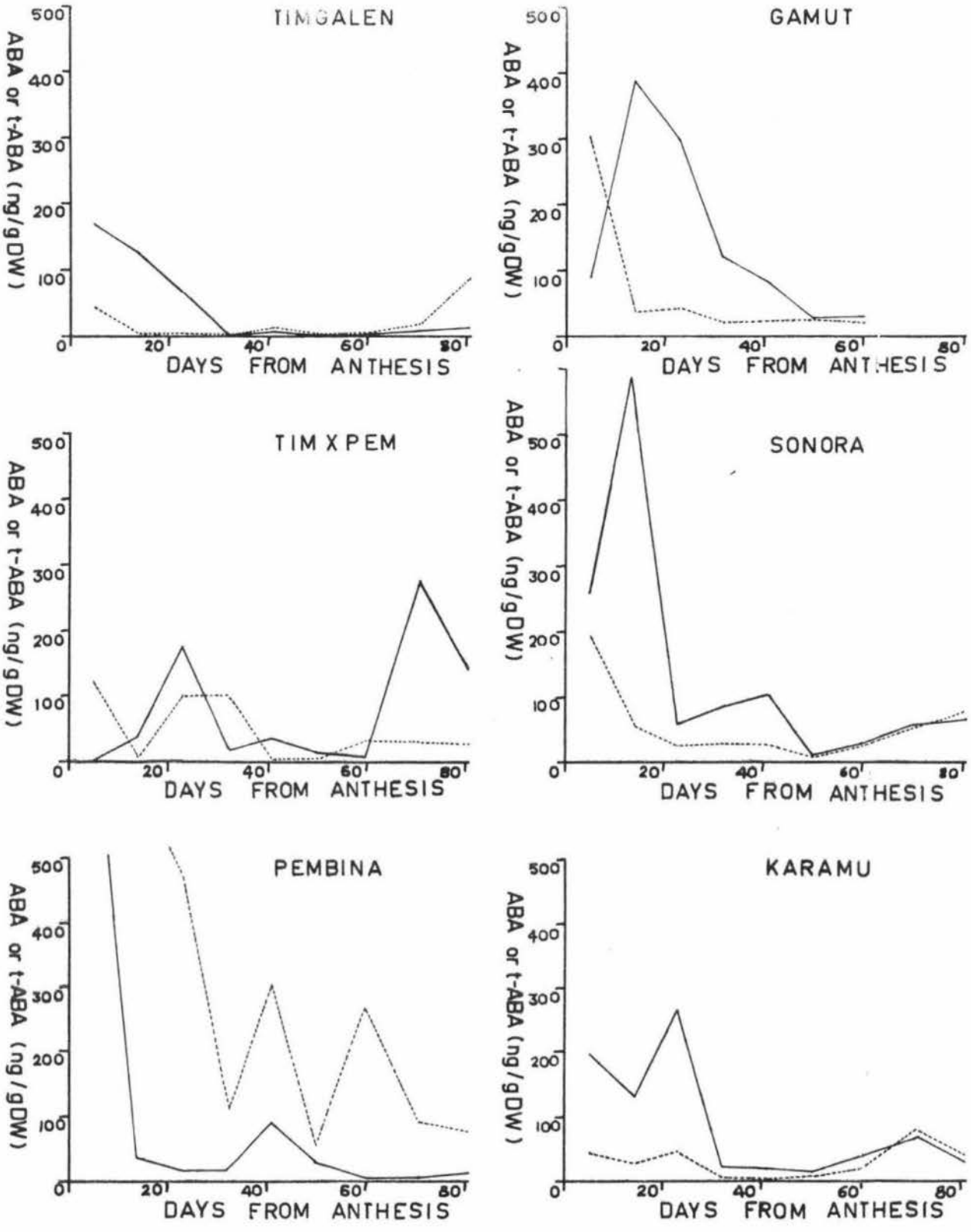


FIGURE 5.6

CHANGES IN CONCENTRATION OF ABSCISIC ACID AND 2-trans ABA PER GRAM DRY WEIGHT IN DEVELOPING WHEAT GRAINS

(—— ABA ; ----- 2 trans ABA)

contributed to the differences in ABA levels among the genotypes.

5.4 Discussion

The highest levels of ABA have been found in the phase of grain development after gibberellins, cytokinins and IAA had disappeared (Wheeler, 1972). The action of ABA may be important during subsequent development, particularly as it would not be counteracted by the other hormones (King, 1976). Because ABA inhibited the synthesis or activity of enzymes such as alpha-amylase (Hemberg, 1975), it was suggested that there may be a causal connection between the decreasing activity of the pericarp alpha-amylase of developing grain and the increasing ABA content (Radley, 1976). However, the present results for Pembina, Timgalen, Karamu, Sonora and Gamut, showed relatively high ABA levels much earlier than the decline in activity of pericarp alpha-amylase would have been expected. In these cultivars, the decline to low levels of pericarp alpha-amylase activity did not occur until 20 days after anthesis in warm (20-30^{o}C) environments nor until 45 days in cool ones (12-18^{o}C) (Gordon, 1975; Olered, 1976; Cross, 1977).

It has also been suggested that insensitivity to gibberellic acid in the morphologically mature aleurone during weeks 5-6 was due to the high levels of ABA (King, 1976). The germinative alpha-amylase response only appeared after ABA levels had decreased (King, 1976). In the present study, there was no apparent relationship between ABA levels and the germinative alpha-amylase responses of the genotypes in a previous study. There was little response before 40 days after anthesis in a warm environment nor before 60 days in a cool one, with a tendency for it to be delayed in Pembina and Karamu (Cross, 1977). However, as discussed previously, the environment of the present study did change considerably during grain development. The inability of immature half-grains or isolated aleurone to produce alpha-amylase until the grain has been dried (Bilderback, 1971; King, 1976) also suggested that it was not ABA alone which controlled premature starch degradation.

A role of ABA may be in inducing dehydration of developing grain. The general pattern of events in several genotypes and environments was:- ABA reached its maximum amount per grain, then the grain water content decreased sharply and dry matter accumulation ceased a few days later (King, 1976; Radley, 1976). It is not known what causes the sudden decrease in water content in wheat grains (Evans and Wardlaw, 1976). Xylem trachaery elements from the rachilla to the pericarp of wheat

discontinuous (Zee and O'Brien, 1970a). Xylem and phloem transfer cells occur in the node where the glumes and grain are attached (Zee and O'Brien, 1971a). Phloem cells are anatomically continuous from the rachilla to the vascular bundle in the pigment strand and labelled assimilates from the leaves rapidly accumulate in the crease (Saleri and Shannon, 1975). The accumulation of adcrusting substances, which may be relatively hydrophobic fatty acids, may function in the control, and eventual sealing off the symplastic and apoplastic routes of nutrients entering the developing grain in the fourth and fifth weeks after anthesis (Zee and O'Brien, 1970b; Zee, 1976).

However, many lines of evidence from experiments on wheat have suggested that it is not lack of assimilates that causes the cessation of grain growth (Evans and Wardlaw, 1976). The leaves and stems may still be green and there may be ample reserves in the stems. The cessation of starch accumulation in the grains has been considered to be regulated more by factors in the grain than by blockages of the supply of sucrose to it (Jenner and Rathjen, 1975). The sucrose concentration in both the free space and the endosperm cells in grains approaching full weight was higher than at earlier stages. The onset of the declining phase of starch accumulation was, therefore, considered to be due to a fall in the synthetic capacity of endosperm enzymes (Jenner and Rathjen, 1975).

An alternative explanation has been that the decrease in water content may be due to increased loss through the pericarp, which becomes fused to the grain at about that time (Radley, 1976). Pericarp tissues are not readily permeable to water vapour or CO_2 during most of the period of grain development and the time when they become permeable may depend on the rate at which they are digested (Radley, 1976). ABA was available at the right time for being a possible regulatory agent for the process (Radley, 1976). However, application of ABA to developing gtain hastened grain water loss by 2-3 days in only some samples (at 15 and 31 days after anthesis but not 21, 26 or 36) (King, 1976).

The changes in grain water content, grain dry weight and abscisic acid for each genotype in the present study are illustrated in figure 5.7. In each case the decrease in water content occurred before the increase in dry matter ceased, as observed by Radley (1976) and King (1976). But the decrease in abscisic acid levels did not invariably precede the decrease in water content, which did not support the hypothesis that ABA may have a role in grain dehydration. However, only one assay at each time of grain sampling was performed for each genotype, and the grains developed in a changing environment. Further

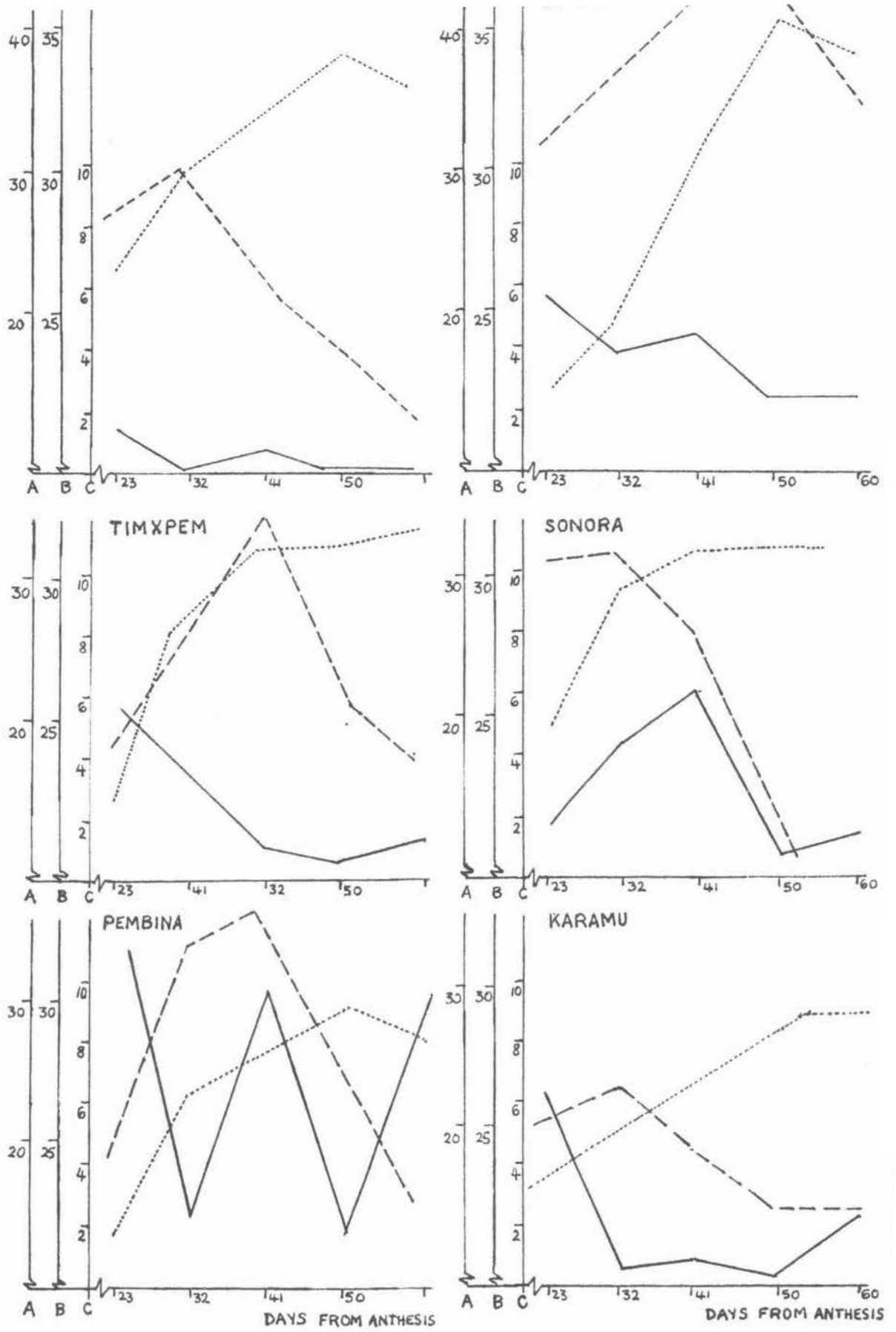


FIGURE 5.7

CHANGES IN DEVELOPING GRAINS OF SIX WHEAT GENOTYPES.

A. WATER CONTENT IN mg PER GRAIN (— — —)

B. DRY MATTER IN mg PER GRAIN (·····)

C. ABSCISIC ACID IN ng ABA + ng t-ABA PER GRAIN (——)

investigation of the relative sequence of events, in diverse genotypes grown in controlled environments, is needed to clarify the role of abscisic acid in developing and germinating grain.

CHAPTER 6

DISCUSSION

6.1 Control of Dormancy in Wheat

Dormancy in seeds with mature embryos probably arises from metabolic or physical restraints imposed by covering tissues, or from lack of integration of interlocking metabolic systems required for germination (Taylorson and Hendricks, 1977).

6.1.1 Metabolic Control

The principal components of integrated metabolism are ATP and the turnovers of NAD⇌$NADH_2$ and NADP⇌$NADPH_2$ (Taylorson and Hendricks, 1977). All are involved in the initial stages of stimulation of metabolic activity during early imbibition, which occur mainly in the embryo. Dormancy could be caused by inhibition of ATP formation, imbalance in the ratio of $NADPH_2$ to $NADPH_2$ or by alteration of their rates of oxidation (Taylorson and Hendricks, 1977). The level of NADP may be important as many enzymes of metabolic control points require it as a cofactor.

6.1.1.1. The Respiratory Pathways and Dormancy

Both the supply of ATP and the turnover of the nicotinamide adenine dinucleotide cofactors result mainly from respiratory activity. During the initial stages of germination, seeds may need to operate the pentose-phosphate pathway and stimulation of its activity may break dormancy (Roberts, 1973). The pathway is associated with an oxidase which is relatively insensitive to terminal oxidase inhibitors (KCN, Na N_3, CO, H_25 and NH OH). Dormant seeds are less capable of operating this pathway than non-dormant seeds, although they show high glycolytic activity which involves cytochrome oxidase. This enzyme has a high affinity for oxygen and is fully saturated at a very low partial pressure (Roberts, 1973). Any treatment reducing the competition for oxygen between the oxidase of the pentose-phosphate pathway and cytochrome oxidase will tend to alleviate dormancy. Such treatments may include puncturing or removing seed-coat tissues,

increasing oxygen pressure or adding respiratory inhibitors of cytochrome oxidase (Roberts, 1973). Dormancy in oats, barley and rice grains may involve this type of control of the respiratory pathways (Roberts, 1973).

In addition to the dormancy-breaking activity of the terminal oxidase of the pentose-phosphate pathway, the oxidation of NADPH by any other agent would have the same effect (Roberts, 1973). Some enzymes of the shikimic acid pathway, which produces the phenolic precursors of flavonoid pigments, oxidise $NADPH_2$. If they they are active during imbibition, their oxidation of $NADPH_2$ could stimulate the pentose-phosphate pathway. But their activity would also result in increased levels of phenolic acids and flavonoids, many of which are inhibitory to growth and germination (Gross, 1975), through their effect on the supply of oxygen (Côme and Tissaoui, 1973). It is possible that germination might depend partly on a balance between stimulation of the pentose-phosphate pathway and the synthesis of inhibitors.

There is no obvious hypothetical connection between the control of the respiratory pathways during imbibition, and the associated dormancy and grain-redness of wheat. The simple inheritance of grain-coat colour would imply that there is a possibility of a maximum of three different enzymes (or isozymes of a single different enzyme) in the red grains. They would probably be active specifically in the polymerisation of flavonoids, because white-grains apparently have the same levels of the precursors as red grains (Gordon, 1975; Cross, 1977) but apparently did not have the polymers (Chapter 4). Such enzymes might be active solely in the testa and only from around the time of the colour-change until senescence of the grain-coat tissues. It seems unlikely that this type of condensation would occur during imbibition to prevent the embryo germinating. However, the enzyme involved in the polymerisation has not yet been isolated or identified. Studies on the phenolases and peroxidases have not been sufficiently specific or sensitive to detect its activity.

The polymerising enzyme of red grains might cause the activation of flavonoids, and these might inhibit the synthesis of ATP (Popovici and Reznik, 1976). Alternatively, the action of these phenolases might diminish the pool of particular flavonoids. If there was a feed-back inhibition of that pool on the first enzymes of the shikimic acid pathway (later ones e.g. phenylalanine ammonia lyase may be controlled by substrate supply (Margna, 1977)), the phenolase activity

might result in increased activity of that pathway. The increased activity could lead to a buildup of inhibitors.

In charlock and sycamore seeds, a slow rate of oxygen supply to the interior of the seed did result in the formation of an inhibitor. If it was not for the inhibitor, the oxygen supply through the coat would be sufficient for germination (Côme and Tissaoui, 1973). It is not known whether such a system occurs in wheat. Although embryos of dormant red grains are not dormant, it is not known whether an inhibitor is formed in them during imbibition which prevents subsequent germination.

Control of dormancy by the relative interactions with activity of the shikimic of flavonoid pathways, would require a diversity of metabolic activity. It is not actually known whether any of the appropriate enzymes are active during dormancy. It seems improbable that all the necessary systems would be active in dormant grains. As any hypothesis based on these types of control would be complex and difficult to investigate experimentally, it would be sensible to investigate (in order to eliminate) alternative mechanisms first.

6.1.1.2 Hormonal Control of Dormancy

Dormancy could also be due to the failure of metabolic integration at the level of enzymic control (Taylorson and Hendricks, 1977). Many of the enzymes involved in the degradation of the endosperm in germinating grain are activated or synthesised in the aleurone layer. That activity is, at least partly, induced by hormones particularly gibberellins, from the embryo. The type of dormancy associated with gibberellin insensitivity may be due to the lack of integration of processes resulting from some inability to metabolise gibberellin. Gibberellin-insensitivity and the consequent inability to synthesis alpha-amylase have been found in only a few genotypes. It is probably not the general mechanism of control of dormancy in wheat.

Phenolic acids and tannins have been shown to be specific inhibitors of gibberellin-induced enzyme synthesis in barley seeds (Jacobsen and Corcoran, 1977). It is possible that the presence of tannins in the testa of red grains may inhibit gibberellin-induced enzyme synthesis in the adjacent aleurone layer. However, alpha-amylase activity was relatively late in the sequence of germinative events

(Gordon, 1977). The grain-coats of dormant red wheats contained inhibitors of germination of wheat embryos (Miyamoto *et al*, 1961). These inhibitors were extracted by water at room temperature, but were not extractable in hot water, acetone or 95% alcohol (Miyamoto *et al*, 1961). The solubility properties indicated they were probably not flavanols, condensed or hydrolysable tannins (Ribéreau-Gayon, 1972; Windholz, 1976).

Flavan-3-ol inhibited the germination of excised wheat embryos (Stoy and Sundin, 1976) but not that of whole grain (Gordon, 1975). Whole grains of white, red non-dormant and red-dormant genotypes all contained similar concentrations of flavanols (Gordon, 1975; Cross, 1977; Chapter 4). The implication is that monomeric flavonoid compounds are probably not involved in imposing dormancy during late grain development.

It is not entirely clear which other hormones are involved in early germinative processes, as discussed in Section 2.5. It is not known whether some balance among the levels of the hormones during development would result in dormancy. Abscisic acid is not an irreversible inhibitor with an inductive action; it may be readily leached from tissues to permit the resumption of growth (Milborrow, 1974). The present and previous results (King, 1976) did not indicate any relationship between ABA levels and grain dormancy. However, the extractions were of whole grain, which did not take into account the distribution within the grain. Only the ABA in the embryo and possibly only in particular cell compartments may inhibit germination (Milborrow, 1974). The embryos of a red-grained wheat have been found to contain relatively more ABA than their endosperm, but comparable estimates for white grains were not made (King, 1976).

Previous estimates of ABA content may have been inaccurate if the extracts were in solution for several days prior to assay. In that situation, the conversion of an ABA precursor may have lead to an apparent increase in ABA content (Sweetser and Valvars, 1976). On the other hand, there may have been a consistent under-estimation of the amount of "bound" ABA (King, 1976). The levels of endogenous ABA in the various tissues of dormant and non-dormant grains under germinative conditions have not been measured.

The interactions of peroxidase activity and auxin levels in the embryo, and the effects of kinetin or alpha-amylase activity have been considered to control dormancy in wheat (Gaspar *et al*, 1977).

In rice with intact hulls, there also appeared to be a critical level of peroxidase activity, as well as of oxygen uptake, below which dormancy decreased (Navasero *et al*, 1975). However, the changes associated with these activities occurred relatively late in the germinative sequence. They were considered to be responsible for allowing germination to occur ("permissive" in the terminology of Khan, 1975), rather than being a dormancy-imposing mechanism (Gaspar *et al*, 1977).

6.1.1.3 Summary

The evidence for any general mechanism of dormancy in wheat involving control at the metabolic and/or hormonal levels is not strong at the moment. The germinability of mature embryos excised from genotypes of all degrees of dormancy in standard germination tests is an argument against those types of control. If they had acted during grain development, then differences in the germinability of embryos from the various genotypes would be expected. If they had acted when germinative conditions were encountered, the same type of result would be expected.

It could be argued that germination of isolated embryos may give a distorted representation of processes in intact grains. Tests have also been made on whole grains with cracks in the senescent tissues of the grain-coats. The subsequent germination rates in both red- and white-grained genotypes gave additional support to the case against direct control by metabolic or homronal processes. A wound-response to the cracking of the senescent outer tissues would not be expected, provided that the under-lying aleurone was not damaged.

There does not appear to be any obvious link between metabolism or hormone levels in developing wheat grains and activity associated with pericarp pigmentation. Any direct involvement of the pigment-synthesising systems with dormancy would apparently require the re-establishment of metabolic activity, which is considered an improbable means of preventing germination. Possibly the pigment may have more indirect effects. It may shield the 2-trans isomer of abscisic acid from photo-isomerisation and maintain it as a continuing source of ABA in dormant grains. However, it is more probable that its role in controlling dormancy is by affecting the permeability of the testa, which would indirectly affect many metabolic processes required for germination.

6.1.2 Grain-Coat Restraints

6.1.2.1 Composition with respect to grain dormancy

Generally it is the grain-coat of wheat which is associated with dormancy at and after harvest-ripeness. Wheat grains with prolonged dormancy apparently always have the red pigment in the colour-layer of the testa. However, the period of dormancy in a red-grained wheat is not invariably prolonged to harvest-ripeness e.g. Sonora. As well as the differences in colour, there were structural differences in the testas of red and white grains. The testa of red grain appeared thicker and denser in light-micrographs (Bradbury *et al*, 1965a) and in scanning electron-micrographs (Belderok, 1976). It was not wrinkled in mature red grains and so was probably less capable of expansion without rupture during imbibition and may have been more restrictive to embryo growth (Wellington, 1956). The thickness of the covering layers varied over the grain, but relative thickness was not correlated with anatomical position of measurement taken (Bradbury *et al*, 1956b).

The grain-coats of other cereals were also involved in imposing dormancy. In sorghum, where red-coat colour was due to anthocyanins (Haslam, 1977), white and red-coated grains contained an average of 3.6% catechin tannins and had an average germination rate of 25%. However brown-coated grains had an average tannin content of 10.2% and less than 2% germination (Harris and Burns, 1970). Those germination test results might have confounded dormancy and embryonic immaturity. However, the difference found in the tannin content of dormant and non-dormant varieties was a different situation from wheat, where the contents were similar in all the genotypes examined (Gordon, 1975; Cross, 1976; Chapter 4.) In rice, excised embryos germinated readily. Dehulling the grain was partly effective in breaking dormancy, while breaking the grain-coat was completely effective (Roberts, 1961; Navasero *et al* , 1975).

6.1.2.2 Role of testa proteins

The dense, homogeneous material in the testas of both red and white grains prior to harvest-ripeness may be high molecular weight proteins (Belderok, 1976). Such proteins would swell when the grain-coat was soaked in water and possibly make the testa impermeable to oxygen. During ripening, before or after harvest-ripeness, these

proteins would be degraded in conjunction with a decrease in swelling capacity and an increase in oxygen permeability (Belderok, 1976). The relative duration of the dormant period would depend on the rate of degradation of the proteins. A week after harvest-ripeness, the dense homogeneous appearance of the testa had disappeared in white grains (82% germination), but was retained in red grains (2% germination) (Belderok, 1976).

On the basis of Belderok's hypothesis, a difference in proteolytic activity in the grain-coats between red- and white-grained wheats, during late maturation and around harvest-ripeness, would be predicted. The nature and role of proteolytic enzymes in dormant red and non-dormant red and white-grained wheats have been investigated. During maturation proteolytic activity, which occuted mainly in the grain-coat, decreased (Kruger and Preston, 1976). A proteolytic enzyme degraded proteins in the grain-coat, presumably including those of the testa, ready for translocation to the endosperm while build-up of storage proteins was occurring there (Kruger and Preston, 1976).

Under germinative conditions, increased proteolytic activity was observed, in both a dormant red-grained variety and in a white-grained one, after only 4 hours imbibition (Kruger and Preston, 1976). During that time, the increase was probably due to the removal of inhibitors rather than to synthesis. It would have occurred mainly in the starchy endosperm and in the aleurone layer, which remains adhering to the testa during endosperm degradation (Fincher and Stone, 1974). The proximity of proteolytic enzymes to the testa and the shortness of the time from the beginning of imbibition until their activation in the grains would suggest that proteins alone are not responsible for dormancy in the red-grained wheat.

The proteolytic activities of mature grain of a number of genotypes of varying dormancy and colour have been investigated. Although the proteolytic activity in some red-grained genotypes tended to be lower, there was no consistent correlation between dormancy in their genotypes and their proteolytic activities (Kruger and Preston, 1976). The evidence from studies of proteolytic activity during grain development and germination is considered to be inconsistent with the testa-protein hypothesis of grain dormancy (Belderok, 1976).

6.1.2.3 Role of testa pigments

The red pigment of the testa is probably a flavanoid polymer or condensed tannin. A major property of both hydrolysable and condensed tannins is their ability to combine with proteins and other polymers such as cellulose and lignin. The cross-links between macromolecules occur in any or all of several ways. Hydrogen bonds may form between the phenolic groups of tannins and receptor groups (-NH-, -CO- or -OH) of the other polymer. Ionic bonds may occur between anionic phenolic or carboxylic groups in the tannins and cationic groups in proteins (e.g. the amino group of lysine). Covalent links may form by the reaction of quinones, which may be either part of the tannin structure or be produced by oxidation, and a reactive group in the protein or other polymers (Ribereau-Gayon, 1972). These latter bonds are of great importance as they give great stability to a protein tannin complex. The size of the tannin is important in determining the stability of the complex. It must have sufficient phenolic groups to form the cross-links, yet be small enough to allow sufficiently close orientation to other polymers (Ribereau-Gayon, 1972). The monomeric and dimeric flavonoids do not have tannin-like properties. Tannins with molecular weights between 500 and 2000 probably form the most stable complexes with proteins (Ribereau-Gayon, 1972).

Condensed tannins in the testa of red-grained wheats may complex with adjacent proteins during maturation of the grain. The tannin-protein complex would be largely resistant to proteolytic enzymes but could still swell on imbibition and cause dormancy by its impermeability to oxygen. A piece of indirect evidence for tannin-protein complexing in red wheat is that their bran does not have marked astringent properties, although astringency increases with the molecular size of the condensed tannin (Haslam, 1977). Protein-tannin complexes were formed in

the seed coats of *Pisum elatius*. During their maturation, phenolase activity rose steadily and, as barriers were broken, quinones were formed and tanned the seed-coat proteins (Marbach and Mayer, 1975). *P. sativum*, which has more permeable seed-coats, had lower levels of diphenols (Marbach and Mayer, 1975).

In forming complexes with proteins, σ-semiquinone radicles or σ-quinones or their oxidised polymerisation products may form covalent links with amine, thiol, thiol ether and indole groups; and possibly with imidazole group and peptide bonds (Davies *et al*, 1975). Bonds are more frequently formed with the residues of the amino acids lysine and tryptophan as well as with the sulphur-containing ones (cystine, cysteine and methionine) (Davies *et al*, 1975). The grain-coat proteins of non-dormant (white or red) and dormant wheats therefore may differ in composition. The unripe grains of a white cultivar and of a red non-dormant cultivar have been found to have low levels of sulphur and insignificant changes in the levels occurred during ripening and storage (Belderok, 1976). However, two red dormant varieties had high sulphur contents in their testas before harvest ripeness and the levels decreased gradually during ripening and after-ripening (Belderok, 1976).

6.1.2.4. The testa and oxygen permeability

The tannin-protein complex hypothesis would also predict that red grain-coats are less permeable to oxygen than white ones. The permeability barrier would have to be quite efficient as almost all the embryos isolated from both red and white grains during late development could germinate in an atmosphere of less than 5% oxygen (Durham and Wellington, 1961). The germination of intact white-coated grains, harvested at various stages of late development, was directly correlated with increasing oxygen concentration of the atmosphere up to the level in air (21%). However, very few intact red-coated grains germinated in atmospheres with concentrations of oxygen up to that in air, and only slightly more germinated at 40% oxygen (Durham and Wellington, 1961). The covering layers of white grains had no inhibitory effect on oxygen permeability, while those of red-grains apparently prevented oxygen, at concentrations of up to 40%, from reaching the embryo. (Durham and Wellington, 1961). This evidence is consistent with the further evidence that an oxygen permeability barrier exists in red grain-coats is provided by the increased germination

after grain-coats were ruptured. In germination tests on hand-threshed barley, rye and triticale grains sampled throughout development, only those grains showing a rupture in the pericarp-testa when harvested would germinate (Gordon, 1970). Unruptured grains from the same immature ears would not germinate until the grain-coat was split with a scalpel (Gordon, 1970). The proportion of ruptured grain-coats in barley near harvest-ripeness was 1-2%, and some grain-coats were split in all the cultivars of rye and triticale examined, whether grown in the field or glasshouse (Gordon, 1970).

In the two cultivars of wheat examined, no splitting or premature germination was found in either situation. However, relatively high percentages were observed in a commercial sample of wheat (Gordon, 1970). Up to 94% of mechanically-harvested samples of red-grained wheat cultivars had no apparent dormancy at harvest-ripeness (Gordon, pers. comm. 1977). The high germination rates of mechanically-harvested grain could be a result of cracking of the grain-coats during the process. Such results would not necessarily reflect the dormancy of un-harvested samples, in which a much higher proportion of grains would be expected to have intact grain-coats.

Exposure of the embryo at the micropyle or of the endosperm at the distal end resulted in increased germination of red grains (Wellington, 1956). Comparison of the germination rates after complete removal of permeability barriers, by excision of the embryos, has been made for grains of white, red non-dormant and red dormant cultivars (King, 1976; Stoy and Sundin, 1976). In all the cases, the germination rate for excised embryos was higher than for intact grains in the first samples (King, 1976; Stoy and Sundin, 1976). The trend was more prolonged for cultivars with greater dormancy (Stoy and Sundin, 1976). The implication of all the results was that some permeability barrier may exist during early development of wheat grains. However, Miyamoto *et al*, (1961) found no significant difference in oxygen uptake among a variety of red dormant, red-non-dormant and white grains during the first ten hours of imbibition. An explanation compatable with concept of an oxygen permeability barrier in the red grains still exists. Phenolic groups of condensed tannins are capable of absorbing oxygen (Côme, 1970; Taylorson and Hendricks, 1977). The aging of solutions if di- and tri-hydroxy phenols during exposure is due to this type of oxidation (Kruger, 1976b). It is possible that the oxygen taken up by the dormant red grains (Miyamoto *et al*, 1961) was bound by phenolics

in the testa and so was still not available to the embryo; whereas that absorbed by the white grains was free to diffuse through to the embryo. The non-dormant red grains may have had fewer phenolic groups available to bind oxygen.

Apple seeds, previously exposed to cold inside the fruit to break embryo dormancy, germinated poorly at 20°C, because the numerous phenolic compounds present in their grain-coats combined with oxygen preventing it from reaching the embryo (Côme, 1970). The seeds germinated better after an ultra-violet light treatment which partially destroyed the phenolics (Côme, 1970).

A major gap in the evidence for the tannin-protein complex hypothesis is that it is not known whether proteins in the testa persist during the dormant period of red grains (Belderok, 1976). If they do persist, then they must be protected from attack by the proteolytic enzymes located there (Kruger and Preston, 1976), possibly by action of the tannins. Alternatively, the proteolytic enzymes could be inhibited during the period of grain development before pericarp senescence. However, no inhibitors of proteolytic enzymes were found in the testa, although an inhibitor of the endosperm proteolytic enzymes built up and remained at a constant level from about 24 days after anthesis (Kruger and Preston, 1976).

Even if it is subsequently shown that the proteins in testas of red grains do not persist during the dormant period, the tannin-complex idea may still be valid. Also, a non-complexed condensed tannin would still be capable of absorbing oxygen and could possibly even have more phenolic groups potentially available. In any case, tannins have the potential ability to form complexes with non-protein macromolecules during late maturation of the grain. As it dehydrates, the contents of pericarp cells become disorganised and cellular structure is almost obliterated in the testa. When the testa becomes disorganised, the condensed tannin polymers may be able to complex with the lignins and celluloses of the cell-walls. Alternatively, the condensed tannins may be synthesised outside the plasma-lemma of the cells, like cell-wall polymers, and be free to complex with them.

Complexing of tannins with cell-wall polymers or proteins may explain the greater mechanical toughness observed in red grain-coats (Wellington, 1956). After dehydration, white grains of one variety had wrinkled surfaces whereas red grains of one variety were smooth (Wellington, 1956). Bonding between macromolecules of the red grain-coat

as it dehydrates could result in its contraction. During early imbibition, swelling of a white grain would simply remove wrinkles, whereas increasing tension might result in a red grain-coat (Wellington, 1956). Removal of the epidermis hastened germination of both the red and white grains (Wellington, 1956). The removal may have damaged the testa, allowing oxygen to diffuse in more readily. Germinating embryos excised from both dormant and non-dormant grains readily split the adhering grain-coat, which indicated that mechanical toughness of the coat is not a barrier to germination (Miyamoto *et al*, 1961).

6.1.2.5 Variability of association of testa pigments and dormancy

Estimates of dormancy are method-dependent, as discussed previously. In addition to the effects of possible cracking of grain-coats during harvesting, ordinary germination tests confound embryo immaturity with dormancy. Estimates of sprouting damage based on alpha-amylase activity may lead to erroneous estimates of non-dormancy, if the activity due to any residual pericarp isozymes is not distinguished from that of germinative isozymes (Gale, 1976). It is necessary the changes in processes associated with dormancy, germinability and sprouting damage throughout the entire period of grain development. In the present study, and related ones (Gordon, 1975; Cross, 1977) the various processes were investigated throughout development, using methods which avoided confounding the effects of several processes in the estimation of dormancy.

Apparent non-dormancy at harvest-ripeness in some red-grained genotypes may have been the result of grain-coat cracking during harvesting. However, others like Sonora have lost their dormancy before they are harvested. There are several possible explanations for their relatively short period of dormancy. The condensed tannins may have fewer phenolic groups available for complexing or oxygen absorbtion, if the hydroxyl groups were acylated or methylated at some stage of flavonoid synthesis. The possible reduction in the number of sulphur-containing amino-acids in the pericarp proteins of non-dormant grains (Belderok, 1976) may result in fewer positions potentially available for stable covalent bonding to tannins (Davies *et al*, 1975). Another possibility is that factors which cause the break-down of permeability

barriers in all red-grained genotypes do so more rapidly in the ones that are non-dormant at harvest-ripeness.

The variability in the duration of dormancy in one genotype grown in different environments is possibly also due to influences on metabolism and synthesis during development. The synthesis of polymeric flavonoid pigments in the testa may depend on a balance between timing of appearance or rate of action their synthesising enzyme(s) and the timing of dehydration and senescence of the pericarp, which would inactivate the enzymes. If a protein-tannin complex is formed, there would also be a race to form it before the pericarp proteolytic enzymes degrade the protein. These types of interactions would result in differences in the permeability of the testa in the various environments, which would lead to differences in the duration of dormancy.

6.1.2.6. Loss of dormancy and the after-ripening process

The loss of dormancy would be expected to occur concurrently with increasing permeability of the grain-coat. In white grains, the permeability would probably increase as senescence of the grain-coat tissue progressed and there would be no barrier to germination by harvest-ripeness. In red-grained wheats, the increase in permeability would be delayed until the elapse of a period of after-ripening following the attainment of harvest-ripeness. After-ripening would not be necessary for germination if the grain-coat was cracked during harvesting.

If permeability to oxygen in intact grain-coats is caused by tannin-protein complexing, then after-ripening could involve the gradual breakdown of the complex and degradation of the proteins. As the complex is degraded, if the condensed tannins are also broken down, there may be a loss of sub-units, which may be volatile. Phenolic acids have an aromatic benzene ring and have been implicated in allelopathic effects and occur as the essential oils and scents of flowers (Whittaker, 1970). There were significant losses of flavanols during drying and storage of wheat grain (Gordon, 1975; Cross, 1977, Chapter 4).

The outer tissues of immature fruit are often rich in phenolics of various sorts, which may act as germination inhibitors and which disappear with ripening (Haslam *et al*, 1977). The flavanol concentration of whole wheat grains decreased rapidly in the first two weeks after anthesis. However, it apparently rose again slightly as harvest-ripeness was approached (Gordon, 1975; Cross, 1977, Chapter 4). It is not clear

why the rise occurred, but it might be related to the beginning of an after-ripening process which broke down flavonoid polymers. Another possible process during after-ripening is the saturation of the oxygen-absorbtion capacity of the tannins, so that oxygen could permeate further into the grain.

The causes of break-down or increase in permeability of the testa are unknown. The lag-phase between the decrease in protein-bound sulphur content and the increase in germinability indicated that another factor, besides the disruption of the pericarp proteins, also affected the termination of dormancy (Belderok, 1976). Both proteins and compounds containing phenolic acids are disrupted by ultraviolet light. Apple seeds germinated better after an ultra-violet treatment which partially destroys the phenolic compounds in the grain-coat, which are responsible for the inhibition of germination.

Photo-induced modifications of proteins result from their direct absorbtion of light (mainly due to aromatic residues) and/or from sensitised reactions, notably photo-oxidation (Laustriat and Hasselman, 1975). Ultra-violet light has produced free carbon-type and sulphur-type radicals from a great variety of proteinaceous material (Androes *et al*, 1972). It was assumed that the disruption of cystine was a secondary process brought about by energy absorbed by an adjacent aromatic group such as tyrosine (Androes *et al*, 1972). The histochemical response of protein-bound disulphide and thiol groups found in the grain-coats of dormant red grains disappeared during after-ripening, indicating disruption of the proteins (Belderok, 1976).

The coats of wheat and other seeds produced free radicals from a limited number of centres, on exposure to intense ultra-violet light. The stable free radicals produced were sensitive to light (Windle, 1972). The identity of the centres of radical production were unknown, but the results were considered to eliminate chlorophyll and phenolic compounds (Windle, 1972). However, extractions were carried out with hot ethyl alcohol, which may not have removed high molecular weight tannins, because these require some water in the extraction medium (Ribéreau-Gayon, 1972). The seeds or isolated coat-pieces did not produce the radicals if they were wetted (Windle, 1972). Under conditions where the pericarp of wheat was wet, no after-ripening occurred (Belderok, 1976).

6.1.2.7 Summary

A dry seed-coat is more or less permeable to oxygen, depending on its structure, but imbibition may result in a poor oxygen supply to the embryo (Côme and Tissaoui, 1973). Oxygen must traverse the imbibed coat in solution to reach the embryo. The presence of phenolic hydroxyl groups or hydrated proteins will delay its passage (Côme and Tissaoui, 1973; Belderok, 1976).

The dormancy of red-grained wheats may be due to the oxygen-absorbing properties of the phenolic hydroxyl groups of the condensed tannin pigments. In which case, they would have to be saturated before oxygen could permeate further into the grain to reach the embryo. Alternatively the red pigments may complex with high molecular weight proteins in the immature testa, preventing their degradation by proteolytic enzymes. Swelling of the proteins during imbibition could create an oxygen permeability barrier until either they were degraded or the grain-coat was ruptured.

The interactions of biosynthetic pathways leading to tannin synthesis with other metabolic processes during development and maturation of the grain may explain the variability of association between redness and dormancy at harvest-ripeness among genotypes. It may also explain variations in the duration of dormancy of a single genotype in different environments.

6.2 Future Research

It is considered desirable that the simpler hypotheses regarding the availability of oxygen to the embryo in dormant and non-dormant wheats should be investigated further. Only when they are shown to provide an insufficient explanation for dormancy will it be really worthwhile to investigate more complex metabolic and hormonal control systems which may exist.

6.2.1 The Pericarp Pigments

Conclusive evidence of the nature of the pericarp pigments is still required. With new techniques (Bate-Smith, 1975, 1979; Haslam, 1977), it may even be possible to obtain precise information of their structures. Even serial extractions of the sort attempted in the present work would provide useful information, if carried out over the period of grain

development covering the colour-change, in a variety of genotypes (Ribéreau-Gayon, 1972). Paper chromatographic methods might prove useful in investigating the phenolic and flavonoid precursors and low MW oligomers (Ribéreau-Gayon, 1972; Walker, 1975).
This type of study would require a large number of plants to allow for a sufficiently large number of grains in the samples from which the pericarp pigments would be extracted. Also, tests of germination and dormancy would have to be performed. Dissecting microscope examination for grain coat cracks before testing.

6.2.2 The enzymes of pericarp pigment biosynthesis

The activity of the enzymes assumed to be involved in the polymerisation of pericarp pigments should be investigated in the various grain tissues during development to harvest-ripeness. Separation of the isozymes of phenolase and peroxidase from coats of white and red grains, by techniques such as poly-acrylamide gel electrophoresis (Kruger, 1976) may reveal differences. It could prove useful to use closely related genotypes differing in grain-colour and dormancy e.g. Sonora 64A and Sherbati - Sonora; Karamu and Condor (McEwan, 1976). This type of investigation is necessary to show how the differences between grains of different colour occur, so the action of any other associated differences which might be causing dormancy may be brought out.

6.2.3 Pericarp proteins

The fate of pericarp proteins during dehydration to harvest-ripeness and during maturation of grain should be determined, in relation to dormancy. Preliminary studies might involve histo-chemical examination of section stained for tannins or for proteins. However, complexing of functional groups may prevent reaction with the dye. The efficiency of staining might be improved by applying the stain in an acid medium to hydrolyse bonds. An alternative approach would be to hydrolyse the pericarp or testa by boiling in acid (Ribéreau-Gayon, 1972). The extract would need to be partitioned in petroleum ether to remove chlorophyll. The amino acids and tannin precursors would have to be separated. This might be possible on PVP columns by elution at various pH's. The eluate could be assayed for proteins and tannins (Swain and Hillis, 1959). Scanning electron micrographs showing the structure

of the testa layers during development to harvest-ripeness and during after-ripening in various genotypes could be interesting.

6·2.4 Oxygen Permeability of the Grain-coat

The oxygen permeability of the grain-coat of red and white grains should be measured directly for grains at various stages of dehydration and after-ripening in both imbibed and non-imbibed grain (Côme, 1973). Techniques for measuring the rate of oxygen diffusion through a seed coat have been discussed (Porter and Wareing, 1974). Alternatively, it may be possible to devise a colour-test by using an oxidation-reduction (redox) indicator in an enclosed container to which the only access of air would be through a detached grain-coat.

A suitable closed system could comprise a spectrophotometer tube entirely filled with the indicator solution, sealed with a cap (made airtight with e.g. wax) with hole(s) of a suitable standard diameter over which the detached grain-coats could be fixed with xylene or coloured nail varnish. A suitable indicator would be an aqueous solution of methylene blue which had been decolourised by titration with zinc powder and dilute sulphuric acid. The colour would be gradually restored to the solution, if oxygen diffused through the grain-coats). After a suitable time, the sealed tubes would be shaken and read in a spectrophotometer at one of the absorption maxima for methylene blue (667.8 or 609.3 nm).

The effects of various experimental treatments on permeability, dehydration and dormancy could also be investigated in a range of genotypes. These could include:- forced premature dehydration of detached grain compared with undehydrated detached grain (King, 1976); the effects of applications of abscisic acid, as it may have a role in pericarp senscence (Radley, 1976); the exposure of the grains to ultra-violet radiation, as this may hasten the breaking-down of phenolics (Côme, 1970) and proteins (Androes *et al*, 1972; Windle, 1972); the effects of keeping the mature grain-coats moist, which has been observed to prevent after-ripening (Belderok, 1976).

Experiments on the germination of intact grains and ones with the testa broken, in atmospheres with various concentrations of oxygen (Durham and Wellington, 1961) could be repeated in a wider range of genotypes. Changes during after-ripening could also be measured. Germination of embryos excised from grains of the various genotypes should be measured at each of the experimental concentrations, and

possibly at some lower ones.

Manometric measurements of oxygen uptake under germinative conditions (Miyamoto *et al*, 1961) should be made in a variety of genotypes during after-ripening. The grains should be surface sterilised (1% hypochlorite bleach for 15 min.) before use and the system should include potassium hydroxide to remove carbon (Porter and Wareing, 1974).

6.3 Some Implications for Breeding for Resistance to Sprouting Damage

The simple genetic systems involved in dormancy in wheat are those controlling grain-coat colour and gibberellin insensitivity. Red grain-coat colour and dormancy have not been separated in a number of crosses and backcrosses (Freed, 1976). If dormancy is due to intrinsic properties of the condensed tannin pigments in the testas of red grains, then it would not be possible to breed a white wheat with a dormancy mechanism of this type. If white grains are considered highly desirable, low germinative alpha-amylase response could be bred in via the gene for gibberellin insensitivity in the grain (Bhatt *et al*, 1976; Gale, 1976). Germination tests need to be designed to separate embryo immaturity from dormancy and be performed on grains with uncracked coats (Gordon, 1975).

Breeding for low activity of alpha-amylase in mature pericarps is a continuing necessity. However, developments in baking technology, such as microwave ovens, may reduce the problem of alpha-amylase (Gale, 1976). It may be desirable to breed for high levels of amylase inhibitors, which are proteinaceous with a high lysine content (Buonocore *et al*, 1977) and for inhibitors of proteolytic enzymes to prevent degradation of both the pericarp and endosperm matrix proteins.

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A3.1 BASIC STATISTICS FOR ALL DATA FOR % GRAIN MOISTURE

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
N	47	49	49	38	38	48
$\overline{X}$	40.7872	40.3467	42.5918	36.7368	40.8684	42.8750
s.e. X	23.8414	24.7340	25.4124	21.8381	24.9899	24.7950
$\overline{Y}$	54.5085	45.7959	45.8061	54.1421	47.5132	48.7063
s.e. Y	19.1919	20.8294	21.5488	20.5585	19.2727	19.2878

A3.2 BASIC STATISTICS FOR ALL DATA FOR % EMBRYO MATURITY

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
N	45	48	43	41	47	53
$\overline{X}$	38.0609	41.5625	37.8605	39.7805	39.3404	41.1509
s.e.X	24.0609	25.7127	25.2583	24.5454	24.9863	24.1704
$\overline{Y}$ (logit)	-3.5495	-2.7940	-3.6176	-4.9256	-3.9888	-5.8142
s.e. Y (logit)	3.7028	3.7849	3.8541	3.0464	3.9634	2.1075

A3.3 BASIC STATISTICS FOR ALL DATA FOR % DORMANCY

	GAMUT	TIMGALEN	TIMxPEM	PEMBINA	SONORA	KARAMU
N	43	48	43	40	46	48
$\overline{X}$	36.5116	41.5625	37.8605	39.6957	39.6957	46.5000
s.e. X	23.1160	25.7127	25.2583	25.1421	25.1421	22.1234
$\overline{Y}$ (logit)	4.8115	2.0773	5.5504	4.9321	4.9321	7.4911
s.e. Y (logit)	6.0933	6.6983	5.0404	6.1350	6.1350	5.2523

A3.4 BASIC STATISTICS FOR RESTRICTED DATA SET (14-29 MAY) FOR % EMBRYO MATURITY

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
N	22	21	19	20	18	29
$\overline{X}$	32.9545	33.0000	31.2105	31.7000	30.7778	37.8621
s.e. X	18.6100	19.2795	19.4296	19.0376	20.5700	19.0820
$\overline{Y}$ (logit)	-5.5278	-5.1307	-5.6475	-7.1245	-6.8736	-7.9950
s.e. Y (logit)	4.5767	4.4144	5.3286	3.7387	3.9245	2.7161

A3.5 BASIC STATISTICS FOR RESTRICTED DATA SET (14-29 MAY) FOR % DORMANCY

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
N	22	19	19	18	18	23
$\overline{X}$	32.9545	31.2105	31.2105	30.1667	30.1667	34.1304
s.e. X	18.6100	19.4296	19.4296	19.4369	19.4369	19.0366
$\overline{Y}$ (logit)	5.1082	1.9976	6.4734	7.66969	6.4978	8.4093
s.e. Y (logit)	7.8650	4.1556	3.4960	5.5078	3.8409	

A3.6 ESTIMATED STATISTICS FOR EQUATIONS (LOGIT Y = $\beta_0 + \beta_1 X$) DESCRIBING CHANGES IN % EMBRYO MATURITY (EM) FOR RESTRICTED DATA SET (14-29 MAY)

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
β_0	-12.2806	-11.8764	-12.9225	-11.8307	-11.7082	-10.4952
s.e β_0	0.5945	0.4783	0.7111	0.6039	0.5777	0.4862
β_1	0.2049	0.2044	0.2331	0.1485	0.1571	0.0660
s.e. β_1	0.0304	0.0237	0.0351	0.0303	0.0271	0.0243
R^2	0.6942	0.7970	0.7224	0.5715	0.6778	0.2152
$F_{regression}$	45.4112 (NS)	74.6159 (NS)	44.2399 (NS)	24.0064 (NS)	33.6655 (NS)	7.4050 (NS)
$\sigma_{y.x}$	2.5932	2.0404	2.8889	2.5144	2.2961	2.4503
Xat 50% EM	59.93	58.10	55.44	79.67	74.53	159.02

(NS) - non-significant at the 5% level

A3.7 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF REGRESSION STATISTICS OF EQUATIONS FOR % EMBRYO MATURITY IN RESTRICTED DATA SET

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	----	0.5297	0.6925	0.5309	0.6905	2.3247
		NS	NS	NS	NS	*
TIMGALEN	.0078	---	1.8738	0.0593	0.0593	2.0251
	NS		(*)	NS	NS	(*)
TIM x PEM	.6116	0.1750	----	3.5358	1.4096	2.8178
	NS	NS		**	NS	**
PEMBINA	1.3094	1.4530	1.8245	----	0.1466	0.9597
	NS	NS	(*)		NS	NS
SONORA	1.1690	1.3139	1.7805	0.2116	----	1.6065
	NS	NS	(*)	NS		NS
KARAMU	3.5646	4.0777	3.9147	0.2846	2.5036	----
	**	**	**	NS	*	

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - 1.10 > P > 0.05

* - 0.05 > P > 0.01

** - 0.01 > P

A3.8 ESTIMATED STATISTICS FOR EQUATIONS (LOGIT Y = $\beta_0 + \beta_1 X$) DESCRIBING CHANGES IN % DORMANCY (D) FOR RESTRICTED DATA SET (14-29 MAY)

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
β_0	13.8365	9.4838	12.1192	11.5140	12.4742	10.4494
s.e. β_0	1.0810	1.6049	0.5616	0.6474	1.0305	0.8406
β_1	-0.2649	-0.1809	-0.1265	-0.1981	-0.0598	
s.e. β_1	0.0553	0.0791	0.0277	0.0320	0.0507	0.0420
R^2	0.5344	0.3511	0.7153	0.4949	0.4888	0.0878
$F_{regression}$	22.9543	9.1089	42.7222	15.6773	15.2984	2.0202
	(NS)	(NS)	(NS)	(NS)	(NS)	(NS)
$\sigma_{y.x}$	4.7145	6.5192	2.2814	2.5611	4.0592	3.7549

(NS) - non-significant at the 5% level

A3.9 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG PAIRS OF REGRESSION STATISTICS OF EQUATIONS FOR % DORMANCY IN RESTRICTED DATA SET

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	----	2.2494	1.4103	1.8432	0.9122	2.4735
		*	NS	(*)	NS	*
TIMGALEN	0.2590	----	5.2232	1.1731	1.5631	0.5330
	NS		*	NS	NS	NS
TIM x PEM	1.3582	0.2588	----	0.7569	0.3514	1.6526
	NS	NS		NS	NS	NS
PEMBINA	2.1662	1.3290	1.2854	----	0.7890	0.7193
	*	NS	NS		NS	NS
SONORA	0.8904	0.4449	0.3419	1.1943	----	1.5226
	NS	NS	NS	NS		NS
KARAMU	2.9536	2.0109	2.4071	0.2638	2.1008	----
	**	(*)	*	NS	*	

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - $0.10 > P > 0.05$

* - $0.05 > P > 0.01$

** - $0.01 > P$

A3.10 ESTIMATED NUMBER OF DAYS TO MEDIAN AND 10% DORMANCY FOR RESTRICTED DATA SET

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
Days to 50% D	52.23	39.53	66.99	91.02	62.97	174.74
s.e.	18.64	28.02	14.05	25.87	22.66	116.90
Days to 10% D	60.53	48.79	79.14	108.39	74.06	211.49
s.e.	19.07	28.47	14.87	19.89	15.82	139.11
Days to HR	88.4	75.0	78.8	77.5	88.0	84.3
D10-HR	-18	-25	0	30	-14	128

A3.11 ESTIMATED t-STATISTICS FOR DIFFERENCES AMONG ESTIMATES OF "DAYS TO MEDIAN DORMANCY" AND "DAYS TO 10% DORMANCY" FOR RESTRICTED DATA SET

10%D \ 50%D	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
GAMUT	----	0.3774	0.6323	1.2165	0.3660	1.0349
		NS	NS	NS	NS	NS
TIMGALEN	0.3455	----	0.88886	1.3501	0.6565	1.1248
	NS		NS	NS	NS	NS
TIM x PEM	0.7696	0.9623	----	0.1086	0.0341	0.9151
	NS	NS		NS	NS	NS
PEMBINA	1.7369	1.7190	0.1812	----	0.8156	0.7013
	(*)	(*)	NS		NS	NS
SONORA	0.5461	0.7789	0.0363	1.3508	----	0.9386
	NS	NS	NS	NS		NS
KARAMU	1.0751	1.1465	0.9460	0.7356	0.9816	----
	NS	NS	NS	NS	NS	

SIGNIFICANCE SYMBOLS

NS - non-significant at the 10% level

(*) - 0.10 >P

APPENDICES FOR CHAPTER 4

A4.1 CRITERIA FOR PHENOL COLOUR SCORES

SCORE	DESCRIPTION
1	Grain-coat totally unstained
2	Brush-end of grain stained dark-brown
3	Brush-end and embryo-end of grain stained dark brown
4	30% of grain-coat stained
5	40% of grain-coat stained
6	50% of grain-coat stained
7	60% of grain-coat stained
8	70% of grain-coat stained
9	80% of grain-coat stained
10	90% of grain-coat stained

A4.2 BASIC STATISTICS FOR DATA FOR PHENOL COLOUR SCORE

	GAM	TIM	T x P	PEM	SON	KAR
N	36	36	36	33	36	35
$\overline{X}$	55.8333	55.8333	55.8333	53.8485	55.8333	55.4000
S_x	17.0855	17.0855	17.0855	16.3843	17.0855	17.1330
$\overline{Y}$	-0.3972	0.0867	-0.0923	-0.5694	0.2366	-0.5533
S_y	1.2974	1.7612	1.4002	1.4065	1.2247	1.3600

A4.3 BASIC STATISTICS FOR DATA FOR FLAVANOL CONCENTRATION (µg/gDW

	GAMUT	TIMGALEN	TIM x PEM	PEMBINA	SONORA	KARAMU
N	43	42	42	38	35	43
$\overline{X}$	41.8889					
S_x	24.6444					
$\overline{X^2}$	2350.7778					
S_x^2	2183.8054					
Y	212.9930	219.8500	186.7929	203.8789	175.7200	194.0093
S_y	160.7937	201.9860	125.3240	179.0223	156.6177	122.1163

APPENDIX 5.1

PVP COLUMNS

5.1.1. Preparation and maintenance

Polyvinyl pyrrolidone (PVP) was used to remove phenolic and other organic acid impurities (Glen *et al*, 1972; Biddington and Thomas, 1976) during the purification of ABA samples. An insoluble form of PVP, Polyclar AT (125-150 mesh), was shaken with distilled water and the supernatant decanted off ten times to remove the fine particles. The PVP was resuspended in distilled water and degassed under vacuum. It was poured into glass columns of 1.5 cm i.d. with sintered glass discs in the base. The excess water was run out and the packed column height adjusted to 15 cm. Approximately 8.5 g of dry PVP is required for packing columns of this size, and adequately, cleans up samples of the size used in the experiment. The columns were washed with 0.013 M phosphate buffer adjusted to pH 3.0 with 10% v/v HCl. The packing must be kept wet at all times to prevent the formation of channels through it.

The volume of buffer needed to elute ABA and the cytokinins zeatin, isopentenyl adenosine and isopentenyl adenine was determined for each separately. One cm^3 of each 500 $\mu g/cm^3$ solution was layered onto the top of the column with a pipette, the buffer flow was started and the eluate was collected in 5 cm^3 fractions. The ultra-violet absorbance of the fractions were measured at 265, 270, 254 and 254 nm respectively on a Varian Techtron-635 spectrophotometer and are shown in Figure A5.1. The fractions found to contain all four compounds, which were cm^3's 50-130, were collected as a bulk fraction for the plant extracts. Columns prepared by the above method were reproducivle, in that the fractions in which the bulk of the ABA was eluted were the same for different columns.

time a column had been used for purifying a plant sample, it was cleaned by dropping 250 cm^3 of 8M urea through to release retained contaminants. The top 2-3 cm of PVP was also replaced at intervals and the column rechecked for the elution fractions of ABA. The urea was cleaned from each column before reuse, by siphoning through at least 2L of distilled water via microtubing from a large reservoir. Thorough cleaning is important as urea or some contaminant in it has the same retention time in the HPLG xipax column as ABA. Finally the columns were washed with 250 mls of 0.013 M phosphate buffer ready

for the next sample. As phosphate buffer is a medium for fungal growth, it cannot be left in the columns when they are not being used.

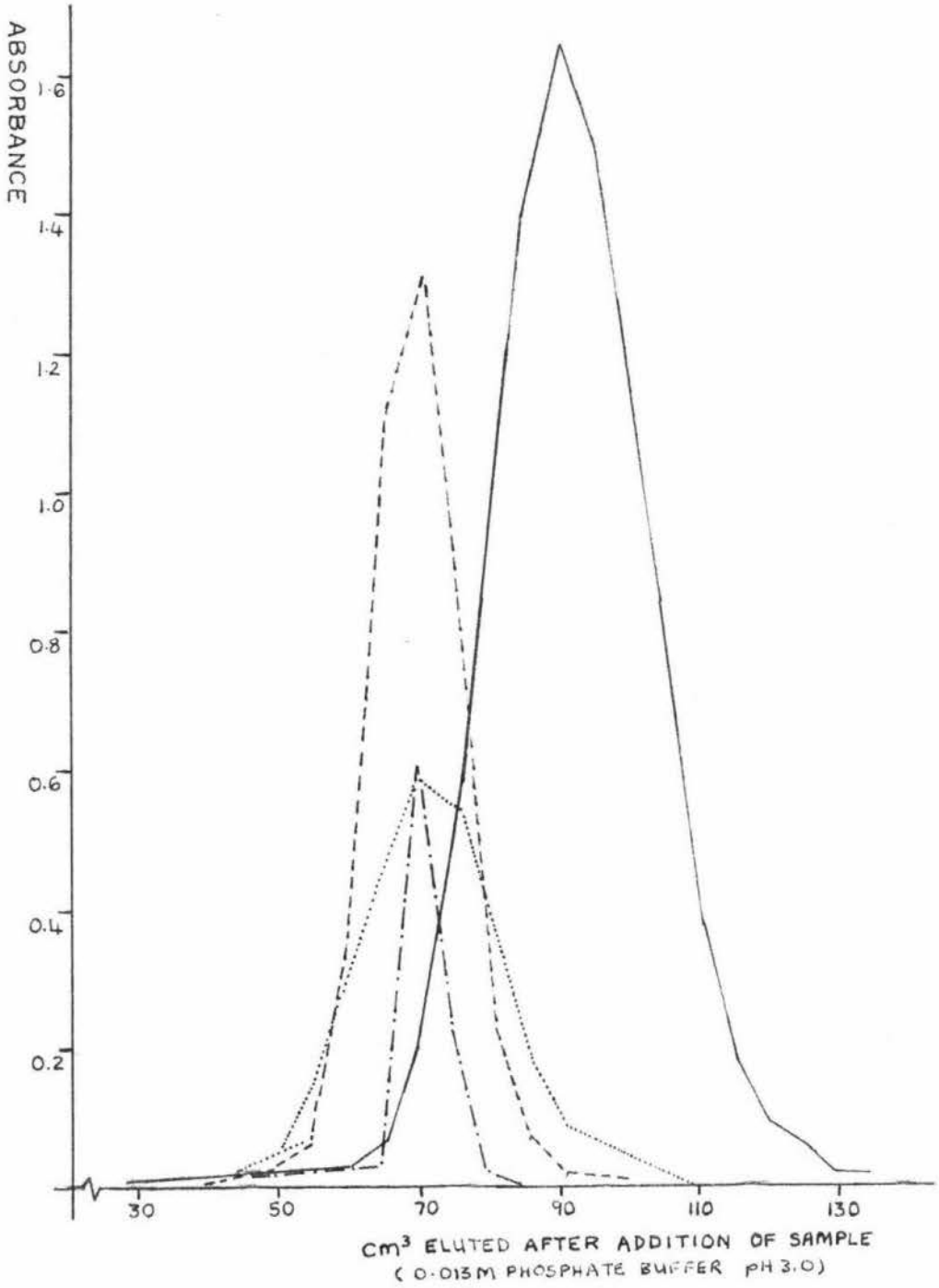


FIGURE A5.1.

ELUTION OF CYTOKININS AND ABSCISIC ACID FROM A POLYVINYL PYRROLIDONE COLUMN

——— absorbance of abscisic acid (ABA and t-ABA) at 265nm.

..... absorbance of N6Δ^2-isopentenyl adenosine (iPA) at 270nm.

– – – absorbance of N6'-[Δ^2-isopentenyl] adenine (2iP) at 254nm.

—·— absorbance of zeatin at 254nm.

APPENDIX 5.2

THE HPLC COLUMN

5.2.1. Column Preparation

Two empty stainless-steel precision-bore columns, 1.0 m and 0.5 m long, with 2 mm internal diameter, were washed using hot detergent solution and a pull-through cleaner, rinsed in hot water and acetone, and dried. The clean columns were connected by 5 cm of clean stainless-steel precision-bore capillary tubing, with a 2 mm i.d. A teflon plub was placed in the lower terminator. The completed column was dry-packed manually by the modified tap-fill method. Aliquots of 100-200mg of packing were introduced via a funnel into the column which was vertically tapped on the floor and rapped on the side at the approximate level of the packing about 80-100 times (2-3 times/sec) after each addition. The packing was further settled by 15-20 sec. of very gentle vertical tapping. The full column was gently tapped for a further 5 min. The method produces efficient columns with a well consolidated, homogeneous bed structure (Kirkland, 1972). The packing was Z_1 pax-SCX strong cation exchange pellicules (Du Pont Instruments), which is a sulphonated fluoropolymer shell bonded to glass beads. It is highly stable chemically and has a cation exchange capacity of about 3.5 μequiv/g (Sweetser and Vatvars 1975).

The connecting tubing was bent in a U-shape so that the column could be connected to the HPLC pump unit and to the optical unit. Bending of narrow-bore columns does not affect their efficiencies provided that the coil radius to column radius ratio is greater than 130, to minimize channelling. (Nadden *et al*, 1971). Distilled water, adjusted to pH 1.7 with concentrated HNO_3 was thoroughly degassed in a vacuum with magnetic stirring. If present, dissolved gases could form bubbles under the pressure in the column and affect its properties. It was pumped through at 2000 psi, to remove air and for final consolidation of the packing, which settled less than 2mm. Then a small quantity of teflon wool was packed in the top of the column so that the top of the column would not be disturbed during use.

A water-jacket 2 cm diam. plastic tubing was built over the straight parts of the column. It was bound, with metal hose clips, against split plastic tubing fitted around the column's ends. The bend in the column was immersed in an 800 ml beaker of water containing

a Gallenkamp metal cooling coil. Plastic tubing of 1 cm diameter connected the various parts of the water jacket to one another and to a pump in a controlled temperature water-bath. The joins were sealed with plastic glue. The system controlled the temperature satisfactorily but heat loss could be significant at higher temperatures and a more heat-resistant glue would be required.

5.2.2 Column Parameters

The chromatographic separation of sample components is measured by resolution. Resolution denotes both column selectivity and column efficiency. Selectivity is a function of the stationary and mobile phases and accounts for the separation of the maxima of two peaks. Efficiency is a function of the operating parameters and is a measure of zone spreading or narrowness of peaks. The column parameters are selected to optimize resolution and speed. Capacity is another consideration in cases where the material is to be collected for further experiments or identification.

The column design parameters are length, internal diameter, support material, mobile and stationary phases. Sweetzer and Vatvars (1975) found that a 3 m x 2.1 mm i.d. column packed with Zipax SCX pellicules eluted with distilled H_2O at pH 1.7 with HNO_3 , gave good selectivity for ABA with operating parameters of 50^oC and a 40 cm_3/hr flow rate.

5.2.3 Operating Parameters

As the column length available was 1.5 m, the operating parameters of flow rate, temperature and pH were experimented with to improve resolution. It was found that raising the pH to 2 had little effect; increasing the flow rate to increase speed reduced separation; lowering the temperature from 50^oC to 27^oC improved peak separation. The conditions finally selected were pH 1.7 HNO_3, a flow rate of 20 ml/hr and 27^oC. All samples and standard ABA solutions were run under these conditions. The pressure was 590 psi. At pH 1.7 it was found that some sample components, especially in young samples, were strongly retained and the recorded baseline was unstable for a long time. It was necessary to speed their elution by flushing the column with 0.05 M $(NH_3)_2\ PO_4$, adjusted to pH 7.0 with $NH_3H_2PO_4$, at 40 cm^3/hr

after the ABA peaks were eluted. Even so, the turn around time between samples was still usually longer than an hour.

5.2.4. Column Efficiency

Column efficiency is quantitatively described by the number of theoretical plates, N. $N = 16 (x/y)$, where x is the distance from injection to peak maximum and y is the length of the baseline of the peak as delineated by two tangents to the peak. The height equivalent to a theoretical plate, HETP, also measures column efficiency. HETP = N/L where L is the length of the column. Column efficiencies may be compared when all operating parameters such as mobile phase, solute, temperature, flow rate and sample size are the same (Brown, 1973). Sweetser and Vatvars (1975) used a comparable system with a mechanically packed column. Their HETP was approximately 0.96 mm compared with 0.99 for the column used here.

5.2.5. Preparation of Solutions

The appropriate amount of each solvent or solution used in the extraction and purification of abscisic acid was evaporated to dryness and taken up in 120 µl of pH 1.7 HNO_3 . Each was analysed on the HPLC at 20 ml/hr and 27°C to check that neither the solvents, the salts nor any impurities in them had retention times similar to those of tABA or ABA. All chemicals used were Analar grade and the diethyl ether was freshly distilled. All glassware was cleaned at the beginning and throughout the experimental work by being scrubbed in detergent, rinsed thoroughly in running tap water and rinsed three times in distilled water. No peaks, which could have been confounded with the t ABA or ABA peaks, were found due to any of these causes.

2.5.1 Phosphate Buffer (0.013 M at pH 3.0)

KH_2PO_4 (MW 136.09) 17.692 g/1

K_2HPO_4 (MW 174.18) 2.264 g/100 cm^3

Make up in the ratio of 95 ml KH_2PO_4 to 5 ml K_2HPO_4, which gives pH 5.6. Acidify with 10% v/v conc HCl to bring the pH down to 3.0 (approximately 3.5 ml). Dilute 10 times before use. Keep stored in the cold.

2.5.2 Phosphate-Citrate Buffer (pH 5.3)

K_2HPO_4	4.485g
citric acid monohydrate	2.547g

Dissolve in 250 ml distilled water. For use, dilute ten times and add 5 g sucrose. The pH should be 5.3. Check with pH meter and adjust if necessary.

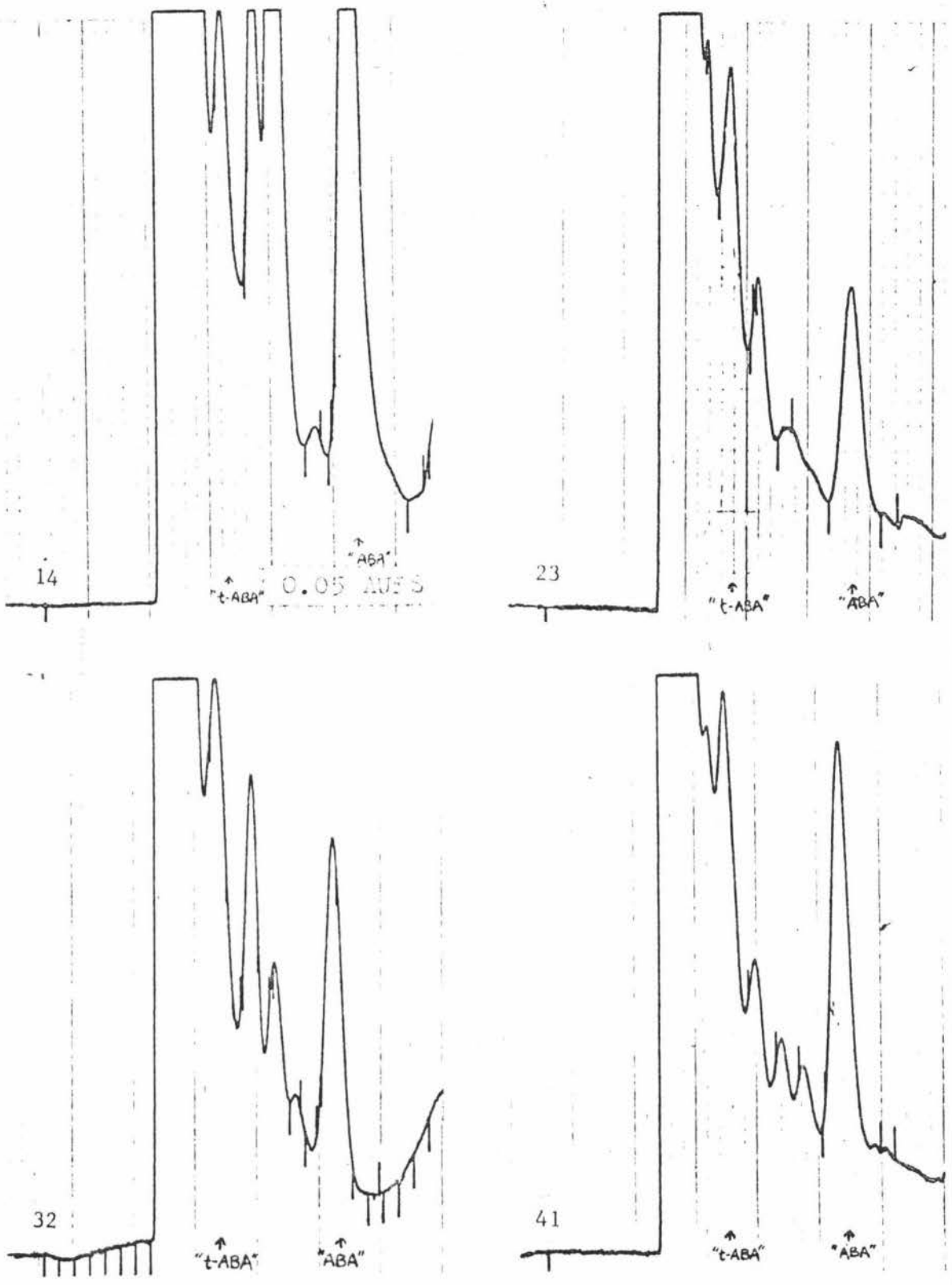
2.5.3 Nitric acid at pH 1.7.

Use pH meter. It takes approximately 1 cm^3 of conc HNO_3 per litre of distilled water. Make up freshly and degas thoroughly before use under vacuum in a desiccator with magnetic stirring.

2.5.4 Purification of Diethyl ether.

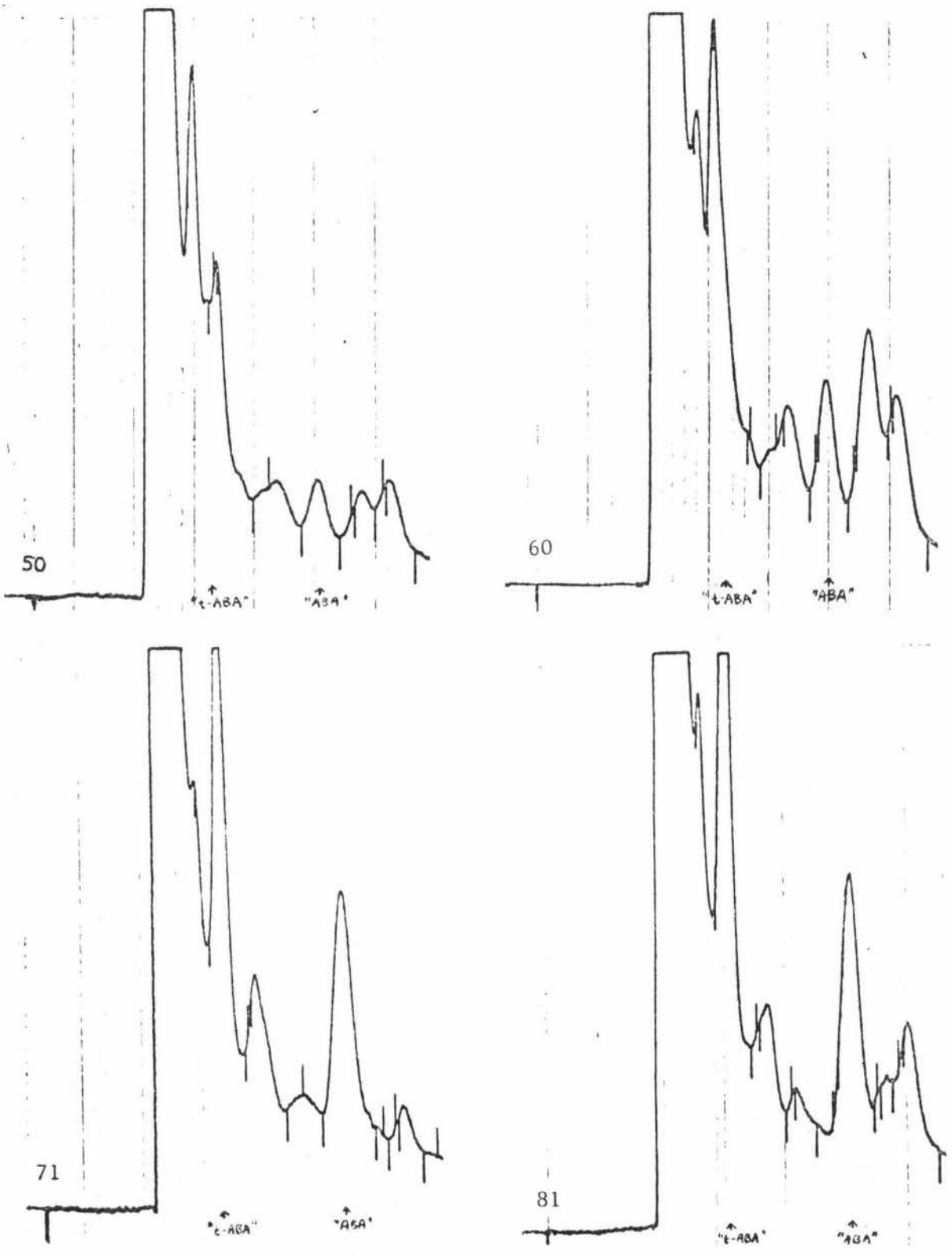
Add 0.5 g CaO and 2.5 g Ferrous Sulphate per litre of impure ether to a dry bottle to prevent them sticking to the side. Fill with diethyl ether and shake vigorously for half a minute. Add 25 cm water, per litre of ether and shake for 3 min. to remove precipitates. Let settle, then transfer the clear liquid to round-bottomed flask and distill at 35°C in a closed system. It is dangerous to continue the distillation to a small volume as any peroxide impurities left will become concentrated and are potentially explosive. The diethyl ether distilled by this method is not absolute, as some water may still be present in it. Store diethyl ether in tightly sealed containers in the cold and dark and use within 3 days.

A.5.3 HPLC CHROMATOGRAMS OF EXTRACTS FROM DEVELOPING GRAINS OF WHEAT CULTIVAR "SONORA" (harvested X days after anthesis)



Chromatographic conditions were: 1.5 m x 2.1 mm i.d. Zipax-SCX column with distilled water (pH 1.7 with HNO_3) mobile phase: 27°C, 590 p.s.i., and a flow of 20 cm^3/hr. ISCO recorder chart speed was 15 cm/hr with 0.02 absorbance units full scale.

HPLC CHROMATOGRAMS OF EXTRACTS FROM DEVELOPING GRAINS OF WHEAT CULTIVAR "SONORA" (harvested X days after anthesis)



Chromatographic conditions were: 1.5 m x 2.1 mm i.d. Zipax-SCX column with distilled water (pH 1.7 with HNO_3) mobile phase: 27°C, 590 p.s.i., and a flow of 20 cm^3/hr. ISCO recorder chart speed was 15 cm/hr with 0.02 absorbance units full scale.

A5.4 ESTIMATED CONTENT OF tABA AND ABA IN ng PER GRAIN, FROM HPLC ANALYSIS

SAMPLE	GAMUT		TIMGALEN		TIMxPEM		PEMBINA		SONORA		KARAMU	
Days from Anthesis	tABA	ABA	tABA	ABA	tABA	ABA	tABA	ABA	tABA	ABA	tABA	ABA
5	0.87	0.23	0.17	0.67	0.35	0.0	3.82	3.15	--	--	0.08	0.38
14	0.46	5.23	0.03	1.40	0.05	0.28	9.98	0.27	2.22	2.98	0.24	1.06
23	0.61	4.93	0.05	1.29	1.97	3.47	10.6	0.38	0.53	1.33	0.92	5.29
32	0.55	3.18	0.00	0.00	3.04	0.51	2.0	0.35	0.93	2.92	0.15	0.63
41	0.99	3.57	0.42	0.13	0.08	1.42	9.56	2.67	0.98	4.85	0.00	0.77
50	1.17	1.26	0.13	0.04	0.21	0.56	2.01	1.12	0.26	0.38	0.04	0.63
60	0.94	1.38	0.08	0.10	0.92	0.23	10.84	0.13	1.17	1.20	0.55	1.73
71	--	--	0.76	0.20	1.09	9.50	4.19	0.13	2.05	2.21	3.46	3.02
81	--	--	3.39	0.45	0.91	5.07	3.34	0.19	3.67	3.29	1.48	1.17

A5.5 ESTIMATED CONTENT OF t ABA AND ABA ng PER GRAM DRY WEIGHT, FROM HPLC ANALYSIS

SAMPLE	GAMUT		TIMGALEN		TIM x PEM		PEMBINA		SONORA		KARAMU	
Days from Anthesis	tABA	ABA	tABA	ABA	tABA	ABA	tABA	ABA	tABA	ABA	tABA	ABA
5	304.8	80.9	42.4	168.0	121.6	0.0	2171	1787	--	--	44.0	195.7
14	33.8	288.3	2.53	125.4	6.2	37.3	1357	37.2	193.5	259.4	23.0	128.4
23	36.9	298.8	2.54	64.5	100.9	177.9	472	17.2	23.3	59.0	45.5	262.2
32	21.3	122.0	0.00	0.0	102.7	17.1	105.6	18.5	27.4	86.4	5.7	23.8
41	22.6	81.6	12.36	3.9	2.1	36.7	315.6	88.3	21.1	104.3	0.0	21.3
50	22.9	24.7	3.0	0.8	5.1	13.5	53.9	29.9	7.3	10.7	0.9	15.5
60	20.3	29.8	2.0	2.3	32.1	7.96	268.9	3.1	25.5	26.1	13.3	41.4
71	--	--	19.0	5.3	31.7	277.4	89.9	2.8	55.6	59.9	79.3	69.3
81	--	--	85.8	11.4	26.0	144.9	74.5	4.2	75.5	67.6	34.2	27.2

A6.1 ORIGINAL DATA FOR CHAPTERS 3 AND 4

Column	Description
I	Genotype Index
J	Age of Grain Sample (Dayst from anthesis
K	Replicate Index (Block number)
1	Fresh weight (g/10 grains)
2	Oven Dry Weight (g/10 grains)
3	% grain moisture (Fresh weight basis)
4	% germination in standard test
5	% germination in dormancy-breaking test
6	% dormancy
7	Sodium Hydroxide Colour Score
8	Phenol Colour Score
9	Flavanol Level (µg/10 grains)
10	Flavanol concentration (µg/gDW)
11	Phenolase activity with catechol substrate (absorbance untis/min/25 grains
12	Phenolase activity with catechol substrate (absorbance units/min/gDW)
13	Phenolase activity with flavan-3-ol substrate (absorbance units/min/20 grains)
14	Phenolase activity with flavan-3-ol substrate (absorbance units/min/gDW)

DATA FOR DEVELOPING GRAINS OF WHEAT CULTIVAR "GAMUT"

	1	2	3			4	5	6	7	8	9	10	11	12	13	14	IJ K
401	.1149	.0251	78.2	0	0	0	0	100	0	1			8	132			4051
402	.0797	.0156	80.4	0	0	0	0	100	0	1	7.8	385.1	8	132			4052
403	.1292	.0244	81.1	0	0	0	0	100	0	1	16.3	460.3	4	93			4053
404	.0881	.0184	79.1	0	0	0	0	100	0	1	10.6	533.4	7	162			4054
405	.0964	.0185	80.8	0	0	0	0	100	0	1	18.6	709.7	4	56			4055
406	.1158	.0243	79.0	0	0	0	0	100	0	1	17.2	713.1	3	42			4056
407	.3909	.1098	71.9	0	0	0	0	100	0	1			7	25	4	28	4141
408	.1920	.0467	75.7	0	0	0	0	100	0	1	22.4	376.3	9	32	6	34	4142
409	.2083	.0519	75.1	0	0	0	0	100	0	1	20.5	259.5	9	65			4143
410	.	.	.	0	0	0	0	100	0	1	.	.	14	100			4144
411	.3391	.0953	71.9	0	0	0	0	100	0	1	21.4	225.1	14	41			4145
412	.	.	.	0	0	0	0	100	0	1	30.8	483.5	12	35			4146
413	.5310	.1971	62.9	0	0	0	0	100	0	1	.	.	12	28			4231
414	.5767	.1726	70.1	0	0	0	0	100	0	1	31.3	217.7	11	26			4232
415	.3597	.0998	72.2	0	0	0	0	100	0	1	39.7	322.9	2	5			4233
416	.3647	.1056	71.0	0	0	0	0	100	0	1	42.5	315.7	1	2			4234
417	.	.	.	0	0	0	0	100	0	1	27.5	173.5	13	45			4235
418	.4611	.1403	69.6	0	0	0	0	100	0	1	41.6	217.9	15	52			4236
419	.8035	.3250	59.6						0	1			13	22	23	42	4321
420	.5874	.2377	59.5	0	0	0	0	100	0	1	23.8	88.3	15	25	36	60	4322
421	.3546	.1018	71.3	0	0	0	0	100	0	1	32.2	149.5	4	6	25	35	4323
422	.5517	.2033	63.2	0	0	0	0	100	0	1	35.5	139.6	4	6			4324
423	.4527	.1335	70.5	0	0	0	0	100	0	1	50.5	159.2					4325
424	.6041	.2063	65.9						0	1	72.1	289.0					4326
425	.7886	.4038	48.8	0	17	0	85	100	0	2	.	.	14	14	37	42	4411
426	.6516	.2784	57.3						0	1	51.0	142.2	16	15	39	54	4412
427	.6479	.2901	55.2	2	4	10	20	50	2	1	40.7	150.6	4	4	42	55	4413
428	.6324	.2810	55.6	0	0	0	0	100	0	1	55.2	163.8	10	11			4414
429	.7462	.3468	53.5	1	5	5	25	80	0	1	48.1	113.1	10	14			4415
430	.5912	.2854	51.7	0	17	0	85	100	0	2	59.9	182.2	17	25			4416
431	.7962	.4316	45.8	4	12	20	60	67	3	7	.	.	9	7	62	59	4501
432	.8349	.4649	44.3	3	10	15	50	70	0	3	56.2	107.1	11	8	33	33	4502
433	.7144	.3891	45.5	6	11	30	65	54	3	6	52.9	107.4	12	12	48	46	4503
434	.7642	.4240	44.5	0	3	0	15	100	3	6	50.0	106.2	15	15			4504
435	.7382	.3802	48.5	5	5	25	25	0	2	4	54.7	106.1	7	7			4505
436	.9943	.5124	48.5	10	17	50	85	41	2	4	76.3	160.0	0	1			4506
437	.7397	.4390	40.7	11	11	55	55	0	4	8	.	.	9	8	31	30	4601
438	.7483	.4316	42.3						4	5	43.0	88.0	13	11	42	40	4602
439	.8113	.4450	45.1	4	5	20	25	20	3	4	41.1	89.2	13	13	45	51	4603
440	.6515	.3593	44.9	11	10	55	50	0	2	6	46.3	94.5	16	16			4604
441	.5769	.2688	53.4	6	10	30	50	40	2	6	64.1	123.6					4605
442	.7994	.4427	44.6	7	15	35	75	53	2	6	66.9	129.0					4606
443	.4101	.3312	19.2						4	8			10	23	44	42	4711
444						9		45	5	6			10	23	30	33	4712
445									3	8	53.8	102.7	18	18	49	44	4713
446	.6485	.4466	31.1	6	16	30	80	63	3	6	53.8	111.5	27	27			4714
447									3	6	64.6	150.2					4715
448	.7682	.4470	41.8	2	0	10	0	0	3	6	51.0	88.0					4716
449	.4661	.3805	18.4	11	16	55	80	44	4	7			13	10	16	14	4811
450									5	6			14	10	32	26	4812
451	.3179	.2735	14.0						4	6	71.2	215.4			74	65	4813
452	.4989	.4048	18.9	2	12	10	60	83	3	8	67.9	147.2					4814
453	.4935	.3873	21.5	3	14	15	70	79	3	6	74.0	126.3					4815
454	.5455	.4482	17.8	15	15	75	75	0	4	6	76.3	135.1					4816

DATA FOR DEVELOPING GRAINS OF WHEAT CULTIVAR "TIMGALEN"

	1	2	3			4	5	6	7	8	9	10	11	12	13	14	IJ K
301	.1342	.0289	78.5	0	0	0	0	100	0	1			6	83			3051
302	.1216	.0286	76.5	0	0	0	0	100	0	1	12.5	433.7	11	153			3052
303	.1297	.0306	76.4	0	0	0	0	100	0	1	33.6	977.2	4	49			3053
304	.1516	.0328	78.4	0	0	0	0	100	0	1	22.4	653.5	6	74			3054
305	.1044	.0240	77.0	0	0	0	0	100	0	1	33.1	845.6	2	24			3055
306	.1323	.0327	75.3	0	0	0	0	100	0	1	26.1	594.6	2	24			3056
307	.3730	.1157	69.0	0	0	0	0	100	0	1			11	36	18	85	3141
308	.3241	.0829	74.4	0	0	0	0	100	0	1	8.3	81.7	13	43	31	46	3142
309	.3160	.0885	72.0	0	0	0	0	100	0	1			0	4	5	23	3143
310	.3672	.0948	74.2	0	0	0	0	100	0	1	29.9	242.2	1	4			3144
311	.4251	.1183	72.2	0	0	0	0	100	0	1	29.9	323.5	0	4			3145
312	.6323	.2524	60.1	0	0	0	0	100	0	1	38.3	279.2	4	17			3146
313	.4814	.2135	55.7	0	1	0	5	100	0	1			19	20			3231
314	.5252	.2326	55.7	1	3	5	15	67	0	1	19.5	89.8	16	27			3232
315	.4838	.2262	53.2	3	1	15	5	0	0	1	43.0	165.5	2	3			3233
316	.5755	.2445	57.5	1	0	5	0	0	0	1	51.4	202.8	6	10			3234
317	.4965	.2240	54.9	2	0	10	0	0	0	1	49.1	195.7	7	11			3235
318	.	.	.	0	0	0	0	100	0	1	.	.	9	15			3236
319	.5726	.2897	49.4	0	0	0	0	100	0	2	.	.	12	15	50	74	3321
320	.5974	.2954	50.6						0	1	6.4	21.5	21	27	38	55	3322
321	.6233	.3076	50.6	1	4	5	20	75	0	2	8.7	26.4	4	20			3323
322	.5983	.3203	46.7	0	0	0	0	100	0	1	51.4	190.3	10	3			3324
323	.6671	.3338	50.0						0	1	39.2	101.4	3	32			3325
324	.5450	.2540	53.4	1	0	5	0	0	0	1	66.5	223.8	8	3			3326
325	.6375	.3576	43.9	0	0	0	0	100	3	6			24	26	63	95	3411
326	.5328	.3050	42.8						0	3	17.7	53.4	27	29	66	81	3412
327	.6458	.3058	52.6	6	12	30	60	50	3	2	23.3	107.5	9	12	45	68	3413
328	.5472	.2918	46.7						0	2	57.6	165.3	11	19			3414
329	.5594	.3397	39.3	2	5	10	25	60	2	4	42.5	104.8					3415
330	.5568	.3173	43.0	0	0	0	0	100	3	8	54.7	199.9					3416
331	.7762	.4429	42.9	1	3	5	15	67	3	6	.	.	22	20	45	47	3501
332	.6424	.4224	34.2	3	7	15	35	29	2	7	11.5	28.2	20	27	49	50	3502
333	.6231	.3625	41.8						3	7	43.9	102.3	11	10	43	53	3503
334	.8424	.4890	42.0	4	6	20	30	33	5	5	63.2	131.2	15	14			3504
335	.5601	.3811	32.0	3	9	15	45	67	3		58.5	134.4	4	20			3505
336	.4323	.3173	26.6	1	7	5	35	86	3	5	59.9	175.0	10	22			3506
337	.5844	.3668	37.2	15	15	75	75	0	4	7	.	.	22	34	25	22	3601
338	.	.	.	7	7	35	35	0	3	8	18.6	47.1	28	27	32	41	3602
339	.3677	.3082	16.2	4	8	20	40	50	5	7	55.7	140.8	10	10	34	41	3603
340	.7116	.4322	39.3	9	10	45	50	10	5	7	69.8	138.4	14	14			3604
341	.6971	.4459	36.0	5	10	25	50	50	3	7	71.2	143.1	19	20			3605
342				2	10	10	50	80	3	7	72.1	185.8	21	22			3606
343	.5601	.4263	23.9	3	12	15	60	75	4	7			37	34	54	60	3711
344	.4835	.3714	23.2	2	20	10	100	10	3	8	54.3	137.5	48	44	26	33	3712
345	.4853	.4028	17.0	6	12	30	60	50	4	7			5	5	61	69	3713
346									5	8	79.5	186.4	9	9			3714
347	.2956	.2530	14.4	0	14	0	70	100	6	8	75.4	203.5					3715
348				6	11	30	55	45	4	7	70.2	188.4					3716
349	.4238	.3648	13.9	15	15	75	75	0	4	7			33	33	60	71	3811
350	.4019	.3449	14.2	9	17	45	85	47	3	9	89.9	221.8	31	31	62	74	3812
351	.3552	.3091	13.0	15	17	75	85	12	5	8	78.2	214.9			34	46	3813
352	.4192	.3612	13.8	9	17	45	85	47	5	8	83.8	184.4					3814
353	.4622	.3976	14.0	9	12	45	60	25	6	8	64.6	172.8					3815
354	.4661	.3805	18.4	15	18	75	90	17	5	9	85.2	218.4					3816

DATA FOR DEVELOPING GRAINS OF WHEAT CULTIVAR "TIX X PEM"

	1	2	3			4	5	6	7	8	9	10	11	12	13	14	IJ K
501	.1156	.0290	74.9	0	0	0	0	100	0	1	.3	9.00	13	215			5051
502	.0774	.0188	75.7	0	0	0	0	100	0	1	23.3	99.5	13	215			5052
503	.1283	.0301	76.5	0	0	0	0	100	0	1	11.1	406.0	7	106			5053
504	.	.	.	0	0	0	0	100	0	1	.	.	7	106			5054
505	.0729	.0155	78.7	0	0	0	0	100	0	1	9.7	556.0					5055
506	.1407	.0340	75.8	0	0	0	0	100	0	1	20.5	605.7					5056
507	.2255	.0567	74.9	0	0	0	0	100	0	1	9.7	98.9	11	41	3	18	5141
508	.4130	.1120	72.9	0	0	0	0	100	0	1	34.1	320.0	13	48	1	6	5142
509	.2085	.0528	74.7	0	0	0	0	100	0	1	20.9	261.8	4	25	3	11	5143
510	.	.	.						0	1	.	.	7	44			5144
511	.2490	.0669	73.1	0	0	0	0	100	0	1	29.4	438.7					5145
512	.3630	.1070	70.5	0	0	0	0	100	0	1	40.7	314.4					5146
513	.4755	.2056	56.8	0	0	0	0	100	0	1	15.8	73.7	24	68			5231
514	.4013	.1286	68.0	0	0	0	0	100	0	1	34.1	234.3	19	54			5232
515	.4550	.1823	59.9	0	0	0	0	100	0	1	38.8	182.5	4	8			5233
516	.4510	.1321	70.7	0	0	0	0	100	0	1	.	.	10	19			5234
517	.4133	.1237	70.1	0	0	0	0	100	0	1	41.1	274.2					5235
518	.	.	.	0	0	0	0	100	0	1	27.0	198.7					5236
519	.4825	.2512	48.0						0	1	6.4	26.4	10	17	37	70	5321
520	.5314	.2475	53.4	0	0	0	0	100	0	1	35.0	148.1	15	25	36	75	5322
521	.6238	.3670	41.2	0	0	0	0	100	0	1	49.1	114.8	9	13	32	59	5323
522									0	1			16	24			5324
523	.6327	.2585	59.1	0	0	0	0	100	0	1	58.5	215.8					5325
524	.4873	.2328	52.2	0	0	0	0	100	0	1	38.8	161.3					5326
525	.6204	.3212	48.2	2	4	10	20	50	5	5	13.9	40.7	26	29	30	35	5411
526	.7498	.3788	49.5	0	1	0	5	100	0	2	60.6	149.2	20	23	31	48	5412
527	.7232	.3369	53.4	0	1	0	5	100	0	1	54.3	149.5	9	11	45	55	5413
528	.	.	.	0	0	0	0	100	9	1	.	.	3	16			5414
529	.7763	.3328	57.1						0	3	59.4	159.6					5415
530	.5347	.3075	42.5						7	1	57.1	157.1					5416
531	.5257	.3109	40.9	1	2	5	10	50	5	6	11.5	34.7	17	16	40	43	5501
532	.6924	.3802	45.1	3	6	15	30	50	5	3	55.7	147.4	20	19	50	63	5502
533	.6742	.3685	45.5	0	2	0	10	100	7	6	46.3	114.4	9	9	42	58	5503
534	.5554	.3206	42.3	3	10	15	50	70	8	7	.	.	13	13			5504
535	.5977	.3350	44.0						3	4	58.0	133.1					5505
536	.3731	.2151	42.3	9	5	45	25	0	8	6	51.9	157.4					5506
537	.5566	.3426	38.4					75	5	6	22.3	58.1	7	6	37	45	5601
538	.7217	.4912	31.9	6	7	30	35	14	5	7	37.8	78.5	8	7	50	63	5602
539	.3425	.2873	16.1	3	9	15	45	67	7	7	54.7	140.1	7	7	44	57	5603
540	.5648	.3765	33.3	0	2	0	10	100	9	7	.	.	13	13			5604
541	.2477	.2108	14.9	13	20	65	100	45	3	6	79.1	176.5					5605
542	.7596	.4448	41.4	2	11	10	55	82	8	7	57.1	148.0					5606
543	.4207	.3387	19.5	2	10	10	50	80	7	9	45.3	103.6	124	29	38	72	5711
544	.6988	.4279	38.8	3	5	15	25	40	4	7	31.7	130.3	22	26	39	64	5712
545	.4372	.3393	22.4	0	5	0	25	100	7	8	52.8	134.7			56	82	5713
546	.5005	.4166	16.8	5	7	25	35	29	3	7	.	.					5714
547	.3958	.3192	19.3						5	7	66.9	166.7					5715
548	.2545	.2137	16.0						3	7	67.4	237.2					5716
549	.4200	.3342	20.4	6	14	30	70	57		7	.	.			32	41	5811
550	.3743	.3018	19.4	2	8	10	40	75	5	8	.	.			32	40	5812
551	.2430	.2091	14.0						5	8	42.5	176.9			46	50	5813
552	.4442	.3800	14.5						8	8	.	.					5814
553	.4452	.3821	14.2	17	20	85	100	15	5	7	62.2	152.7					5815
554	.3158	.2676	15.3	6	16	30	80	63	8	7	63.6	168.1					5816

DATA FOR DEVELOPING GRAINS OF WHEAT CULTIVAR "PEMBINA"

	1	2	3			4	5	6	7	8	9	10	11	12	13	14	IJ K
601	.0772	.0158	79.5	0	0	0	0	100	0	1	.3	14.1	5	99			6051
602	.0741	.0147	80.2	0	0	0	0	100	0	1	27.5	999.9	6	118			6052
603	.	.	.						0	1	.	.	24	582			6053
604	.0770	.0152	80.3	0	0	0	0	100	0	1	13.4	616.1	21	509			6054
605	.0811	.0164	79.8	0	0	0	0	100	0	1	17.7	882.8					6055
606	.0697	.0140	79.9	0	0	0	0	100	0	1	.3	18.					6056
607	.1862	.0462	75.2	0	0	0	0	100	0	1	11.5	160.0	6	42	4	18	6141
608	.1951	.0405	74.2	0	0	0	0	100	0	1	32.2	578.2	1	7	0	0	6142
609	.	.	.						0	1	.	.	8	62	1	8	6143
610	.2368	.0602	74.6	0	0	0	0	100	0	1	20.9	347.9	1	8			6144
611	.2906	.0847	70.9	0	0	0	0	100	0	1	34.1	445.0					6145
612	.3012	.0809	73.1	0	0	0	0	100	0	1	15.3	221.5					6146
613	.	.	.	0	0	0	0	100	0	1	20.0	160.4	7	26			6231
614	.3771	.1068	71.7	0	0	0	0	100	0	1	48.6	437.0	7	26			6232
615	.	.	.						0	1	.	.	0	0			6233
616	.4341	.1313	69.8	0	0	0	0	100	0	1	36.0	235.0	7	26			6234
617	.3465	.1005	71.2	0	0	0	0	100	0	1	35.0	271.1					6235
618	.3350	.0977	70.8	0	0	0	0	100	0	1	29.4	216.0					6236
619	.5074	.2474	51.2	0	0	0	0	100	0	1	13.9	70.5	2	5	5	13	6321
620	.4963	.1921	61.3	0	0	0	0	100	0	1	45.3	206.4	0	0	26	67	6322
621	.	.	.						0	1	.	.	4	13	31	82	6323
622	.5820	.2244	61.4	0	0	0	0	100	0	1	40.2	179.8	1	36			6324
623	.5790	.2206	61.9	0	0	0	0	100	0	1	42.1	169.7					6325
624	.4736	.1904	59.8	0	0	0	0	100	0	1	36.4	166.5					6326
625	.5652	.2549	54.9	2		10			0	1	16.2	53.6	10	16	39	60	6411
626	.6491	.3147	51.5	0	0	0	0	100	0	2	42.5	157.0	8	12	51	79	6412
627	.	.	.						8	1	.	.	10	14	41	67	6413
628	.5952	.2857	52.0	0	0	0	0	100	7	1	47.7	153.1	6	9			6414
629	.6059	.2878	52.5	0	0	0	0	100	8	2	.	.					6415
630	.5626	.2614	53.5	0	0	0	0	100	9	1	45.8	132.2					6416
631	.5446	.2962	45.6	0	8	0	40	100	4	2	5.9	15.2	15	18	42	50	6501
632	.6333	.3595	43.2	1	2	5	10	50	5	3	51.0	148.3	13	15	39	67	6502
633	.	.	.						9	2	.	.	3	3	34	47	6503
634	.5970	.3600	39.7	0	0	0	0	100	8	2	50.5	129.7	10	10			6504
635	.6210	.3374	45.7	0	0	0	0	100	8	5	49.6	137.5					6505
636	.6006	.3189	46.9	1	1	5	5	0	9	3	26.6	87.8					6506
637	.2006	.1638	18.3	2	8	10	40	75	8	5	.3	1.6	24	30	55	59	6601
638	.7271	.4168	42.7	1	6	5	30	83	5	6	52.4	148.3	24	30	41	53	6602
639	.	.	.						8	6	.	.	13	15			6603
640	.2608	.2107	19.2	2	3	10	15	33	8	5	57.6	156.7	17	20			6604
641	.4212	.3356	20.3	0	3	0	15	100	9	7	45.8	126.5					6605
642	.4707	.3120	33.7	6	12	30	60	50	9	8	45.8	142.5					6606
643	.2503	.2294	8.4	2	7	10	35	71	8	7	13.9	71.6	12	15	22	25	6711
644	.4497	.3273	27.2						7	5	.	.	21	27			6712
645	.	.	.						8	6	.	.	15	17			6713
646	.	.	.						8	8	51.9	115.4	12	13			6714
647	.5406	.3496	35.3	0	0	0	0	100	9	7	50.5	119.7					6715
648									9								6716
649	.	.	.	0	9	0	45	100	9	9	24.2	52.3	10	10	29	33	6811
650	.4313	.3510	20.0		19		95		6	7	.	.	12	12	39	46	6812
651																	6813
652	.	.	.	3	7	15	35	57	8	7	70.2	175.4	17	21			6814
653	.	.	.	0	0	0	0	100	5	8	99.9	258.2					6815
654	.	.	.	5	9	25	45	44	8		.	.					6816

DATA FOR DEVELOPING GRAINS OF WHEAT CULTIVAR "SONORA"

	1	2	3			4	5	6	7	8	9	10	11	12	13	14	IJ K
201	.0917	.0221	75.9	0	0	0	0	100	0	1	.	.	10	167			2051
202	.1326	.0330	75.1	0	0	0	0	100	0	1	.8	23.2	10	167			2052
203	.0852	.0214	74.9	0	0	0	0	100	0	1	9.7	445.8	9	239			2053
204	.	.	.	0	0	0	0	100	0	1	.	.	9	239			2054
205	.0931	.0228	75.5	0	0	0	0	100	0	1	18.1	858.8					2055
206	.1292	.0327	74.7	0		0	0	100	0	1	17.7	416.4					2056
207	.3155	.0855	72.9	0	0	0	0	100	0	1	.	.	11	51	2	11	2141
208	.3042	.0855	71.9	0	0	0	0	100	0	1	39.7	349.3	17	79	0	0	2142
209	.3243	.1071	67.0	0	0	0	0	100	0	1	20.2	183.2	5	19	4	17	2143
210	.	.	.	0	0	0	0	100	0	1	.	.	13	50			2144
211	.3068	.0823	73.2	0	0	0	0	100	0	1	36.9	369.4	15	61			2145
212	.	.	.	0	0	0	0	100	0	1	.	.	25	102			2146
213	.4784	.2173	54.6	0	0	0	0	100	0	1	.	.	12	21			2231
214	.5010	.1767	64.7	0	0	0	0	100	0	1	32.2	119.3	11	19			2232
215	.5738	.2177	62.1	0	0	0		0	0		44.9	206 4	3	5			2233
216	.	.	.	0	0	0	0	100	0	1	.	.	0	2			2234
217	.	.	.	0	0	0	0	100	0	1	.	.	10	18			2235
218	.4688	.1757	62.5	0	0	0	0	100	0	1	31.3	167.7	9	16			2236
219	.5887	.3002	49.0	0	0	0	0	100	8	2	.	.	18	23	23	34	2321
220	.6654	.3654	45.1	0	0	0	0	100	6	3	.	.	15	19	42	52	2322
221	.6932	.3845	44.5	0	0	0	0	100	0	1	.	.	5	5	41	47	2323
222	.6212	.2250	63.8	0	0	0	0	100	0	2	37.5	155.3	1	1			2324
223	.5833	.3099	46.9	0	0	0	0	100	0	1	.	.	15	16			2325
224	.	.	.	0	0	0	0	100	0	2	.	.					2326
225	.6633	.3777	43.1	4	3	20	15	0	7	2	.	.	19	18	15	16	2411
226	.	.	.	0	1	0	5	100	6	6	33.6	94.6	26	25	20	29	2412
227	.6259	.3508	44.0	1	0	5	0	0	7	4	39.2	99.1	6	28	14	15	2413
228	.	.	.	0	1	0	5	100	7	4	41.4	116.9	11	51			2414
229	.6153	.3416	44.5	0	0	0	0	100	7	2	62.2	155.6	9	8			2415
230	.	.	.						8	6	58.0	140.7	9	8			2416
231	.6374	.3715	41.7	6	6	30	30	0	8	5	.	.	17	17	24	28	2501
232	.7246	.4275	41.0	5	7	25	35	29	6	6	13.0	27.5	38	39	12	14	2502
233	.6789	.4178	38.5						8	3	45.3	105.6	17	15	50	57	2503
234	.	.	.						7	5	51.4	112.9	20	18			2504
235	.3748	.2734	27.1	0	0	0	0	100	8	6	56.1	164.3	23	24			2505
236	.4948	.3106	37.2	9	8	45	40	0	7	5	25.6	60.9	31	33			2506
237	.5418	.3693	31.8	7	9	35	45	22	7	5	.	.	23	19	74	82	2601
238	.6077	.3471	42.9	5	9	25	45	44	7	8	.	.	36	30	30	37	2602
239	.4628	.3517	24.0						8	5	60.8	152.0	8	12	38	43	2603
240	.5964	.3646	38.9	5	10	25	50	50	9	5	46.8	113.4	13	20			2604
241	.	.	.	0		0			9	7	77.7	164.7	13	12			2605
242	.4441	.3785	14.8	7	9	35	45	22	8	8	9.7	24.2	20	18			2606
243	.	.	.	7	11	35	55	36	7	6	.	.	36	35	1	2	2711
244	.	.	.	7	8	35	40	13	8	8	66.9	145.0	39	38	21	24	2712
245	.	.	.	2	3	10	15	33	8	6	64.6	146.8	8	7	40	53	2713
246	.	.	.						9	8	52.4	108.7	7	6			2714
247	.4809	.2861	40.5	0	4	0	20	100	8	8	75.4	156.4	27	25			2715
248	.4391	.3328	24.2	0	0	0	0	100	8	8	32.2	70.0	21	19			2716
249	.6863	.4486	34.6	13	20	65	100	54	8	9	.	.	35	31	23	24	2811
250	.4461	.3404	23.7	3	14	15	70	79	8	8	63.7	158.8	33	29	31	42	2812
251	.	.	.						8	9	82.9	181.8			24	25	2813
252	.4451	.3064	31.2	11	20	55	100	45	9	8	90.9	210.6					2814
253	.4207	.3635	13.6	3	19	15	95	84	8	8	90.9	210.6					2815
254	.4238	.3648	13.9	9	17	45	85	47	9	9	58.5	140.7					2816

DATA FOR DEVELOPING GRAINS OF WHEAT CULTIVAR "KARAMU"

	1	2	3			4	5	6	7	8	9	10	11	12	13	14	IJ	K
1				0	0	0	0	100	0	1	N		7	135				1051
2	.0887	.0160	82.0	0	0	0	0	100	0	1	9.2	422.2	7	135				1052
3	.0864	.0220	74.5	0	0	0	0	100	0	1	16.7	711.3	4	81				1053
4	.0707	.0174	75.4	0	0	0	0	100	0	1	13.3	458.0	1	20				1054
5	.1106	.0253	77.1	0	0	0	0	100	0	1		4	8	169	9			1055
6	.0665	.0134	79.8	0	0	0	0	100	0	1	3.6	228.8	7	148				1056
7	.2246	.0628	72.0	0	0	0	0	100	0	1	.	.	2	13	3	15		1141
8	.2897	.0735	74.6	0	0	0	0	100	0	1	28.4	286.5	6	40	22	105		1142
9	.3183	.1025	67.8	0	0	0	0	100	0	1	21.9	210.0	3	13	14	72		1143
10	.2938	.0770	73.8	0	0	0	0	100	0	1	28.8	337.9	3	13				1144
11	.2292	.0582	74.6	0	0	0	0	100	0	1	23.3	234.1	4	15				1145
12	.	.	.	0	0	0	0	100	0	1	27.0	383.1	6	23				1146
13	.4177	.1612	61.4	0	0	0	0	100	0	1			12	27				1231
14	.6068	.2741	54.8	0	0	0	0	100	0	1	18.6	244.0	16	36				1232
15	.3831	.1203	68.6	0	0	0	0	100	0	1	43.0	203.9	2	7				1233
16	.4655	.1875	59.7	0	0	0	0	100	0	1	47.6	267.5	1	4				1234
17	.4358	.1708	60.8	0	0	0	0	100	0	1	48.2	255.9	16	31				1235
18	.4355	.1262	71.3	0	0	0	0	100	0	1	20.9	238.2	14	28				1236
19	.4906	.2419	51.0	0	0	0	0	100	0	1			35	79	20	39		1321
20				0	0	0	0	100	0	1			9	16	29	45		1322
21	.4718	.2358	50.0	0	0	0	0	100	0	1	32.2	150.4	11	20	52	84		1323
22	.4771	.1687	64.6	0	0	0	0	100	0	1	35.4	184.1						1324
23				0	0	0	0	100	0	1	51.9	193.4						1325
24	.5506	.2787	49.4	0	0	0	0	100	0	1	14.8	59.2						1326
25	.4416	.2323	47.4	0	0	0	0	100	0	1			27	41	25	55		1411
26	.5600	.3007	46.3	0	0	0	0	100	7	2	38.8	105.3	35	53	53	69		1412
27	.4534	.2500	44.9	0	0	0	0	100	6	1	46.8	185.6	13	17	47	105		1413
28	.5355	.2950	44.9	0	0	0	0	100	7	2	51.3	145.5	19	25				1414
29	.5227	.2304	55.9	0	1	0	5	100	7	1	35.0	111.3						1415
30	.5486	.2752	49.8	0	0	0	0	100	8	2	44.4	152.1						1416
31	.5655	.3354	40.7	1	0	5	0	100	6	1			64	66	21	28		1501
32	.5472	.3409	37.7	0	0	0	0	100	8	4	43.5	110.4	65	65	41	53		1502
33	.6852	.3858	43.7	0	0	0	0	100	6	1	43.5	150.3	16	16	51	66		1503
34				0	0	0	0	100	7	5	36.9	85.9	15	15				1504
35	.6567	.3614	45.0	0	0	0	0	100	6	4	60.6	163.4	18	22				1505
36	.5985	.3472	42.0	0	2	0	10	100	8	3	48.2	135.6	20	24				1506
37	.5674	.3325	41.4	0	1	0	5	100	5	6			29	29	58	72		1601
38	.5989	.3484	41.8	3	3	15	15	0	8	5	43.0	100.3	41	41	63	74		1602
39	.5246	.3204	38.9	0	0	0	0	100	7	5	57.1	139.8	29	30	52	62		1603
40	.6184	.3697	40.2	0	0	0	0	100	7	6	53.8	128.9	22	22				1604
41	.5605	.3511	37.4	0	0	0	0	100	8	7	79.6	156.3	9	10				1605
42	.6799	.4174	38.6	0	2	0	10	100	7	5	36.0	84.2	10	11				1606
43	.4790	.3272	31.7	0	0	0	0	100	7	9			20	27	65	77		1711
44				0	1	0	5	100		5			17	23	52	61		1712
45	.5389	.3801	29.5	0	0	0	0	100	7	6	59.9	133.2	10	12	52	68		1713
46	.5196	.3162	39.1	0	0	0	0	100	7	6	51.4	139.2	12	13				1715
47	.4890	.3363	31.2	0	1	0	5	100	8	6	45.8	141.3	2	2				1715
48	.5555	.3827	31.1	0	0	0	0	100	9	5	51.4	127.7	13	13				1716
49	.4167	.3292	21.0						7	9			6	6	42	50		1811
50	.4248	.3626	14.6	0	12	0	60	100	8	6	50.9	131.8	17	16				1812
51	.4520	.3735	17.4	1	1	5	5	0	6	7	50.9	131.8	14	14				1813
52	.3570	.3045	14.7	4	4	20	20	0	7	7	74.9	192.4	15	15				1814
53	.5064	.4323	14.6	1	1	5	5	0	8	6	86.2	173.3						1815
54	.4055	.3520	13.2	1	9	5	45	89	9	7	63.4	147.9						1816