

THE PHYSIOLOGY OF FLOWERING

and

SOME ASPECTS ON THE REGULATION OF FLOWERING IN PEAS

a thesis submitted by

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PREFACE

This thesis is divided into two parts:

Part I is a review of the current literature on "The Physiology of Flowering".

Part II contains the results of research done into the environmental conditions and into the role of the cotyledons in regulating the flowering behaviour of two varieties of Pisum sativum.

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...*Julian Chinos*...

ABBREVIATIONS USED IN TEXT

DNA	=	deoxyribose nucleic acid.
EDTA	=	ethylene diamine tetra-acetic acid.
5-FDU	=	5 - fluorodeoxyuridine.
5-FU	=	5-fluorouracil.
GA ₃ (7)	=	gibberellin A ₃ (7)
L.D	=	long day
R.N.A	=	ribose nucleic acid.
S.D	=	short day
S-LD	=	short long day.

PART I

THE PHYSIOLOGY OF FLOWERING.

INTRODUCTION

The process of flowering involves 4 major stages:- a) the differentiation of floral primordia ; b) the differentiation of the individual flower parts ; c) floral maturation ; and d) anthesis. This review will only be concerned with the first aspect, that of the transition from vegetative to reproductive growth, and of the developments which precede this transition. Since the early review by Lang (140), many reviews dealing with certain aspects of flowering have appeared (24, 25, 50, 52, 65, 74, 154, 155, 171, 187, 197, 198, 201, 209, 224, 245, 247, 252, 261), giving an excellent coverage of the field, and the approach of this review is similar in its presentation to that of Searle (209). Similarities can be seen of virtually all the steps involved in the flowering process in other biological functions e.g. photoperiodism implies a response to light and a measurement of time, and the response to the flowering hormone is an example of morphogenesis - perhaps the most fundamental phenomenon of biology. Since floral initiation is basically a morphogenetic response, these changes and causes will be noted first, to be followed by a discussion on substances causing this response, and then to the regulation of these substances.

CHAPTER ONE

NUCLEIC ACID METABOLISM IN THE BUD.

Since meristematic cells are potentially capable of any of the functions of specialised cells, differentiation processes, of which flowering is one aspect, must be controlled by the genetic information of the cells contained in their DNA molecules, either by activating passive genes or by inhibiting active genes. (220).

Synthesis of nucleic acid.

Synthesis of nucleic acid in the bud is concerned with floral induction, and this fact has been confirmed by using nucleic acid inhibitors.

Salisbury, Bonner & Zeevart have shown that 5 - FU can inhibit flower induction in Xanthium if it is applied during the inductive dark period (21, 202) while in Pharbitis, its greatest effect seems to be at the end of the inductive dark period (257). In both these S-D plants 5 - FU has its effect by inhibiting nucleic acid synthesis (48, 49) - and thus bud growth (211)-being incorporated into bud R.N.A. (21). However, while its effect on induction in Xanthium is caused by a repression of R.N.A. synthesis (21), its effect on induction in Pharbitis seems to be caused by an inhibition of DNA synthesis (257). Evans, working with the L.D. plant Lolium temulentum, has shown that 5 - FU was most effective at inhibiting flowering if added at the end of the inductive period, and by using actinomycin - D, an inhibitor of R.N.A. synthesis, and orotic acid, a nucleotide precursor, he showed that its mode of action was by inhibiting the synthesis of R.N.A.,

a similar effect to that in Xanthium (73). Cytological effects with Allium have shown that DNA synthesis is blocked by 5 - FU, and that 5 - FU is incorporated into R.N.A.(12).

5 - FDU is a more specific nucleic acid inhibitor than 5 - FU, inhibiting DNA synthesis by blocking the formation of thymidylic acid (100a). This substance also inhibited floral induction in Xanthium, Pharbitis and Lolium, but whereas the DNA synthesis in the apex seems to be an essential component of induction in Pharbitis (257), this is not the case in Xanthium or Lolium (21, 73). Thymidine, a DNA precursor, can reverse the inhibition of 5 - FDU, but not of 5 - FU. The inhibitory effects of both 5 - FU and 5 - FDU were not permanent ; the buds were once more inducible after the inhibitors had dissipated.

Working with the S.D. plant Cannabis, Heslop - Harrison showed that 2 - thiouracil inhibited floral induction and disrupted cellular differentiation (106). This substance also becomes incorporated into R.N.A. (107, 254). Collins and Salisbury showed that 2 - thiouracil has a similar effect on Xanthium (54).

These results show that the cells of the apical meristem lose temporarily their capacity to respond to the leaf-generated stimuli (18, 212). Even if the floral genes are activated during induction, they cannot produce the characteristic proteins necessary as a result of the modified stages of R.N.A. synthesis. Since dormant buds are unresponsive to the flowering stimulus (198), it seems that flowering can only be induced when DNA and R.N.A. are being synthesised (55). Thomas found in Xanthium that there was an increase in mitotic activity caused by the presence of a floral inducer (238), and he suggested a

DNA regulatory mechanism which is upset in the presence of the floral stimulus (236). Other workers have also found that after induction, mitotic activity increases. (14,90).

A specific floral RNA.

A small amount of work has been done on the analysis of bud nucleic acid before and after induction. Ross (192), using a paper electrophoretic technique, failed to show any difference in the composition of RNA between the vegetative and floral buds of Xanthium. Using histochemical techniques, Gifford and Tepper (90) have shown with Chenopodium that the DNA : histone ratio increases soon after photo-periodic induction, together with a rapid rise in the RNA content. Knox and Evans (132), working with Lolium, and Nongarède et al (175), working with Amaranthus, obtained similar results, again using histochemical methods, and noted that after induction the content of RNA rose sharply, accompanied by a small histone change and an increase in the nuclear and nucleolar size. Although it is known that floral initiation involves the synthesis of bud nucleic acid, (54, 55, 202, 257), and that the meristematic growth rate is proportional to the RNA content (255), the chances of isolating a specific floral RNA seem very small indeed, especially if only a few genes are involved in the actual conversion of the bud from a vegetative to a reproductive state. In fact, the conversion may simply involve a change in the proportions of the same enzyme systems, in which case no new nucleic acid would be formed. However, the results of Gifford and Tepper, and of Knox and Evans, showing a rise in the DNA : histone ratio and an associated rise in RNA, indicate that new genes are being activated.

CHAPTER TWO

CONTROL MECHANISMS IN THE NUCLEUS

Gene activation and repressor action.

In their article, Jacob and Monod (121) bring forward the theory that there are two different functions of a gene, the first to transcribe the structural message via m-RNA, and the second to regulate this transcription. This regulation involves a system of regulator genes (or a transmitting system) and operator genes (or a receiving system), the operator genes receiving a specific cytoplasmic signal in the form of a repressor molecule, which in turn recognises a particular metabolite and a particular operator gene. The metabolite, by some unknown mechanism, can either activate or inactivate the repressor. The repressor molecule seems to be an RNA molecule which - when activated - has such a base sequence that it can combine specifically and reversibly with the operator gene, thus causing cessation of messenger RNA and thus of protein. Upon inactivation of the repressor, the production of the particular protein recommences.

Although this theory is concerned with bacterial systems, a similar system could be involved in floral induction. On arrival at the apex, the floral stimulus could combine with the repressor RNA, inactivating it ; this would thus allow for the synthesis of m-RNA - and thus proteins - concerned with the flowering process.

The role of histones.

The role of the histone proteins in the nuclear control of cell differentiation has become the topic of a great deal of experimenta-

tion and research (20, 33). In 1951, Stedman and Stedman (221) suggested that histones act as gene inhibitors, each histone being capable of suppressing the activities of specific groups of genes. Similar ideas (17, 146) and much evidence (e.g. 2,10,85,115) have been brought forward which support this view. Huang and Bonner (115), working with pea embryo chromatin, have shown that histone, when bound to DNA, increases the stability of DNA, and if the histone is removed, there is an increased rate in RNA synthesis, mainly - as m-RNA (3). Later, working with a specific gene, (or genes), responsible for globulin formation in peas, Bonner et al (19) showed that this gene is only active in vivo in the cotyledons. When the histone was removed from bud chromatin, globulin formation resulted. Other workers have also shown that histone in calf thymus tissue can suppress RNA synthesis (2, 147). However, it is thought that histone structure may not be specific enough to control gene action (17, 124, 125).

It has been suggested that as there are relatively few histone types, a much smaller number than genes, gene activation may in fact be due to an enzymic removal of a type histone (10). Several workers (2,10,117,153) have found that different histone types differ markedly in their ability to repress gene action, lysine-rich histones having a much greater effect than arginine-rich histones. It has been found that the lysine-rich histones play an important role in the structural organisation of chromosomes (241) by binding the chromatin threads together while arginine-rich histones occur more frequently in the diffuse region of the nucleus (153). Since RNA synthesis can occur in loose chromatin (152) but not in dense chromatin (114), this would

explain why lysine-rich histones can suppress RNA synthesis to a greater extent than arginine-rich histones. It has also been shown that certain lysine-rich histones are only formed in cells where DNA is being duplicated (45). Allfrey, Faulkner and Mirsky (1) have shown in vitro that if histones are acetylated or methylated, although they are still complexed with DNA, RNA synthesis is not suppressed. In fact Pogo et al (183) have found that histone acetylation is a pre-requisite for RNA synthesis, and Nohara et al (174) have acetylated histones with an acetylating enzyme. Lately, two groups of workers, one group working with animal tissue (11), and the other group with plant tissue (116), have found a special class of RNA which is intimately associated with histone.

Gene function may in fact be regulated by a reversible acetylation process, in the case of flowering the flowering hormone perhaps being an acetylating agent - or an inhibitor repressing acetylation - of a type histone.

The role of proteins.

It has been shown in animal cells that differentiation can only proceed if the substrates and co-factors necessary for the synthesis of proteins are present. If they are not, then even though a specific gene is activated, the corresponding protein can not be synthesised and therefore differentiation will not ensue. Flickinger calls this a "temporal sequence" in gene action (80), and this may explain some of the results obtained by workers using amino acid analogues as inhibitors. Attempts to isolate specific proteins concerned with flowering have not succeeded as yet. Nitsan, working with Xanthium (172), and Marushige and Marushige, working with Pharbitus (160),

have shown a quantitative but not a qualitative difference in electrophoretic patterns of extracts from vegetative and floral buds. This may mean that it is not that new proteins are being synthesised, but that the balance of enzymes already existing in the bud are altered, thereby causing a change from the vegetative to the floral state. This idea, that a change in the balance of enzymes causes a change in function of the cell, has also been arrived at by working from a theoretical stand-point (64).

Also tying in with this view is the idea of Commoner (56), who suggests that DNA plays two interrelated roles, the first role being that of genetic coding, which occurs in the euchromatic region of the chromosome and is observed as a qualitative difference, and the second role being a regulatory role, occurring in the heterochromatic region of the chromosome and appearing as a quantitative difference. This second role is one by which metabolism is genetically regulated, this regulation being carried out by nucleotide sequestration and thus making them unavailable for other metabolic processes e.g. ATP synthesis. The composition of the residual nucleotide pool will therefore be controlled by the amount of DNA present, and to the base composition of the replicating DNA. This idea, that of the dual role of DNA, is very similar to the theory proposed by Jacob and Monod, mentioned previously (see P.5).

It can be seen therefore, that nucleic acid metabolism is certainly involved in floral differentiation, and that this metabolism must be both controlled and regulated.

CHAPTER THREE

ENDOGENOUS REGULATORS.

Most work on endogenous regulators has been done on plants which respond to only one or a few inductive cycles. Since the site of photoperiodic induction is the leaf (e.g. 197,261), and the site of floral differentiation is the bud, the synthesis of the floral regulators and their transport can be studied separately from the morphogenetic reactions occurring in the bud.

Endogenous floral initiators.

a) Florigens.

It has been shown with the S.D. plant Xanthium, that when the plant is given its induction treatment, a flowering stimulus is produced in the leaves where it can be stored (151), this stimulus being stable and easily transportable under either light or dark conditions (210). Nucleic acid metabolism - and thus protein synthesis (164) - is involved in production of the stimulus, and the donation of methyl groups is involved (54). Using a metabolic inhibitor of animal cholesterol biosynthesis, tris - (2 diethylamino ethyl) phosphate (SK+F 7997-A₃), it has been shown that steroid bio-synthesis is also concerned in the production of the flower stimulus, at least in Xanthium (18), Pharbitis (18,196) and tomato (180). Since these reactions are all enzymatic, specific proteins may be involved.

Hess (108,109,110), working with Streptocarpus weinlandii, has shown that by adding either 2-thiouracil or ethionine to the leaf, flowering can be inhibited without disrupting vegetative growth - results which are quite different to those of Heslop - Harrison, who

found that the addition of 2-thiouracil had a marked effect on vegetative growth (106). (see p.3). The RNA's of the induced leaves had different guanine-adenine ratios, a result which could mean that these anti-metabolites blocked a RNA-protein system associated with the production of the flowering stimulus. In an animal system, ethionine has been shown to inhibit protein synthesis, not by competing with methionine for incorporation into the protein (197), but by decreasing the level of ATP, this being achieved by reacting with the nucleotide to form adenosyl-ethionine (242). Ethionine therefore may cause its inhibitory effect on flowering by interrupting the energy supply in the leaf during the induction process, a finding which supports the idea advanced by Commoner (see p.8).

Many attempts have been made to isolate and identify the flowering stimulus. In 1950, Roberts obtained crystals of a flower - promoting substance from leaves of Xanthium (190), this substance being lipid-like in character (191). However, this experiment has not been successfully repeated. By lyophilising leaves of flowering Xanthium stumarium plants and extracting them with cold absolute methanol, Lincoln et al obtained a crude extract which induced a flowering response in vegetative Xanthium pennsylvanicum plants when added as a lanolin paste (149). By a similar method, a crude extract from leaves of the day neutral flowering Helianthus plant induced flowering in vegetative Xanthium plants (150). As paper chromatograms of the extracts from both plants were similar (161), it seems probable that the inducing substance is the same in both plants, or at least very similar. This substance, which has been named florigenic acid (148), has been shown to be stable to both wide pH and temperature variations (161), as well

as having an acidic character and being water soluble (148,161).

Working with the S.D. grass Rottboellia exaltata, Evans (71) showed that a flowering stimulus was formed in the expanding leaf, and was transported via the assimilation stream in either light or darkness - as in Xanthium (135,210). This stimulus was summated at the apex, a phenomenon which also occurs in sugar-cane (53). Evans found in a L.D. grass, Lolium temulentum, that steroid metabolism may be involved in the synthesis of the floral stimulus (73). With Wardlaw (78), he also found by removing the induced leaf at various intervals that enough stimulus had moved out of the leaf four hours after the critical period to induce flowering, and postulated that the rate of movement of the stimulus was approximately two centimetres/hour. Although this figure differs markedly from the figures of 2.6 - 3.8 millimetres/hour obtained by workers using the S.D. plant Pharbitis nil (120,261) it agrees with the figure obtained by Canny (39) on the rate of movement of the assimilation stream.

Grafting experiments have shown the presence of a floral inducer. With both Bryophyllum, a L-SD plant, (259), and with Pharbitis, a S.D. plant (261), it has been shown that an induced stock can cause a vegetative scion to flower. In fact an induced S.D. stock Kalanchoë can cause flowering in a L.D. scion (Sedum). (261). By intergrafting two species of Cestrum, Griesel has shown that more than one type of floral stimulus is involved in the flowering of this plant (93), and crosses-breeding experiments, which have shown that two floral genes are involved (94, 95), support this statement.

Therefore it can be seen that floral initiation in both S.D. and L.D. plants, both monocotyledon and dicotyledon, is associated with the action of a promotive substance which is produced in the leaves. However whether this substance is universal for all plants or not is still a matter of speculation.

b) Gibberellins.

It has been shown that the gibberellins can induce flowering in L.D. plants and in plants requiring a vernalisation treatment, when these plants are grown under non-inductive conditions (5, 122, 222). With S.D. plants however, gibberellin can promote flowering in some plants (e.g. Pharbitis) but not cause induction (261). It seems highly probable that gibberellin is only indirectly associated with flowering, its primary effect being that of inducing meristematic activity (72, 119, 141, 142, 145). In fact in some plants flowering may have to be preceded by a bolting process e.g. Spinach (123). Working with Petasites hybridus, Wardlaw (244) has shown that gibberellin has no effect on the vegetative apices, but can promote flowering once initiation has occurred. Of the nine different gibberellins known, G.A.₇ is generally the most effective gibberellin in inducing flowering, G.A.₃ also being a very strong inducer (26, 72, 162). An inhibitor of gibberellin action (2-chloroethyl) - trimethyl Chloride (CCC) has been shown to inhibit flowering in both S.D. and L.D. plants, its effect being completely reversed by the addition of G.A.₃ (5, 258) and it is thought that this inhibitor suppresses the formation of gibberellin (5, 145).

Gibberellins are present at the time of induction (173,258). The role of gibberellin may be that of a precursor to florigen (5), or to initiate a sequence leading to floral induction (142). Working with the L.S.D. plant Bryophyllum, Zeevart and Lang (262) have shown that under S.D. conditions, gibberellin regulates the floral stimulus by substituting for the L.D. requirement. It may be concluded however, that the gibberellins, although photoperiodically involved in the flowering process, are not biochemically related to the floral stimulus (256,262). In fact, in Pisum varieties, the presence of G.A₃ inhibited flower formation (8,126,217) perhaps by disorganising the apex. Pictures illustrating this disorganisation have been presented by Sprent (217).

Endogenous floral inhibitors.

The question as to whether flowering is under the regulation of endogenous floral inhibitors is as yet unresolved, evidence still being accumulated for both sides. According to Zeevart (261), inhibition can be divided into two categories, that of a specific type where an actual substance is produced, and that of a non-specific type, the result being caused by the lack of stimulus. For a specific endogenous inhibitor, three main points arise:-

- a) its mode of action may be with relation to the production of florigen, or else at the site of floral differentiation.
- b) it may or may not be transmissible.
- c) if the site of action is the bud, it may either be produced at the bud, or else it may be produced away from the bud.

In the latter case it will need to be transported to the bud.

If the inhibition is of a non-specific variety, again three points arise:-

- a) it may be a general inhibition of metabolic processes
- b) it may interrupt cell division in the apex at a time when the stimulus is present
- c) it may interfere with the translocation of the stimulus (209).

With regard to the translocation effect, Chailakhyan and Butenko (44) working with Perilla, have presented some results which support this idea. By exposing both induced and non-induced leaves to radioactive carbon dioxide ($C^{14}O_2$) and determining the distribution pattern of the photosynthates, they found that if non-induced leaves were present between the induced leaves and the apex they absorbed most of the labelled assimilates, and also in this situation the plant did not flower. Since it was assumed that the stimulus travels with this transport stream, the inhibitory effect of the non-induced leaves would be the result of an interference of this stream to the apex.

Guttridge, on the other hand, working with the cultivated strawberry - a S.D. plant - has brought forward convincing evidence for the presence of a floral inhibitor (97). He showed that flowering is repressed and vegetative growth is enhanced in the one plant if, during its inductive period, a second plant connected to the first by a stolon had its inductive dark period interrupted by light. Thompson and Guttridge (240) showed that a defoliated plant, but not an intact one, would flower in continuous light, while an intact plant would flower in continuous darkness. In long days therefore, the plant was inhibited from flowering by a transmissible inhibitor

which is formed in the leaves. Lesham and Koller (144) confirmed this inhibitor idea, and brought forward evidence suggesting that the inhibitor was stored in the daughter strawberry plants.

Schwabe (206), working with Kalanchoë and other S.D. plants, has shown that intercalating one long day between inductive short days not only stops initiation but actually inhibits it - in the case of Kalanchoë, the inhibition by one long day being equivalent to that of 1.5 - 2 succeeding inductive short days. The effect of several long days is not cumulative. Schwabe postulated an interaction between a stimulus and an inhibitor, the latter being formed during long days and its effect being on the formation - not the action - of the stimulus. It seems likely that in S.D. plants the effect of the dark period is two-fold : in the first case removing the light-dependent inhibitor which interferes with the induced state, or flower hormone production, and second, the formation of the induced state or production of the flower hormone (256).

Thomas (237) found that intercalated long days inhibit flower formation in the S.D. plants Xanthium and Chenopodium, but only if the induction was slight. If the plants were strongly induced, the effect of the intercalated long days was stimulatory. In the former instance, he concluded that the effect was caused by an inhibition of the inductive processes in the leaves, while in the latter case, the effect was caused by an altered sensitivity of the apex to the stimulus.

These results could equally well be explained on the basis of a single inductive substance. This substance, which is dependent on light for its formation, must reach a threshold value before it becomes

effective, and under long light periods, the concentration reaches such a high value that the substance becomes inhibitory, and thus would inhibit flowering. In darkness, this substance is slowly broken down.

Fratianne observed the effects of the leafless, parasitic dodder plant on various host plants (81). He found that under an inductive photoperiod, dodder does not affect the flowering of the host plant. If a dodder bridge was formed between an induced plant (e.g. Glycine - a S.D. plant) and a non-induced plant, the flowering of the induced plant was decreased; the non-induced plant did not flower. He assumed that an inhibitor was produced in the leaves under non-inductive conditions.

Non-inductive conditions in Salvia, another S.D. plant, also causes the production of an inhibitor in the leaves (15). Evans (71), working with the S.D. grass Rottboellia, found that if non-induced leaves were situated below an induced leaf, they still had an inhibitory effect. Since interference with translocation is ruled out, he suggested that the non-induced leaves produce an inhibitor while the induced leaves produce a stimulus. Flowering in Salvia and Rottboellia is therefore regulated by both an inhibitor and a stimulus, both being transmissible and both acting at the apex.

Working with the L.D. grass Lolium (69), Evans showed that flowering in this plant is also regulated by a stimulus and an inhibitor, the former being produced in induced leaves and the latter in non-induced leaves. Although Zeevart (256) suggested that this inhibitory effect by the S.D. leaves could be caused by either their interference with the translocation of the L.D. stimulus, or to

dilution of the stimulus reaching the apex by assimilates from the lower leaves, Evans and Wardlaw (78) later found by using $C^{14}O_2$ that the idea of a sink was in this case not correct, since only a very small proportion of the assimilates moved from the upper leaves to the lower leaves, and that the most feasible idea was that of an inhibitor being produced under non-inductive conditions. Further it was shown that the formation of the stimulus could be carried out under anaerobic conditions, while the production of the inhibitor required oxygen (70). Evans has found that abscisin II inhibits flowering in Lolium (76). He suggested that abscisin II, which is actually the same substance as dormin of woody plants (57), may be the S.D. inhibitor. However, it has been found that abscisin II is effective in inducing the S.D. plants Pharbitis and Chenopodium, but not Xanthium (68).

By growing buds of the S.D. plant Perilla in culture, Raghaven and Jacobs (188) have shown that under S.D. conditions normal flowers were produced, while under L.D. conditions only a rudimentary stage was reached. This suggests that although Perilla requires S.D. conditions for normal floral development, it has an inherent ability to initiate flowers independent of day-length. If small leaves were attached, under S.D. conditions they promoted flowering while under L.D. conditions they inhibited flowering. They therefore concluded that flowering in Perilla is controlled by a balance between an inhibitor and a stimulus.

By intergrafting early and late varieties of Glycine, Kiyosawa and Kiyosawa (131) concluded that flowering in this plant was also determined by a balance between an inhibitor and a stimulator, a

conclusion which Curtis, by intergrafting different varieties of beet, also reached (63).

In 1952, Barber and Paton proposed that a floral inhibitor was present in late varieties of Pisum (9). This was soon confirmed by intergrafting early and late varieties (178,179) and from experiments involving cotyledon excision (126,178). This substance is synthesised in the cotyledons soon after germination (178), moving into the plumule in about two weeks, during which time it can be leached from cuttings (218). It was found that vernalisation and long photoperiodic regimes decreased the amount of inhibitor (178, 217), and Barber gave it the name colysanthin (7). Colysanthin may be involved in gibberellin metabolism (8). Lately it has been found that a short-lived inhibitory substance is present in early varieties also (126).

It can be seen therefore that there is a great deal of evidence present to suggest an inhibitor-regulation of flowering. At the moment however, it is not known what form the inhibitor(s) takes and what its mode of action is.

CHAPTER FOUR

CONTROL OF ENDOGENOUS REGULATORS

The majority of work done on flowering has been concerned with the aspect of photoperiodic control and its underlying mechanism, and recent reviews and surveys have emphasised different aspects of this field (30,65,98,99,105,197,198).

Photoperiodic induction.

Plants have a wide range of photoperiodic responses, ranging from those that are completely insensitive to photoperiod (i.e. day-neutral plants) to those which will respond to only one inductive period (e.g. the S.D. plants Xanthium (210), Chenopodium (62), and Pharbitis (227,257) will flower after one long night period, and the L.D. plants Lolium (75), Anagallis (6), Sinapis (13), and Brassica (88) will flower after being given one short night). In general though, photoperiodically sensitive plants will require repeated inductive cycles before flowering is initiated, and Salisbury has compiled a list of plants, classifying them with respect to their photoperiodic response (198).

Most work on photoperiodic control has been done with plants which are highly responsive to photoperiodic treatment e.g. Xanthium, Pharbitis. It has been known for some time now that the leaves are the site of photoperiodic induction, although in Pharbitis and Anagallis, the cotyledons also are sensitive (6,257), and that induction is caused by the length of the night period, and not the length of the light period (65). For the S.D. plant Xanthium therefore, a day-length of 8.5 hours or less will cause flowering as long as the

dark period is not interrupted by a flash of light (210). In some L.D. plants on the other hand, the plant will flower if the night length is too long, so long as it is interrupted by a light flash. In these cases, too long a period without light will inhibit flowering (261).

Before the inductive night period can begin however, a period of high intensity light is required, in the first instance simply to provide photosynthates, and secondly to convert a photopigment to a physiologically active form, a point which will be discussed a little later in this chapter. It was once thought that a second high-intensity light period was required to stabilise the newly-formed flower hormone or its precursor(s) (154,156), but since then experiments have been conducted which show that this is not the case, at least for Xanthium (210), Pharbitis (227) and Lolium (69).

It can therefore be seen that a S.D. plant will flower if the dark period is greater than a critical value, and it is thought that during this dark period a sequence of reactions occurs which is inhibited - or reversed - by light. On the other hand, with L.D. plants a dark period greater than a critical value will inhibit flowering. This inhibition may be the result of an inhibitor being produced during the dark period which requires a certain amount of light for its removal (261), or that during a long night the concentration of a dark-produced stimulus reaches such a value that it inhibits flowering (105), although many L.D. plants are known to flower under continuous light (105).

Two theories have been brought forward in an attempt to explain this photoperiodic induction. The first theory concerns an endogenous circadian rhythm, while the second involves a photo-receptor pigment. At this stage it seems likely that the actual mechanism of photoperiodic induction involves a combination of both theories.

Endogenous circadian rhythms.

Endogenous circadian rhythms have been observed in a great variety of organisms, ranging from unicellular to highly complex forms, and Sweeney in her review has discussed many examples of these (224). It is now clear that these rhythms play a part in the time perception process, although the mechanism is not at all understood e.g. diurnal fluctuations of chlorophyll content have been observed in Perilla and Rumex (29,167), together with the fixation of carbon dioxide in certain succulants (224), and of carbon dioxide metabolism in plant tissue cultures (251). Although diurnal changes have been observed in plant cell nuclei (31,246), and in the proportion of ribosomes aggregated in active polyribosome form in leaf tissue (51) - both observations suggesting that nuclear DNA-controlled protein synthesis is involved - Sweeney has observed that diurnal rhythmicity is maintained in Acetabularia cells after enucleation - at least as far as photosynthesis is concerned (226). Contrary to Sweeney, Schweiger et al (207) have shown that the circadian rhythm of oxygen balance in the cells is determined by the nucleus, and other workers have shown a photosynthetic rhythm in intact cells of various plants (66,113,129,177). Chance and co-workers (46,47) have shown that the NADH level of yeast cells undergoes a rhythm, both in whole cells and in cell-free extracts, showing that metabolic enzyme systems are involved in the biological

clock, and in fact, rhythms can be disrupted by anaerobic conditions (250).

The interesting factor of the biological clock is that the circadian period is almost temperature independent, ^{having} a Q10 of approximately 1-1.2 (249). Since biochemical reactions have a Q10 of the order of two to three, it seems that biochemical reactions alone do not operate the clock. A model has been brought forward by Ehret and Barlow (67), involving a feed-back system of biochemical steps with relatively temperature-independent biophysical steps (e.g. diffusion); this model has a Q10 of 1.2. Irrespective of the mechanism, it is now known that the clock has the properties of an oscillator (30,31,98,208), it can be rephased by temperature, (208,249), light (181,182,250), and ultra-violet light (225), and can be coupled with its environmental light regime (98).

With respect to flowering, the endogenous rhythm seems to give rise to, and couple with, a sequence of biochemical reactions which vary in their sensitivity to light. In this way, the endogenous rhythm can control the synthesis of the floral stimulus. The "photophile" and "scotophile" phases of the endogenous rhythm as proposed by Bünning (31, 99) can thus be accounted for. If for example the scotophile - or as Hamner has called it, the photophobe - phase of the rhythm was interrupted by a flash of light, the light would inhibit the sequence either by inhibiting a light-sensitive reaction or else by resetting the clock mechanism, thus causing the reaction sequence not to reach completion. This would be caused either by a light-sensitive reaction being made to co-incide with the oncoming light period, or else an essential component

of the reaction sequence would be critically displaced from following reactions of the pathway (181). Similar consequences could be obtained by altering the length of the cycle, and this is substantiated by many experiments in which light flashes have been used at various times during a prolonged dark period.

During a 72-hour cycle (8 hours inductive light period, 64 hours dark period) Hamner (99), using the S.D. plant Glycine max, found that if a light interruption was given when the plant 'expected' darkness (i.e. the photophobe phase i.e. at 24 hour intervals of 14 hours, 38 hours and 62 hours after the beginning of the light period), flowering was markedly inhibited, while light flashes occurring during the 'expected' light periods (i.e. the photophile phase - at 26 hours and 52 hours after the beginning of the light period) enhanced flowering above that of the controls. In other experiments (99), where the cycle length was varied by extending the dark period after 8 hours of light, flowering was enhanced with cycle lengths of 24 hours, 48 hours and 72 hours, and inhibited with lengths of 34 hours and 60 hours.

Coulter (58), using a 72 hour cycle and Glycine max, found similar photophile and photophobe reactions, and with Hamner (59), suggested that an 8-hour photoperiod initiates a fundamental oscillation, the amplitude of which can be increased or dampened by light breaks, depending on when they occur. They found (60) that light falling in the first photophobe phase was twice as inhibitory as in the second photophobe phase, as did Schumate (204), while light falling in the second photophile phase was twice as stimulatory as in the first photophile phase.

It was found by using two successive light interruptions (41), that if each fell in a photophile phase flowering was enhanced, and if each fell in a photophobe phase, flowering was inhibited. If one light interruption fell in a photophile phase, and the other interruption fell in a photophobe phase, the stimulation from the photophile interruption partially overcame the inhibition from the photophobe interruption. Using cycles of different lengths, similar results to those above have been obtained with another S.D. plant Pharbitis, while the L.D. plants Hyoscyamus and Silene showed results similar to, but out of phase with, the S.D. plants (79,99).

Coulter and Hamner, working with Glycine (59), and Takimoto and Hamner, working with Pharbitis (228,229) have suggested that the basic endogenous rhythm is composed of three separate mechanisms:-

- a) an "hour-glass" component. By increasing the dark period, flowering is increased. This component is temperature sensitive.
- b) an endogenous circadian rhythm component, which is initiated by the beginning of a light period. This rhythm is temperature insensitive, although the magnitude may be affected by temperature.
- c) an oscillating mechanism which begins at the commencement of a dark period. This component is also insensitive to temperature.

Mitchell (166) has shown that the flowering response in Xanthium is regulated by an endogenous rhythm, flowering maxima being obtained with cycles of 24 hours, 48 hours and 72 hours, and flowering minima occurring with cycles of 36 hours, 60 hours and 84 hours. These

results suggest that a common clock mechanism is in operation in widely different types of plants.

The phytochrome system.

Plants can discriminate between the quality of light received, and it is now thought that this is the result of a pigment called phytochrome (34). The effects of different light qualities on plant growth have long been observed and in 1959, Butler et al first separated a crude form of this pigment (38). The phytochrome pigment is a soluble cytoplasmic protein, attached to a chromatophore of the bilitriene type (214,215). Phytochrome can occur as two inter-convertible forms, one form (P730 or Pfr) having an absorption maximum at a wavelength of 730 mμ in the far-red region of the spectrum, and the other form (P660 or Pr) having an absorption maximum at a wavelength of 660 mμ in the red region of the spectrum.

Irradiation by far-red light causes the P730 to revert to the P660 form, and red light will cause the P660 form to be converted to the P730 form, perhaps via an intermediate form (27,37). In fact Spruit has observed that phytochrome has four different absorption maxima - at wavelengths of 650 mμ, 670 mμ, 698 mμ and 744 mμ and thinks that perhaps phytochrome can exist in four forms (219). In darkness, P730 slowly reverts to the P660 form, and also decomposes to give a net loss of reversible phytochrome (36). White light appears to be equivalent in its action to red light (34,250) so that at the onset of darkness, the pigment is in the P730 form. In dark-grown seedlings however, all phytochrome is in the stable P660 form (34),



In dark-grown seedlings, it was initially thought that a flash of red light converted all the phytochrome from P660 to P730, there being a 20% reversion to P660 in darkness, 80% of P730 being destroyed. It has now been found however that 20% of the phytochrome remains as P660 (35), the rest of the phytochrome which had been converted to the P730 being enzymatically destroyed (184). This leads to an overall loss in reversible phytochrome (36,136). This enzymatic destruction is correlated with the respiratory rate of the seedlings, being inhibited by respiratory inhibitors, anaerobic conditions and low temperatures (28,32,136). In cauliflower heads however, the reaction $\text{Pfr} \longrightarrow \text{Pr}$ is not affected by anaerobic conditions (35,36).

A prolonged far-red interruption has the same overall effect as a red one, owing to the long absorption tail of Pr in the far-red region of the spectrum (36). It is in the manner of the diagram above that plants respond to red and far-red light, with the corresponding photomorphogenic effects, and many surveys have been made on phytochrome and its involvement in the photoperiodic effect (22,24,103,105,197,198,213). The classic behaviour of a S.D. plant to different light qualities is as follows, the example taken being that of Xanthium.

A brief interruption of red light given near the middle of an otherwise inductive night period will inhibit flowering. If this is followed by a flash of far-red light, flowering is re-promoted. This behaviour will continue for a series of red - far-red interruptions, the quality of the last interruption determining the flowering pattern (22). This behaviour has been observed in a variety of S.D. plants (42,84,127,185). The effect on L.D. plants is the opposite ; a red light interruption will promote flowering, while a far-red light

interruption is inhibitive (22,118).

The actual role of the phytochrome pigment in photoperiodic induction is still not known however. Hendricks and Borthwick have suggested that the phytochrome pigment acts as an enzyme when in the P730 form; inhibiting flowering in S.D. plants by diverting essential intermediates, and enhancing flowering in L.D. plants by reducing some inhibition (105). Price et al have noticed that red light increases the rate of disappearance of starch and sugars, (186) while Gordon suggests that red light can control energy transfer by esterifying phosphate (91). Both these phenomena are reversed by far-red light. Lane and co-workers (139) have detected the presence of phytochrome in green tissue of about twenty plants, but failed to detect it in Chrysanthemum, Perilla and Glycine, all three plants having a strong photoperiodic response. Briggs and Siegelmann found that the highest concentration of phytochrome lies in the meristematic region (28), an idea that agrees with that of Butler and Lane (35), who suggest that phytochrome synthesis occurs in new-formed tissue.

Much of the recent work with L.D. plants has suggested that light breaks in the middle of long nights are rather ineffective in causing induction. A much more efficient method is to supplement the natural day length with artificial light, but only if this supplementary light contains both red and far-red light (4,77,137,138,176,243). Lane, Cathey and Evans, using lights of different red: far-red ratios on a variety of L.D. plants, found that optimal induction required the action of Pfr over a long period each day. The optimal Pfr level was low when the concentration of the products of the high energy reactions during the day was high, and vice versa. High levels of Pfr inhibited induction (137).

In S.D. plants, depending on the experimental conditions, a variety of responses can occur which differ from the classic response.

a) In Xanthium, a long far-red interruption during an inductive night period will inhibit flowering (159). It is thought that under a long period of far-red light a photostationary state is set up between Pr and Pfr, and it is the presence of the Pfr which causes the inhibition, even though the Pfr : Pr ratio would be very small.

b) If Xanthium is given a photoperiod of only two hours light per day, it has been found that a far-red light interruption given at the beginning of the dark period actually inhibits flowering, while at the end of the dark period, flowering is inhibited by a flash of red light. It has been suggested that at the beginning of the dark period, Pfr is involved in a flower-promoting function, the far-red light flash causing reversion of Pfr to Pr before this function is completed. This far-red light inhibition can be reversed by red light (23, 83, 84). This effect has also been observed in Pharbitis and Kalanchoë (82).

c) It has been noted also that if Xanthium is subjected to prolonged dark periods, a far-red interruption does not reverse a red light inhibition (169, 189).

In Chenopodium also, the prolonged far-red light inhibition has been observed (159), Pfr being at a level of 1 - 2% of photoreversible phytochrome (127). Kasperbauer et al have also shown that the rate of dark reversion of phytochrome in vivo is approximately 1% of the total amount of phytochrome present per minute (128). Cumming (62) has shown that increasing the red : far-red ratio at the end of the photoperiod increases the length of the optimum night. He concludes that flowering in Chenopodium is regulated by a P730-dependent hormone, the concentration

of which depends on the amount of P730 remaining after dark reversion to the inactive P660 form. Könitz (134) has shown that a far-red flash during the inductive photoperiod inhibited flowering, although this work was not confirmed by others (133).

It was found that whereas one minute of incandescent light given during the dark period could inhibit Xanthium, or Glycine, this was not the case in Chrysanthemum. In Chrysanthemum, it was found that one minute of fluorescent light could inhibit flowering, and it was suggested that chlorophyll absorbing red light gave a higher red : far-red ratio of light reaching the phytochrome, as this red light inhibition by fluorescent light could be reversed by far-red light. Since incandescent light contains appreciable quantities of far-red light, a light interruption from this source was equivalent to that of a far-red interruption, thus causing no inhibition (42,43).

With Glycine, the story is a little different. As in Hamner's experiment with white light, it was found that red light interruptions given during a dark period greater than 16 hours either enhanced or delayed flowering, depending at which stage of the night period the dark flash was given (i.e. a rhythmic effect), and it was also shown that far-red light did not always reverse this inhibition (40). It was concluded from these experiments that phytochrome only influences the time measurement within the framework of the endogenous clock mechanism. Kalanchoë showed a similar rhythmic response to that of Glycine (32).

With another S.D. plant Pharbitis, Takimoto et al (227,233) have shown that induction can be caused by a photoperiod of white or red light, but not by far-red light. This would be because Pfr is required at the beginning of the dark period (84). In the middle of the dark period

red - far-red reversibility can occur only if the light treatments have a duration of only 30 seconds and are not separated by a dark period. This would explain why other workers have not been able to cause a far-red reversibility (e.g. 105). It is thought that perhaps once Pfr is formed, it has a very fast action (84).

The results of Takimoto and Hamner have suggested that the main time-keeping mechanism in this plant is that of an endogenous clock, this clock having three distinct mechanisms (see above). They found that a red light effect was not reversed by far-red light, but a far-red light effect was reversed by red light, and that the level of Pfr remains relatively constant during the dark period. They conclude from these results that there are two pigment systems in operation, and that phytochrome is concerned with the 'hour-glass' mechanism component, far-red light stopping the mechanism of this component after a certain time (228,229,230,231,232). Mohr has also postulated that another pigment system is also present (168), and Friend (86,87) has carried out experiments with wheat which have tended to support his idea.

It seems therefore that the phytochrome pigment plays some part in the plant's ability to measure time, most likely by being coupled to some oscillator system, the mechanism of which is still not known (31). Hendricks and Borthwick (103,104,105) however believe that the principal measurement of time is the dark conversion of P730 to P660, this process interacting with time-dependent metabolic reactions.

Temperature effects.

a) Thermoperiodic induction

Temperature changes are known to interact with photoperiod by being able to change the amplitude and phase of an oscillation,

and in fact rhythmic responses have been initiated by temperature changes (30,31,98,249). However, only scant information exists to show a thermoperiodic induction equivalent to photoperiod.

Evans, for example, working with Lolium temulentum, has shown that a low temperature given during the inductive period inhibited flowering (69) and Takimoto and Hamner have found a similar effect in Pharbitis (228). Others have found that low temperatures are a pre-requisite for flowering (101,170). The dark reactions of Pfr may be involved here (36,136). With the L.D. plant Hyoscyamus flowering was promoted by a 3-hour period of high temperature given in the middle of the dark period, and inhibited by a cold period; in the S.D. plant Perilla, the effects were opposite (208). Low temperatures could slow down the rates of synthetic reactions, and transportation. However, these results suggest that in certain cases high temperature can substitute for light, and low temperatures can substitute for darkness (208). In the S.D. plant Glycine, the time between flowering maxima was lengthened if the plant was subjected to low temperatures, either during the day or night (99). It seems as though the endogenous clock is in this case sensitive to temperature, although usually it is relatively temperature independent, with a Q₁₀ of about 1.2 (98).

b) Vernalisation.

Chouard has covered this topic very thoroughly indeed in his review of 1960 (50) and so this segment of the review will be confined to reports which have been published since then.

Working with the cereal Petkus rye, Friend and Purvis found that the effects of vernalisation could be reversed by high temperatures (i.e. greater than 15°C). This reversal was prevented by neutral temperatures

(c.a. 12°C), weak light, a restricted water supply, and long vernalisation periods of from ten to twelve weeks (89). They formulated the following diagram:-



Schwabe has suggested that vernalisation in Petkus rye is probably controlled by only one gene. No matter what treatment was given to the parent plant, the new grain was unvernalsed (205). Wellensiek suggested that a devernalisation process may occur at meiosis. He showed that the locus of vernalisation, originally thought to be at the growing tip, was with dividing cells (248). Grant has found that best results are obtained with winter varieties of wheat if they are vernalised for from five to eight weeks (92), a figure which agrees with that of other cereals (50).

Nutritional and hormonal factors.

Hillman has found that heavy metals have an influence on the flowering behaviour of Lemna (112). Lemna perpusilla, a S.D. plant on Hoagland's medium, becomes a day neutral plant, and Lemna gibba, a L.D. plant on Hoagland's medium, is inhibited from flowering if cupric or mercuric ions are present. This effect is reversed when EDTA (ethylene diamine tetraacetic acid), a metal chelating agent, is present (111). The apparent requirement of EDTA for the flowering of Wolffia microscopica, another member of the Family Lemnaceae, may in fact be simply this reversal effect, since the plants were sterilised with mercuric chloride (158). Leaves of Xanthium which had been bleached with streptomycin were found to be incapable of floral

induction (130). Since an albino L.D. wheat variety mutant was quite sensitive to photoperiod (223), it seems that iron, but not the photosynthetic pigments, is required in the induction process. The flowering of Phleum, a L.D. plant, is delayed if the concentration of nitrogen is low (195).

In the tomato and the pea, the effects of kinetin are opposite to gibberellin A3 (253). Flowering is inhibited in tomatoes and enhanced in peas if kinetin is added, and it is thought that in these cases kinetin antagonises growth. It has been shown that both kinetin and gibberellin can replace the red-far-red light effect (141,163), and a few reviews concerning the physiological action of both gibberellins and auxins on flowering have been written (141,143,222). There is a great amount of contradictory evidence concerning the role of auxin. Salisbury found that auxin inhibited flowering in Xanthium if it was applied before translocation of the leaf stimulus, but enhanced flowering after the stimulus had been translocated (199). Evans on the other hand, working with the L.D. plant Lolium (72), found that auxin inhibited flowering if applied at the end of the L.D. photoperiod, but stimulated flowering if given during a S.D., accompanied by a two hour light break during the night. He concluded that the effect of auxin was on processes occurring during the long day.

Zucker et al have found that the concentration of leaf chlorogenic acid in Nicotinia rises just prior to induction, and falls during induction (263). Since phenols inhibit IAA oxidase (102), and since chlorogenic acid is a phenol-type substance, it seems that the removal of IAA is concerned with induction in Nicotinia. However, induction in

Pharbitis is preceded by a drop in the phenol concentration (263).

Lang has suggested that the primary effect of auxin is concerned with the processes leading up to or concerned with the plant's "ripeness to flower" (141). Nitsch and Nitsch (173), using chromatographic techniques on extracts of induced Nicotinia, have found five different peaks of activity, and they think that the middle peak - or substance C - may be an auxin, and the fifth peak - or substance E - may be a gibberellin, a finding which agrees with that of Harada (100).

Griesel and Caplin have been able to photo-induce nodes of Cestrum on an agar medium (96), while floral buds of Aquilegia (234,235) and of Viscaria (16) have been grown in vitro. In all cases hormones have been required in the medium, as well as nutrients.

These results suggest that although certain nutrient or hormone deficiencies may inhibit the expression of the stimulus at critical times, no particular combination of nutrients and hormone can, by itself, initiate floral induction.

CHAPTER FIVE

CHEMICAL CONTROL OF FLOWERING.

A variety of work has been done on flowering by adding metabolic inhibitors to the plant.

By adding the nucleic acid antimetabolites 5-fluorouracil (5-FU) (18,21,73,202,211,257), 5-fluorodeoxyuridine (5-FDU) (18,21,73,257), 2-thiouracil (106,107), and 6-azauracil (193,211,212) to a variety of plants under inductive conditions (both S.D. and L.D.), it has been shown that flowering is inhibited while vegetative growth continues normally. Thompson found that maleic hydrazide, another inhibitor of nucleic acid synthesis, inhibited induction in strawberry (239). CCC ((2-chloroethyl) trimethyl ammonium chloride) is also a growth inhibitor and it is has been observed to inhibit flowering in Pharbitis (258), Bryophyllum (260) and Samolus (55). Apparently CCC causes the inhibition of cell division (258), or inhibits the biogenesis of GA (145). The effect of CCC ^{is} ~~being~~ reversed by applying GA3 (5). Since these anti-metabolites inhibit nucleic acid synthesis and RNA replication, the buds would be induced into a dormant state, and would therefore be unable to perceive the flowering stimulus.

Metabolic inhibitors have also been used on the leaf. Anti-amino acids (55,194), including p-fluorophenylalanine (PFPA) (165,194) have inhibited floral induction in Xanthium, as have also such broad-spectrum enzyme inhibitors such as azide, cyanide and mercuric ions (211). As the inhibition by the anti-amino acids can be reversed by the corresponding amino acids (e.g. in the case of PFPA, with L-phenylalanine), it is thought that these inhibitors interfere with the synthesis of

enzymes in the leaf.

The cobaltous ion (Co^{+2}) inhibits flowering by slowing down the timing mechanism of the biological clock (200,203). Since cysteine and glutathione both reverse this effect, it is thought that -SH - containing enzymes are involved. Loercher and Liverman (157) have found that Co^{+2} inhibits the activity of the enzyme adenosine triphosphatase.

Tris-(2-diethylaminoethyl) phosphate, or SKF 7997-A3, is a substance which inhibits cholesterol synthesis in animals. This substance inhibits the formation of the flowering stimulus in the leaves of Pharbitis (18,196) Xanthium (18), Lolium (1) and the tomato (180), which suggests that steroid metabolism may be associated with the inductive processes in the leaves, and in fact that the flowering hormone may be a steroid.

It can be seen therefore that the more specific the inhibitor used, the more information can be gained concerning the reactions associated with floral induction.

CONCLUSION

This review has been mainly concerned with reports that have been published during the last decade. The main question arising from these recent developments is:- "What is the mechanism regulating the physiological process of flowering?", and more refined biochemical techniques may lead us to the answer.

Histones obviously play a role in differentiation processes, but whether histones can be regulated by a flower hormone and/or an inhibitor is a problem still to be resolved. The results with antimetabolites show that nucleic acid synthesis is involved, and further histochemical studies should show the actual role that nucleic acids play in the flowering process upon the arrival at the apex of the flowering stimulus.

A break-through would be the isolation and characterisation of the flower hormone, since the reactions concerned with its synthesis in the leaf would then be better understood. This hormone is produced in the leaf under favourable environmental conditions of temperature and daylength, and a system of time measurement must be involved. The phytochrome pigment obviously plays a part in this connection, but the reactions with which it is associated are still very obscure. Phytochrome may be a part of an overall endogenous clock mechanism, the reactions of the oscillatory mechanism being as yet completely unknown.

Plant hormones and nutrients play a role in flowering, but it seems that this role is essentially a secondary one.

In summary then, under favourable conditions, a biochemical synthesis of a flowering hormone occurs in the leaves, this hormone being able to transform the meristem from a vegetative to a reproductive condition.

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PART II

SOME ASPECTS ON THE REGULATION OF FLOWERING IN PEAS

CHAPTER ONE

Introduction:

A general review of the literature concerning the endogenous control of flowering by both floral stimulants and floral inhibitors has been presented in the first part of this thesis.

The garden pea, Pisum sativum L., has been extensively used in studies of flowering behaviour (e.g. 1,14,15,18,22,24,25,27,28,30). Varieties of this plant can be divided into two distinct groups categorised by their flowering habit. Late varieties typically flower above the fifteenth node, are capable of being vernalised and respond to photoperiod as quantitative long day plants, while early varieties flower at about the ninth to eleventh node above the cotyledons and are insensitive to vernalisation and photoperiod. Flowering in this plant is under the major control of the specific locus (Sn sn), late varieties possessing the dominant Sn gene which causes later flowering by inducing a response to vernalisation and photoperiod (1).

e.g. (29).

Description	Variety, e.g.	Genetic Constitution.
Late	Telephone, Greenfeast.	Sn Sn
Early	Alaska, Massey.	sn sn

Although transmission of the floral stimulus from the leaves or cotyledons to the apex is a proven partial process of photoperiodic induction (37), the actual hormonal regulation of floral induction in Pisum has been interpreted differently by different workers. Haupt (9,10,11,12) has interpreted his results by suggesting that floral

induction in peas is mediated by the positive action of a florigen, and this theory has been supported by Highkin (13), working with pea-seed diffusates. Moore and Bonde (23) found that pea-seed diffusates could also delay flowering as well as promoting it, and Haupt (12) has also suggested that a flower-inhibiting substance may be present in vegetative plants.

Barber and his associates (1, 3, 26, 27, 29, 30) have studied flowering in several varieties of peas, and have suggested that the Sn gene present in late varieties is responsible for the production of a flower-inhibitor hormone in the cotyledons soon after germination, which Barber has named colysanthin. Flowering in late varieties of peas was thought to be mediated by the destruction of colysanthin, this destruction occurring rapidly at low temperatures and long days (1). Leaching of a flower inhibitor from the cuttings of young seedlings has confirmed this idea (30), as did the work of Johnston (15), using the technique of cotyledon excision. The idea has been brought forward by von Denffer that flowering is controlled by the sudden drop in the production of a floral inhibitor, and not by the production of a florigen (5). Paton (26) has suggested that perhaps a flower-inducing substance (florigen) is a precursor to colysanthin, and that under short day conditions the leaves are able to produce colysanthin, while under long day conditions, they can inactivate this substance, perhaps by transforming it into a flower promoter. Early varieties of peas, on the other hand, are believed not to produce colysanthin.

e.g. precursor \longrightarrow florigen $\xrightarrow[\text{L.D., vernalisation.}]{\text{Sn, S.D., non-vern.}}$ colysanthin.
(from(1)).

Sprent (29) has suggested that the production of flowers in peas and other plants is most likely governed by a balance of flower-promoting and flower-inhibiting substances.

The role of the cotyledon in the regulation of flowering in Pisum sativum has been investigated by a number of workers (1, 3, 9, 10, 11, 12, 15, 19, 27, 28) and an interesting reciprocal influence of the shoot axis on the cotyledons has also been reported (32,36). In particular, it has been shown with certain varieties of peas that flowering can be markedly effected if the cotyledons are excised from young seedlings (1,3,9,10,11,12,15,27).

The work to be described in this thesis is concerned primarily with the role of the cotyledon on the flowering in peas, and in its relationship with the environmental conditions of photoperiod and especially vernalisation.

MATERIALS AND METHODS

Plants:- Two commercial varieties of peas were used in the experiments to be described, the early variety 'Massey', and the late variety 'Greenfeast'.

Seed treatment:- Seeds were selected so that their testas were free from cracks or blemishes. Since the tissue inside the testa of normal healthy pea seeds is sterile, seeds were sterilised in a weak solution of sodium hypochlorite for two to three minutes, followed by several rinses in sterile de-ionized water. After sterilisation all seeds were imbibed in de-ionized water for eight hours at room temperature (about 20°C). Since over short periods (e.g. 8 hours), the leaching action reported by Bonner *et al* (4) and Eyster (6) does not affect subsequent germination, growth and development, and since this method ensures uniform germination, this method was adopted.

Conditions of germination:- The imbibed seeds were planted into a moist vermiculite - gravel mixture (1 : 1) and grown in the departmental phytotron under the controlled environmental conditions of high light intensity, a temperature of $21 \pm 2^{\circ}\text{C}$, and either a long day (16 hours) or a short day (8 hours) photoperiod, supplementary light being given by banks of mixed fluorescent tubes and incandescent globes. For experiments which involved a vernalisation treatment, the imbibed seeds were planted in pure vermiculite and grown at a temperature of 4°C for a period of 4 weeks, unless otherwise stated. In addition to watering, plants were given a regular treatment of a nutrient solution every four days.

Embryo culture:- In experiments concerning embryo culture, the embryos were aseptically dissected from the seed at the end of the 8 hour

imbibition period. In making cotyledon extracts, the cotyledons from 25 plants were ground in 125 mls. of water in a Wareing blender, and then added to 125 mls. of double strength White's medium (34, 35). To this was then added and dissolved 5 grams (= 2.0%) of dextrose and 1.88 grams (= 0.75%) of agar. 10 mls. of the resultant solution was then added to each of 25 test tubes (1.5 x 15 cm), which were then capped and autoclaved. On cooling, a gel was present in the test tubes, each test tube containing 10 mls. of cotyledon extract, equivalent to the extract from one plant. The embryos which are aseptically dissected were planted on this cotyledon extract, one embryo per test tube.

A pilot experiment was carried out which showed that a cotyledon extract equivalent to four plants per test tube had toxic effects, the embryos planted on this extract dying.

Overall, the rate of contamination was low, but additional cultures were prepared for each treatment none the less, and replacements made where necessary.

The seedlings continued to grow in the test tubes under sterile conditions until such time as they had a well-developed root system, and 3 or 4 nodes fully expanded. (Figure 1). They were then transplanted out into tins containing a moist 1 : 1 vermiculite : gravel mixture.

Grafting:- For the experiment involving grafting, plants were grown in moist vermiculite until such time as the epicotyl was a little less than 2 cms. long and the second internode was just visible, about 5 days after planting. A cleft - graft technique similar to that used by Paton and Barber (27) was used, the stock plant being decapitated between the cotyledonary node and the node of the first leaf. A median longitudinal cut for approximately half the length of the stock epicotyl allowed

the wedge-shaped scion to be easily pushed into position. For strapping the cut surfaces together, thin rubber rings cut from bicycle valve rubber tubing is all that is required, the rubber ring being slipped over the stock just before insertion of the scion (Figure 2).

With this method, a figure of 75% successful grafts was obtained, each individual graft being completed in about 1 minute. The plants were then transplanted into a moist 1 : 1 vermiculite : gravel mixture. No marked scion growth occurred for a period of 10 - 14 days, and until apical dominance was restored (a period of 3 - 4 weeks), the cotyledonary buds of the stock produced vigorous basal shoots. These shoots were removed daily until the scion had established dominance, and after that the scions were regularly checked for lateral bud growth.

Scoring:- All plants were grown to anthesis, the results of all experiments being analysed solely in terms of node number of the first flower (N), taking as zero the cotyledonary node.

All time measurements are taken from the beginning of the 8 - hour imbibition time.



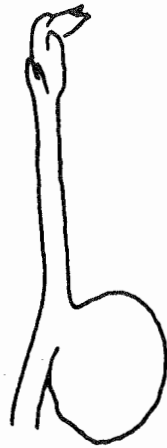
Figure 1.

Conditions of Embryo Culture, 'Greenfeast' seedlings ready for transplanting. The embryos were excised from the seed after 8 hours imbibition, and grown under S.D. conditions (from Johnston (15)).

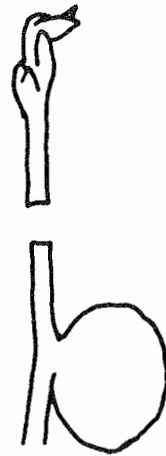
Figure 2.

The grafting technique :- A, intact seedling ; B, decapitation between the cotyledonary node and the first leaf node ; C, wedge-shaped scion and stock with the inserted rubber ring and longitudinal slit ; D, the completed graft.

Figure 2.



A.



B.



C.



D.

CHAPTER TWO

The effect of the cotyledons on the flowering of peas.

Introduction:

The cotyledons of peas are fleshy organs and are the major source of nutrition and of plant growth substances (33) for the young seedlings. Highkin (13) has shown that preparations of pea-seed diffusates have a flower promoting activity. By suggesting that the active principle in the cotyledons may be a flower hormone or hormone precursor, he supports the view of Haupt (9,10,11,12) who has consistently advanced the view that flowering in peas is mediated by the positive action of a florigen. Barber and collaborators (1,3,27,30), using various experimental techniques, have postulated that late varieties of peas contain a mobile flower-delaying substance, colysanthin, in their cotyledons, a hypothesis which has been supported by results recently obtained of Moore (20,21), Sprent (28) and Johnston (15). However, Moore and Bonde (23) found that aqueous extracts of pea seeds from the late variety 'Telephone' could be prepared which had either flower-promoting or flower-inhibiting properties.

It has been shown that removal of cotyledons causes late varieties to flower at an earlier node (16,20,21,27) while in early varieties cotyledon removal after 4 days germination caused a delay in flowering (20). Haupt (9) showed a similar result on the early variety 'Kleine Rheinlanderin' if the cotyledons were removed after 8 hours and the embryos grown on a nutrient culture. Floral initiation in the early variety 'Massey' was delayed if cotyledons were removed between 1 and 4 days after germination, and it was suggested that a floral inhibitor was present in the cotyledons, being rapidly mobilised after germination commences, and being either inactivated or converted to a promotive

substance after about 4 days (15,16).

The experiments discussed in the following pages were carried out for the following 2 reasons:-

a) to obtain further information about the nature of the flower hormone in the cotyledons of a late and an early variety of Pisum sativum.

b) to try and reproduce the results obtained previously (15) by employing a slightly different technique. Instead of removing cotyledons at different stages of development, cotyledon extracts were made at various stages after germination on to which were planted freshly imbibed embryos. Since cotyledons in peas are the source of floral hormone, it was hoped to determine whether, by this method, the pattern of flowering of one variety could be transformed into that of the other.

Experiment 2.1:-

Cotyledon extracts of the early variety 'Massey' and of the late variety 'Greenfeast' were made up in the manner mentioned previously (p. 51) at the stages of the dry seed (= time 0), after 8 hours imbibition (= time $\frac{1}{3}$) and after having germinated for 1, 2, 4 and 6 days. A plain nutrient medium was also set up as a control. Embryos of the variety 'Massey' were excised after having been imbibed in their seed for 8 hours, and placed on the extract, and after transplanting, the seedlings of the individual treatments were randomised, grown to anthesis and scored for the node of first flower.

The results are summarised in Table 2.1 and graphically in Figure 3.

Experiment 2.2:-

Experiment 2.1 was repeated and expanded, using both 'Massey' and 'Greenfeast' embryos and either 'Massey' or 'Greenfeast' cotyledon extracts.

'Massey' extracts were made up at the stages of 3, 5, 6 and 9 days after germination, and 'Greenfeast' extracts were prepared at the stages of 3, 6, 9 and 12 days after germination.

The results are summarised in Tables 2.2 and 2.3, and graphically in Figures 4 and 5.

Results and Discussion:

It has previously been shown that pea seedlings are dependent on the cotyledons as a source of food supply and growth substances up to a week after germination, after which time they can produce their own food material (15,30).

From the results, it can be seen that since variety 'Massey' usually flowers at node 9 or 10, there is a significant inhibition to a mean treatment node 11.50 if the decotyledonised embryo is placed on a plain nutrient agar medium. With variety 'Greenfeast', a plant which under L.D. conditions usually flowers at node 16 or 17, the mean treatment node of first flower has been brought forward to node 15.33 if the decotyledonised embryo is placed on a plain nutrient agar medium (Table 2.2).

On extracts of 'Massey' cotyledons, 'Massey' embryos were delayed in their flowering when the extract was made with cotyledons 2 - 4 days old. (Table 2.1, $P = .01$; Table 2.2, $P = .05$). Although 'Massey' extracts of all ages tested significantly inhibited the flowering of 'Greenfeast' embryos ($P < .001$), the greatest delay in flowering was caused by 3 - day extracts (Table 2.2).

On extracts of 'Greenfeast' cotyledons, 'Massey' embryos were delayed in their flowering when the extract was made with cotyledons 4 days old (Table 2.1, $P = .05$). Although 'Greenfeast' extracts of all ages tested significantly inhibited the flowering of 'Greenfeast' embryos ($P < .001$), the greatest delay in flowering was caused by the 6 - day extracts (Table 2.3).

The deaths of many 'Massey' embryos occurred during the course of these experiments, especially on 3-day cotyledon extracts of both varieties, and on the 6-day 'Massey' cotyledon extract. Since Johnston

(15) found that no cell division (and hence no new node formation) occurred in the embryo until four days after the commencement of germination, some substance may be present in the cotyledons at this stage which causes a shock to the embryo from which 'Greenfeast', but not 'Massey' can recover.

Moore (20) has found that at high light intensities cotyledon excision causes a significant delay in variety 'Massey' peas, and this has been observed in the present experiments. Although many workers (3, 9, 11, 23, 26, 27, 29) have attributed this delay to the removal of a florigen present in the cotyledons, Johnston (15) has produced good evidence for the presence of a temporary inhibitor, which moves from the cotyledons to the plumule 24 - 36 hours after germination. The results presented here show a similar trend to those obtained by Johnston. Extracts of 'Massey' cotyledons at the age of 2 - 3 days can delay the node of first flower of both 'Massey' and 'Greenfeast' embryos. This could be the result of a temporary inhibitory substance, which reaches its maximum level in the cotyledons of 'Massey' after about 3 days of germination, or it could be the result of a flower inducing substance, which surpasses a maximum threshold level after 3 days, and thus becomes inhibitory. After 3 days, a regulatory mechanism becomes fully established which would control the rate of production of this inducing substance.

Since the extracts were autoclaved, the results of these experiments show also that this substance affecting the node of first flower in 'Massey' cotyledons is quite stable to heat extremes.

The results concerning the variety 'Greenfeast' cotyledon extracts

are in agreement with the colysanthin theory of Barber and Paton (3,27). Colysanthin reaches its highest level in the 'Greenfeast' cotyledons 4-6 days after germination, whereupon it begins to move out of the cotyledons into the plumule. The fact that the node of first flower of 'Greenfeast' embryos is brought down when the embryos are placed on a plain nutrient agar medium could mean that colysanthin is present in low quantities in the plumules soon after germination.

Although the results of these experiments show the same general trend as those obtained by Johnston, they are in themselves far from conclusive. Because of this fact, the experiments are presently being repeated, using pure genetic lines instead of the commercial varieties, and also modifying the cotyledon extract media.

TABLE 2.1

Effect of cotyledon extracts of various ages on the flowering of 'Massey' embryos. Data is for node of first flower (FN). The number of plants scored per treatment is given, together with the treatment means and standard errors.

Expt. 2.1

Age of cotyledon extract (days) 'Massey'	FN	No. scored for FN	Age of cotyledon extract (days) 'Greenfeast'	FN	No. scored for FN
Control	11.38 ⁺ .18	8	Control	11.38 ⁺ .18	8
0	11.88 ⁺ .22	8	0	11.40 ⁺ .19	15
1/3	11.92 ⁺ .15 ^x	12	1/3	11.35 ⁺ .15	17
1	11.69 ⁺ .12	16	1	11.90 ⁺ .23	10
2	12.15 ⁺ .19 ^{xx}	13	2	11.41 ⁺ .12	17
4	12.23 ⁺ .16 ^{xx}	13	4	12.14 ⁺ .18 ^x	14
6	11.40 ⁺ .51	5	6	11.71 ⁺ .13	14

The significance of difference between the means of the control and the treatment means is indicated at the .05 level of probability (x), at the .01 level of probability (xx), and at the .001 level of probability (xxx).

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Figure 3.

Scatter diagram showing the effects of different cotyledon extracts on the node of first flower of 'Massey' embryos. (Experiment 2.1). Treatment means and standard errors are shown in each case.

Figure 3.

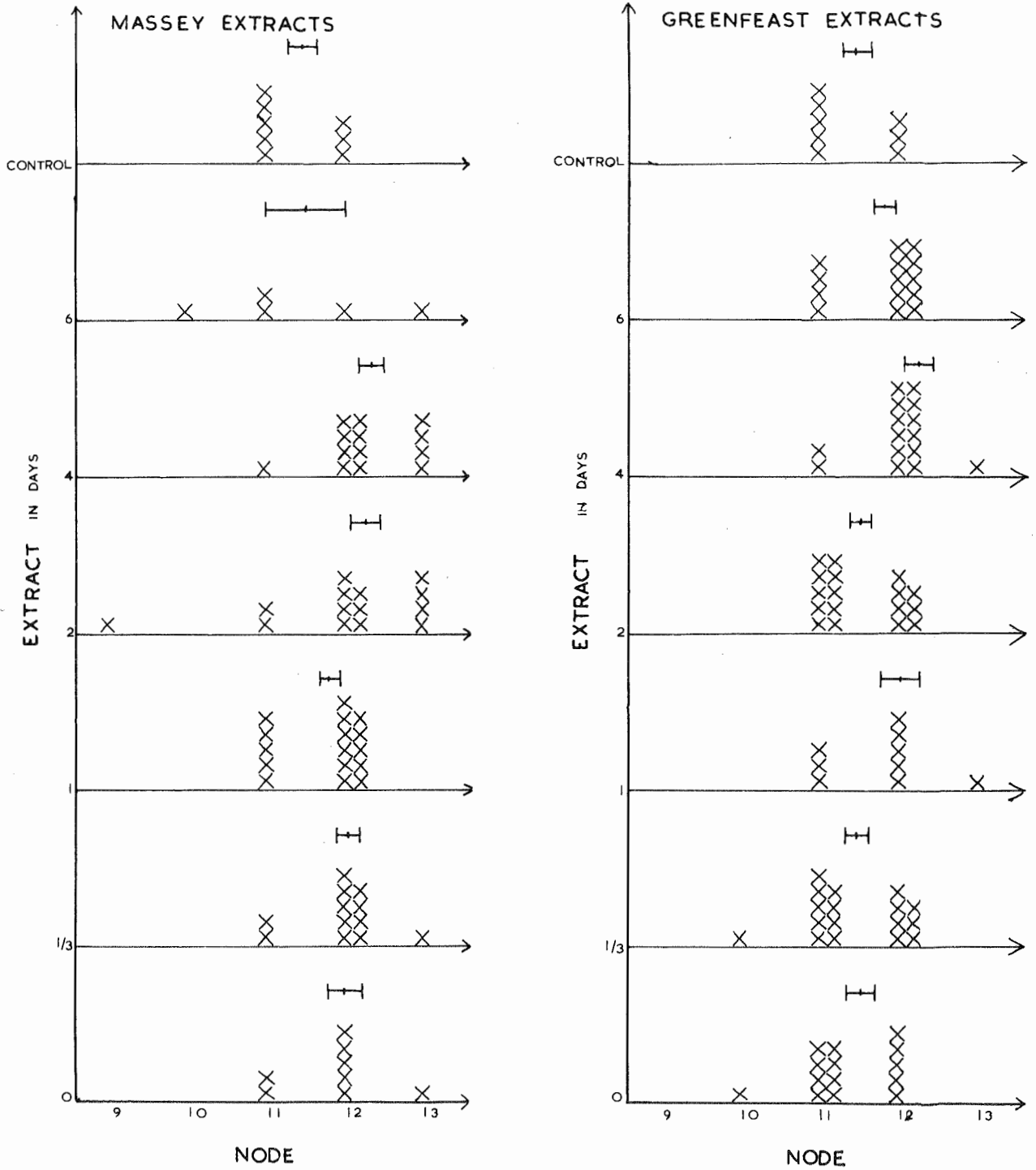


TABLE 2.2

Effect of Massey cotyledon extracts of various ages on the flowering of 'Massey' and 'Greenfeast' embryos.

Data is for node of first flower (FN). The number of plants scored per treatment is given, together with the treatment means and standard errors.

Expt. 2.2

'Massey' embryos			'Greenfeast' embryos		
Age of cotyledon extract (days) 'Massey'	FN	No. scored for FN	Age of cotyledon extract (days) 'Massey'	FN	No. scored for FN
Control	11.50 ⁺ .17	18	Control	15.33 ⁺ .12	33
3	12.50 ⁺ .50	2	3	16.50 ⁺ .17 ^{xxx}	18
5	11.67 ⁺ .19	12	5	16.14 ⁺ .18 ^{xxx}	22
6	-	0	6	16.22 ⁺ .14 ^{xxx}	23
9	11.93 ⁺ .10 ^x	29	9	16.11 ⁺ .12 ^{xxx}	36

The significance of difference between the means of the controls and the treatment means is indicated at the .05 level of probability (x), at the .01 level of probability (xx), and at the .001 level of probability (xxx).

Figure 4.

Scatter diagram showing the effects of 'Massey' cotyledon extracts on the node of first flower of both 'Massey' and 'Greenfeast' embryos. (Experiment 2.2). Treatment means and standard errors are shown in each case.

Figure 4.

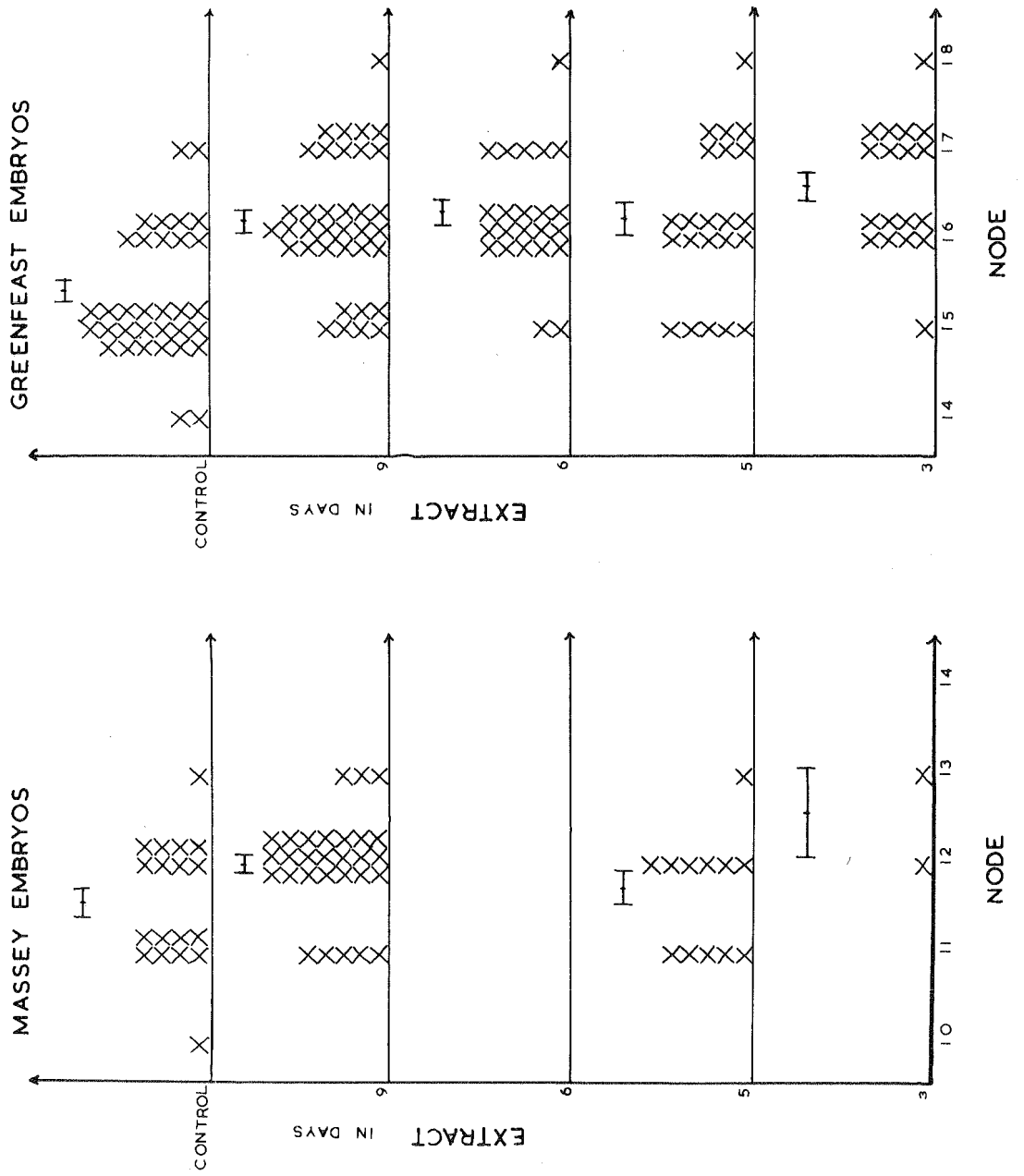


TABLE 2.3

Effect of 'Greenfeast' cotyledon extracts of various ages on the flowering of 'Massey' and 'Greenfeast' embryos.

Data is for node of first flower (FN). The number of plants scored per treatment is given, together with the treatment means and standard errors.

Expt. 2.2

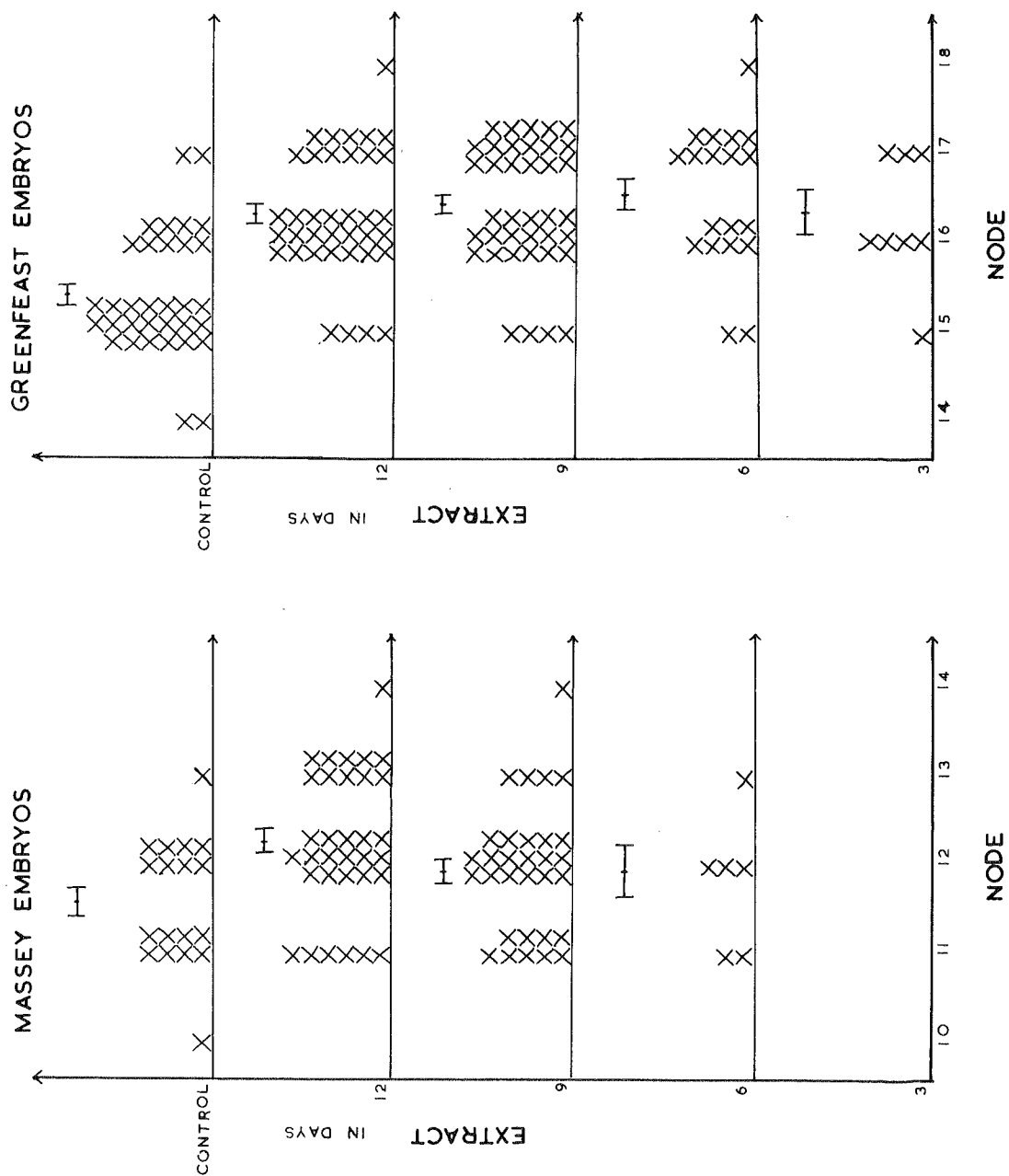
'Massey' embryos			'Greenfeast' embryos		
Age of cotyledon extract (days) 'Greenfeast'	FN	No. scored for FN	Age of cotyledon extract (days) 'Greenfeast'	FN	No. scored for FN
Control	11.50 ⁺ .17	18	Control	15.33 ⁺ .12	33
3	-	0	3	16.25 ⁺ .25 ^{xxx}	8
6	11.83 ⁺ .30	6	6	16.45 ⁺ .17 ^{xxx}	20
9	11.84 ⁺ .14	32	9	16.34 ⁺ .11 ^{xxx}	38
12	12.18 ⁺ .13 ^{xx}	33	12	16.24 ⁺ .11 ^{xxx}	37

The significance of difference between the means of the control and the treatment means is indicated at the .05 level of probability (x), at the .01 level of probability (xx), and at the .001 level of probability (xxx).

Figure 5.

Scatter diagram showing the effects of 'Greenfeast' cotyledon extracts on the node of first flower of both 'Massey' and 'Greenfeast' embryos (Experiment 2.2). Treatment means and standard errors are shown in each case.

Figure 5.



CHAPTER THREE

The effect of vernalisation on the flowering of Pisum sativum

Introduction:

In the garden pea, Pisum sativum L., the first flower is formed on a precise, genetically defined node for each variety, and as explained previously, pea varieties can be divided into two categories, depending on their flowering habit. Early varieties are insensitive to vernalisation and act as day-neutral plants, while late varieties show a positive response to vernalisation and behave as quantitative long day plants (1,14,23,27).

Barber (1) and Paton (26) have suggested that flowering occurs in late varieties when the cotyledon inhibitor, colysanthin, is destroyed, and suggest that the vernalisation and photoperiod reactions compete with one another for the colysanthin substrate. Although gibberellin can reverse the vernalisation effect, (22) it is not identical with colysanthin (1); and high temperatures given after the vernalisation treatment can partially annul the promotive action (23). Moore and Bonde (23), working with the late pea variety 'Dwarf Telephone', have suggested that vernalisation reduces auxin activity and thus promotes flowering, a concept which ties in with the idea of Galston (7), who suggests a functional association between a growth hormone (auxin) and a flowering hormone (e.g. florigen). However, Leopold and Guernsey (18) have shown that flowering in 'Alaska' peas can be promoted if low temperatures follow treatment of the seeds with auxin.

It has been shown in rye that germination is dependent on the reaction of the embryo - and not of the endosperm or aleurone layer - to

vernalisation (8). Moore and Bonde (24) have proposed the theory that vernalisation and cotyledon excision in peas may be explained on a common basis, and Paton, using grafting experiments, has suggested that there is less inhibitor present in vernalised than in unvernalsed stocks (26).

Thus the current information regarding the regulation of flowering in peas by vernalisation is marked by conflicting interpretations and the series of experiments reported in the following pages were done in an attempt to understand the vernalisation reaction in a clearer light.

The experiments had a three-fold purpose:-

- a) to try to determine the site of action of the vernalisation reaction.
- b) to try to determine whether the vernalisation reaction is associated with the cotyledonary inhibitor.
- c) to try to determine any relationship between the vernalisation reaction and the photoperiod response.

Experiment 3.1:

A full factorial experiment was performed involving grafting, two pea varieties, two vernalization treatments and two photoperiod regimes.

- M - the early flowering variety 'Massey'.
- G - the late flowering variety 'Greenfeast'.
- U - unvernallized
- V - vernalized for 4 weeks at 4°C.
- P8 - an 8-hour photoperiod.
- P16 - a 16-hour photoperiod

(e.g. $\frac{\text{MU}}{\text{GV}}$ means an unvernallized 'Massey' scion grafted on to a vernalized 'Greenfeast' stock).

The plants were randomised within each photoperiod regime, grown to anthesis and scored for the node of first flower.

The results are summarised in Tables 3.1, 3.2, and 3.3, and graphically in Figures 6, 7 and 8.

Results:

Prior to germination, six nodes have usually been laid down in the dry embryo, and no further nodes are formed until 4 days after soaking, after which time node formation occurs rapidly (Johnston).

Since variety 'Massey' flowers at about node 9 or 10, the results of the grafting experiment involving 'Massey' scions will not be considered in any detail in this discussion, because by the time of grafting, the flowering node of 'Massey' plants has in all probability been laid down. However, this point was not actually confirmed by dissection. Further experiments involving embryo grafting are needed to clarify this position. The discussion concerning the grafting experiment (Experiment 3.1) will therefore be confined to the results involving 'Greenfeast' scions.

a) Grafting lowers the node of first flower in both vernalized and unvernallized plants, especially under L.D. conditions ($P < 0.001$). Under S.D. conditions, the effect of grafting is only slight ($P < 0.8$ and $P < 0.7$ respectively). (see table 3.3 I).

b) Vernalization decreases the flowering node significantly ($P < 0.001$), in both intact and self-grafted plants, and under either photoperiod regime.

c) Comparison of cross-grafting plants involving a similarly treated scion on a vernalised and an unvernallised stock (see table 3.3 II), shows that vernalising the stock has a small effect on the flowering node, but generally, this effect is not very significant.

d) By comparing cross-grafted plants involving a vernalised and an unvernallised scion on a similarly treated stock however, it can be seen that the effect of vernalizing the scion is very significant, in either photoperiod regime (see table 3.3 III).

e) Under S.D. conditions, unvernallized 'Greenfeast' scions on a 'Greenfeast' stock all flower at about the same node (i.e. GU 23.4, $\frac{GU}{GU}$ 23.1, $\frac{GU}{GV}$ 22.7 - see table 3.2), irrespective of the treatment to the stock, and vernalised 'Greenfeast' scions on 'Greenfeast' stocks flower in a similar manner (i.e. GV 20.5, $\frac{GV}{GU}$ 20.6, $\frac{GV}{GV}$ 20.4 - see table 3.2).

f) Under L.D. conditions, the effect of self-grafting is quite evident, as is the effect of cross-grafting (i.e. GU 16.90, $\frac{GU}{GU}$ 15.61, $\frac{GU}{GV}$ 14.17 and GV 14.53, $\frac{GV}{GU}$ 12.79, $\frac{GV}{GV}$ 12.90 - see table 3.2).

Discussion:

These results tie in with the theory of an inhibitor - colysanthin - being produced in the cotyledons of late varieties of peas. The presence of colysanthin at the apex determines a threshold value which the floral inducing substance, produced in the leaves, must attain before it can be effective. This inducing substance is dependent on light for its formation, more inducer being produced in long days than in short days.

a) Grafting disrupts the passage of the inhibitor from the cotyledons to the apex until full physiological union has been restored (27), and thus the threshold value will be lowered. Under S.D. conditions, physiological union is restored and the original threshold value is almost regained before the stimulus can reach the threshold value. Under L.D. conditions, enough stimulus is produced to reach the lowered threshold value before full transport of colysanthin is restored.

b) The effect of vernalization is to lower the threshold level set by colysanthin, and it could achieve this in a variety of ways:-

- 1) it could halt or decrease the rate of production of colysanthin
- 2) it could affect the mobilisation and transportation of colysanthin.

3) it could cause the destruction of colysanthin at the apex, perhaps by initiating a reaction which produces a colysanthin-destroying substance.

4) it could make the apex less sensitive to colysanthin

or 5) it could perhaps make the apex more sensitive to the inducer.

c) Since the effect of vernalizing the stock is generally small, it seems that the amount of colysanthin present in unvernallized and vernalized stocks is similar. The small effect observed could be the result of a decreased rate of colysanthin production, which picks up once the period of vernalization is over.

d) Vernalization of the scion gives a highly significant effect, the threshold value being greatly decreased. The major site of action of the vernalization response would therefore seem to be at the apex and not in the cotyledons (b-3, mentioned previously).

e) Since under S.D. conditions, all grafted 'Greenfeast' scions flower at approximately the same node as the ungrafted control, irrespective of the treatment to the stock, this backs up the statements made previously. The original threshold level can be regained before the stimulus reaches the required level, and although the rate of colysanthin production may be delayed during the vernalization treatment, it picks up after completion of the vernalization treatment, the lower threshold value still being too high for the amount of inducer produced up to that time.

f) This is not the case under I.D. conditions. The lowered threshold value caused by grafting can be attained, since the inducer is reaching the apex at a much faster rate than is the colysanthin. The decreased rate of production of colysanthin caused by vernalizing the cotyledons

causes a further lowering of the flowering node, as can be observed under the S.D. conditions. That it cannot be observed under L.D. conditions is most likely because 'Greenfeast' has a certain node (12 or 13) below which it will not flower. Up to this node, it is in a juvenile state, and once this node is laid down, the plant attains its "ripeness to flower".

If it is assumed that the cotyledons of 'Massey' peas do not contain colysanthin then the comparison between stocks of the two different varieties carrying similar scions supports the idea that vernalization causes a temporary decrease in the rate of production of the inhibitor in the cotyledons. Since the threshold value is brought down, under L.D. conditions the scions will not differ much in their flowering node, as the decreased threshold level is reached by the level of the inducer before the colysanthin supply can raise the level again. Under S.D. conditions, the colysanthin from the 'Greenfeast' stocks can raise the threshold level, and therefore a significant difference in the node of first flower would be expected between the scions. However, further evidence must be obtained regarding 'Massey' scions and stocks before an assumption such as this can really be considered.

It therefore seems that vernalization has its major effect in the scion by decreasing the threshold level set by colysanthin. It causes a minor effect in the cotyledons by decreasing the rate of production of colysanthin. This minor effect may be part of a general slowing down of metabolism.

TABLE 3.1

The effect of vernalization, grafting and photoperiod, and their interaction, on the flowering behaviour of 'Massey' scions

Data is for node of first flower (FN). The number of plants scored per treatment is given, together with the treatment means and standard errors.

Graft type	SD		LD	
	FN	no. scored	FN	no. scored
MU	9.74 ± 0.10	19	10.00 ± 0.30	19
MU	9.91 ± 0.18	12	9.90 ± 0.18	10
MU				
MV	10.00 ± 0.12	10	10.00 ± 0.10	12
MU				
MV	10.38 ± 0.12	16	10.06 ± 0.13	18
MU	9.67 ± 0.24	9	9.91 ± 0.21	11
MV				
MV	10.00 ± 0.11	13	9.92 ± 0.08	13
MV				
MU	10.00 ± 0.45	5	10.14 ± 0.29	14
GU				
MV	10.33 ± 0.23	9	10.00 ± 0.08	19
GU				
MU	10.67 ± 0.44	9	9.94 ± 0.16	17
GV				
MV	10.19 ± 0.14	16	9.89 ± 0.07	19
GV				

Figure 6.

Scatter diagram showing the effects of grafting, photoperiod, vernalization, and their interaction on the node of first flower of 'Massey' scions. Treatment means and standard errors are shown for each case.

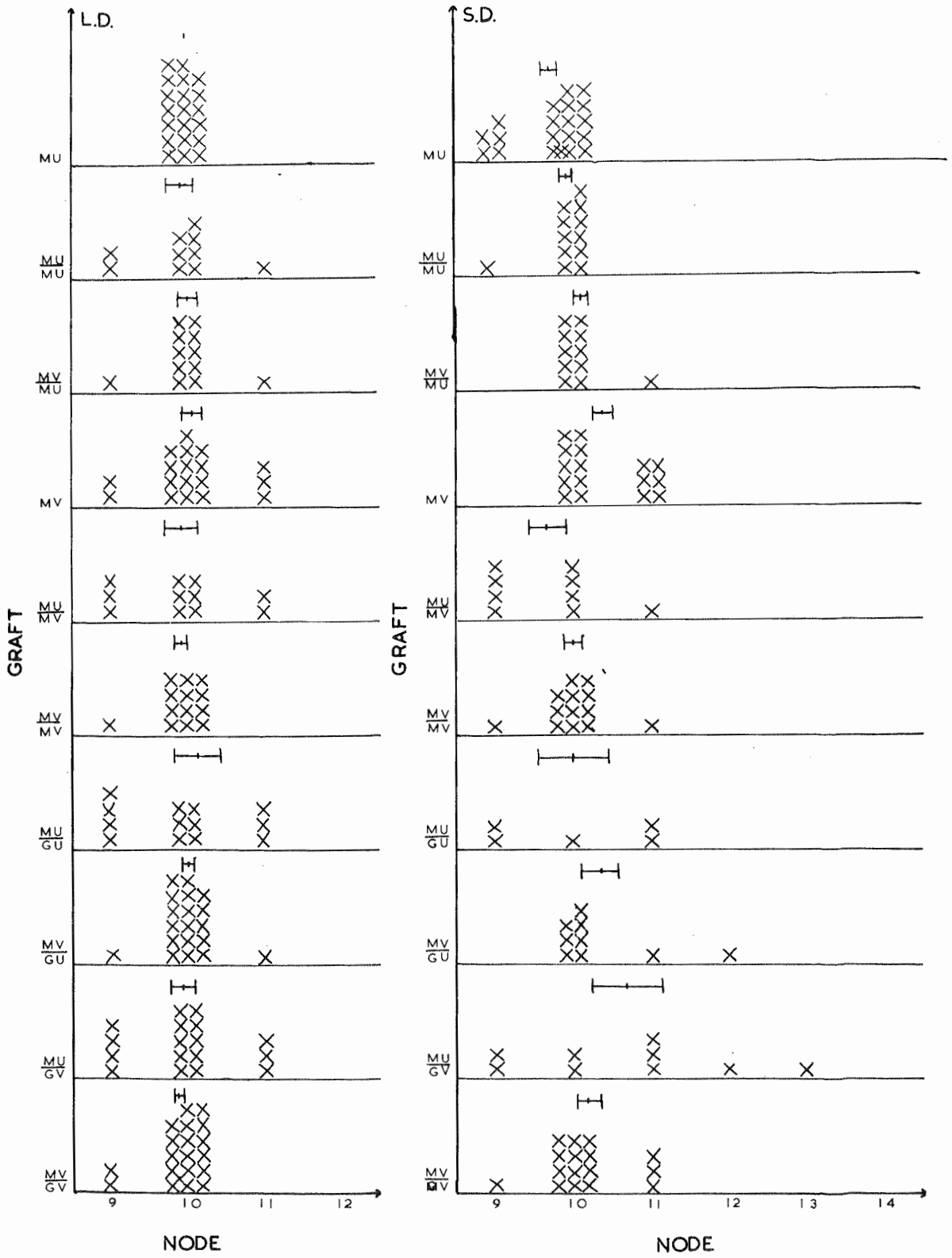


Figure 6.

TABLE 3.2

The effect of vernalization, grafting and photoperiod, and their interaction, on the flowering behaviour of 'Greenfeast' scions.

Data is for node of first flower (FN). The number of plants scored per treatment is given, together with the treatment means and standard errors.

Graft type	SD		LD	
	FN	no. scored	FN	no. scored
GU	23.35 [±] 0.30	20	16.90 [±] 0.18	20
GU GU	23.13 [±] 0.23	8	15.6 [±] 0.21	18
GV GU	20.67 [±] 0.20	18	12.79 [±] 0.14	19
GV	20.50 [±] 0.21	20	14.53 [±] 0.12	19
GU GV	22.71 [±] 0.29	14	14.17 [±] 0.19	18
GV GV	20.42 [±] 0.20	24	12.90 [±] 0.17	20
GU MU	19.86 [±] 0.54	7	13.88 [±] 0.09	8
GV MU	18.10 [±] 0.52	10	12.67 [±] 0.14	12
GU MV	20.71 [±] 0.47	7	13.37 [±] 0.22	19
GV MV	16.91 [±] 0.97	11	12.67 [±] 0.14	18

Figure 7.

Scatter diagram showing the effects of grafting, vernalization, and their interaction on the node of first flower of 'Greenfeast' scions under S.D. conditions. Treatment means and standard errors are shown for each case.

Figure 8.

Scatter diagram showing the effects of grafting, vernalization, and their interaction on the node of first flower of 'Greenfeast' scions under L.D. conditions. Treatment means and standard errors are shown for each case.

TABLE 3.3

The tests of significance between different graft types, under both photoperiod regimes. The method of calculation is demonstrated in Appendix 1.

I	Comparison	SD	LD
	GU - GV	xxx	xxx
	GU - GV GU - GV	xxx	xxx
	GU - $\frac{GU}{GU}$	0.6 < P < 0.7	xxx
	GV - $\frac{GV}{GV}$	0.7 < P < 0.8	xxx

II	Comparison	SD	LD
	$\frac{GU}{GU} - \frac{GU}{GV}$	0.2 < P < 0.3	xxx
	$\frac{GU}{GU} - \frac{GU}{MU}$	xxx	xxx
	$\frac{GV}{GV} - \frac{GV}{GU}$	0.4	0.6
	$\frac{GV}{GV} - \frac{GV}{MV}$	xxx	0.3
	$\frac{GU}{MU} - \frac{GU}{MV}$	0.2 < P < 0.3	0.1 < P < 0.2
	$\frac{GV}{MU} - \frac{GV}{MV}$	0.3	1.0
	$\frac{GU}{GV} - \frac{GU}{MV}$	xxx	xx
	$\frac{GV}{GU} - \frac{GV}{MU}$	xxx	0.5 < P < 0.6

III	Comparison	SD	LD
	$\frac{GU}{GU} - \frac{GV}{GU}$	xxx	xxx
	$\frac{GU}{GV} - \frac{GV}{GV}$	xxx	xxx
	$\frac{GU}{MU} - \frac{GV}{MU}$	x	xxx
	$\frac{GU}{MV} - \frac{GV}{MV}$	xx	x

The significance of difference between the means of the graft treatments is indicated at the 0.05 level of probability (x), at the 0.01 level of probability (xx), and at the 0.001 level of probability (xxx).

Experiment 3.2:

After 8 hours imbibition, seeds of the pea variety 'Greenfeast' were given vernalization treatments of 0, 1, 2, 3 and 4 weeks. At the end of the cold treatment, half of the seedlings in each treatment had their cotyledons removed, the other half remaining intact. The seedlings were then placed in the phytotron and subjected to a photoperiodic treatment of either 8 hours (S.D.) or 16 hours (L.D.), the individual treatments being randomised within each photoperiodic regime. Seedlings which had had their cotyledons removed at the stages of 0, 1, 2 and 3 weeks were grown on a nutrient agar medium until ready for transplanting. The plants were then randomised within each photoperiod regime, grown to anthesis and scored for the node of first flower.

The results are summarised in Tables 3, 4, 3.5 and in Figures 9, 10 and 11 and can be explained in the same terms as in the previous discussion.

Results and Discussion:

1. The effect of photoperiod is very highly significant ($P \ll 0.001$ - Table 3.5). Plants under L.D. conditions flower at a lower node than those under S.D. conditions, irrespective of the vernalization or cotyledon treatment (Figure 11). This is to be expected, since more stimulus is produced in the leaves in long days than in short days, and therefore the threshold level set by colysanthin will be reached sooner. Also under L.D. conditions, the threshold level may be less than under S.D. conditions, since by the time floral initiation occurs, not all of the colysanthin from the cotyledons has arrived at the apex. Under S.D. conditions, the slower rate of production of stimulus allows time for all of the colysanthin to arrive at the apex.
2. The effect of removing cotyledons after completion of the vernalization treatment is highly significant ($P \ll 0.001$ - Table 3.5). Plants which have been decotyledonised flower at a lower node than intact plants, irrespective of the photoperiod or vernalization treatment (Figure 11). This is to be expected since by removing the cotyledons the source of colysanthin is also removed, and therefore the threshold level will be lowered. Some colysanthin will still be present in the plumule however, since the cotyledons are intact during the vernalization treatment, but at a much lower concentration from that in intact plants.
3. The effect of vernalization is highly significant ($P \ll 0.001$ - Table 3.5). As the time of the vernalization treatment is increased, there is a decrease in the node of first flower, irrespective of the photoperiod or cotyledon treatment (Figure 11). This is also to be expected if vernalization has its effect in inactivating or destroying colysanthin at the apex, as the threshold level set by the colysanthin

level would be decreased.

a) Vernalization for 1 week has little effect in intact plants, but has a marked effect in decotyledonised plants. It seems therefore that the reactions set in motion by vernalization require about 1 week of cold treatment before becoming operative. At the high colysanthin concentrations in intact plants, these reactions make little difference to the threshold level, and require a vernalization period of 2 weeks or more before being able to lower the threshold level. In decotyledonised plants however, the threshold level is much reduced, as not much colysanthin has reached the plumule, and although the vernalization reactions are occurring at a very low rate, they can still show a marked effect.

b) In decotyledonised plants, vernalization had its maximum effects after 2 weeks, whereas for intact plants the node of first flower is still decreasing at 4 weeks vernalization, this being because of the reduced amount of colysanthin present in the decotyledonised plants. It appears that under L.D. conditions, vernalization of decotyledonised plants is effective after 1 week, but this could be because it has brought the node of first flower down to the minimum value - the "ripeness to flower" threshold. Although a little colysanthin may still be present in the plumule after 1 week, destroying it will have no effect on the flowering node.

4. On this argument therefore, one would expect an interaction between photoperiod and vernalization, and between photoperiod and cotyledon status. The analysis of variance shows that this is the case ($0.01 < P < 0.05$ - table 3.5). That these interactions are significant may be the result of the extremely large photoperiod effect. Long days decrease the flowering node by enhancing the rate of production of

inducer, while both cotyledon removal and vernalization decrease the flowering node by lowering the effective threshold level.

5. If the theory that the effect of vernalization is to inactivate or destroy colysanthin, a highly significant interaction between vernalization and cotyledon status would be expected, and this was found to be the case ($P \ll 0.001$ - table 3.5).

6. The third order interaction was also found to be significant. All three treatments individually lower the flowering node, and there is also the very large interaction between vernalization and cotyledon status, and this would explain the order of significance of the third order interaction.

Discussion - general:

The original theory proposed by Paton (26) and Barber (1) and Sprent (29) was that an inhibitor (colysanthin) was produced in the cotyledons of late-flowering pea varieties, which was selectively destroyed or converted into a flowering stimulus by long days and a cold treatment. Paton went one step further by suggesting that the inhibitor acts as a precursor to a floral stimulus (florigen) (26).

In the light of the present experiments, this theory needs to be modified and in fact flowering in peas can be explained in a much less complicated way, by assuming that there are two distinct independent mechanisms regulating flowering.

a) The first mechanism involves colysanthin. Colysanthin sets a new threshold level at the apex above that of the intrinsic "ripeness to flower" threshold, which is set by the amount of inducer substance produced by the leaves. Before the inducer can be effective, it must reach this increased level. Vernalization has a direct effect on the new threshold level, lowering it by inactivating or destroying colysanthin.

b) The second mechanism involves the floral inducer. Increasing the photoperiod increases the amount of inducer produced and therefore the threshold level is attained sooner.

In this way, the complex idea of the inhibitor acting as a precursor to the inducer, and of the interconvertibility of the two substance, can be avoided.

Stanfidd et al observed a vernalization effect in the late variety 'Dark Skin Perfection', if the night temperature fell to 4°C (the day temperature being 13°C) (31). Moore and Bende have also observed that vernalization causes a significant lowering of the flowering node in late

pea varieties, but only if the vernalization treatment lasted for longer than 10 days (22). Vernalization had its maximum effect after 28 days (23). These results are in accord with those obtained in the present series of experiments.

The vernalization reaction may be associated with gibberellin biosynthesis (2). Lockhart has found in 'Alaska' peas that a natural gibberellin factor is produced at the stem tip (19). Since applied gibberellin delays flowering in peas (15, 28), if colysanthin was involved in the biosynthesis of gibberellin, perhaps as a precursor, then vernalization could enhance flowering by destroying or inactivating colysanthin or by stopping a reaction in the gibberellin biosynthesis pathway. Moore and Bonde (22) have found that gibberellin applied after a vernalization treatment to the late variety 'Dwarf Telephone' completely reversed the vernalization effect.

Further experiments involving vernalization of the excised embryo are being planned at the moment in order to clarify further the mechanism of the vernalization effect.

Table 3.4

The significance of difference between the means of the controls and the treatment means is indicated at the 0.05 level of probability (x), at the 0.01 level of probability (xx) and at the 0.001 level of probability (xxx).

TABLE 3.4

The effect of the length of the vernalization period, cotyledon excision and photoperiod regime, and the interaction of the three on the flowering of 'Greenfeast' plants.

Data is for node of first flower (FN). The number of plants scored per treatment is given, together with the treatment means and standard errors.

Experiment 3.1

Long Days					
Cotyledons intact			Cotyledons excised		
Length of vernalization treatment in weeks.	FN	No. plants scored for FN	Length of vernalization treatment	FN	No. scored for FN
0	16.90 ⁺ -0.21	20	0	14.94 ⁺ -0.15	18
1	16.70 ⁺ -0.21	20	1	13.00 ⁺ -0.25 ^{xxx}	12
2	15.80 ⁺ -0.12 ^{xxx}	20	2	13.64 ⁺ -0.25 ^{xxx}	14
3	14.75 ⁺ -0.18 ^{xxx}	20	3	13.50 ⁺ -0.50 ^{xxx}	2
4	14.42 ⁺ -0.16 ^{xxx}	19	4	12.50 ⁺ -0.22 ^{xxx}	6
Short Days					
0	24.35 ⁺ -0.21	20	0	21.43 ⁺ -0.25 ^{xx}	14
1	24.00 ⁺ -0.25	20	1	20.22 ⁺ -0.27 ^{xxx}	18
2	22.26 ⁺ -0.29 ^{xxx}	19	2	18.45 ⁺ -0.16 ^{xxx}	11
3	21.10 ⁺ -0.24 ^{xxx}	20	3	18.00	1
4	20.11 ⁺ -0.21 ^{xxx}	18	4	18.57 ⁺ -0.20 ^{xxx}	7

Figure 9.

Scatter diagram showing the effect of the length of the vernalization treatment and photoperiod on the node of first flower of intact 'Greenfeast' plants. (Experiment 3.1). Treatment means and standard errors are shown in each case.

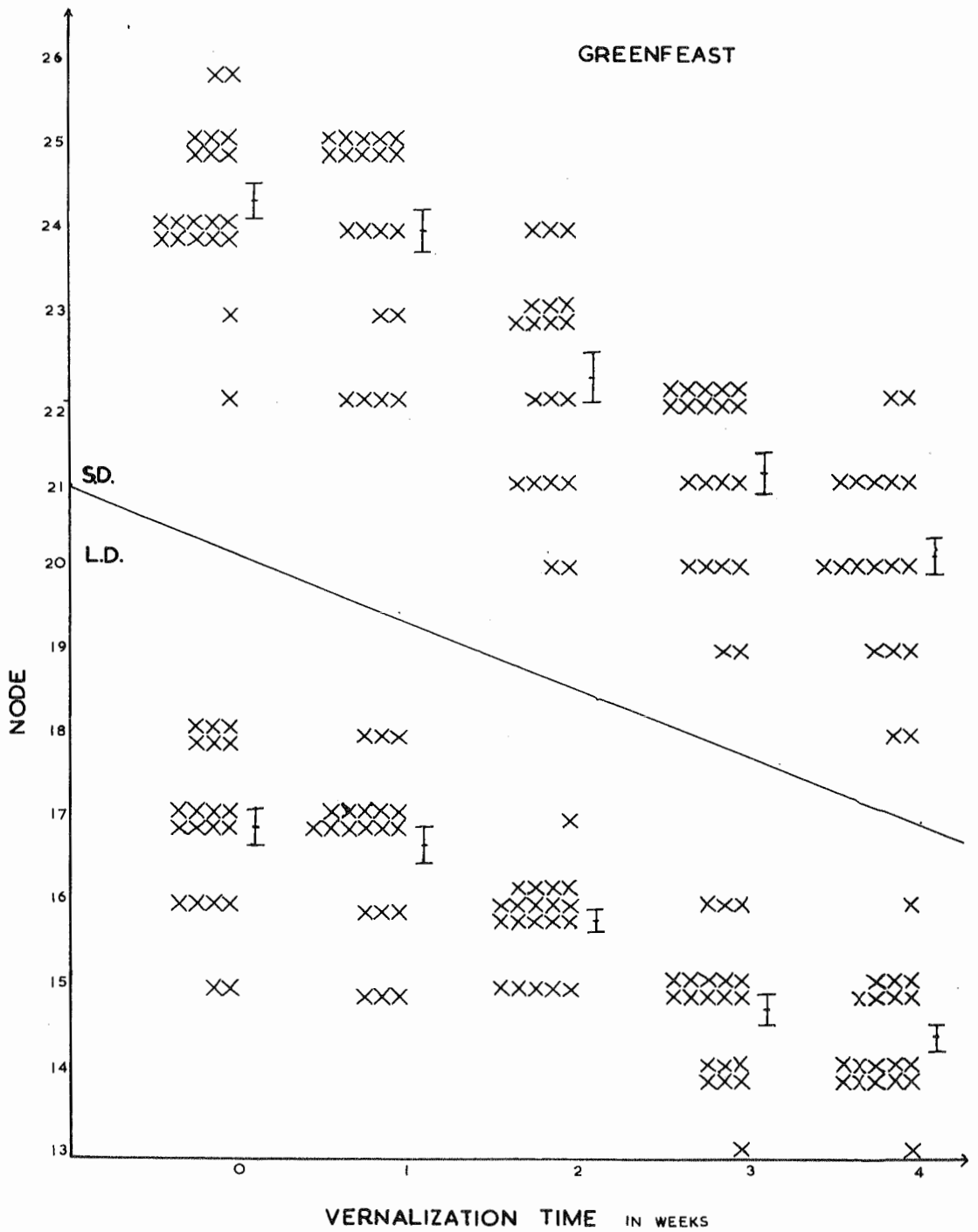


Figure 9.

Figure 10.

Scatter diagram showing the effect of the length of the vernalization treatment and photoperiod on the node of first flower of de-cotyledonised 'Greenfeast' plants (Experiment 3.1).

Treatment means and standard errors are shown in each case.

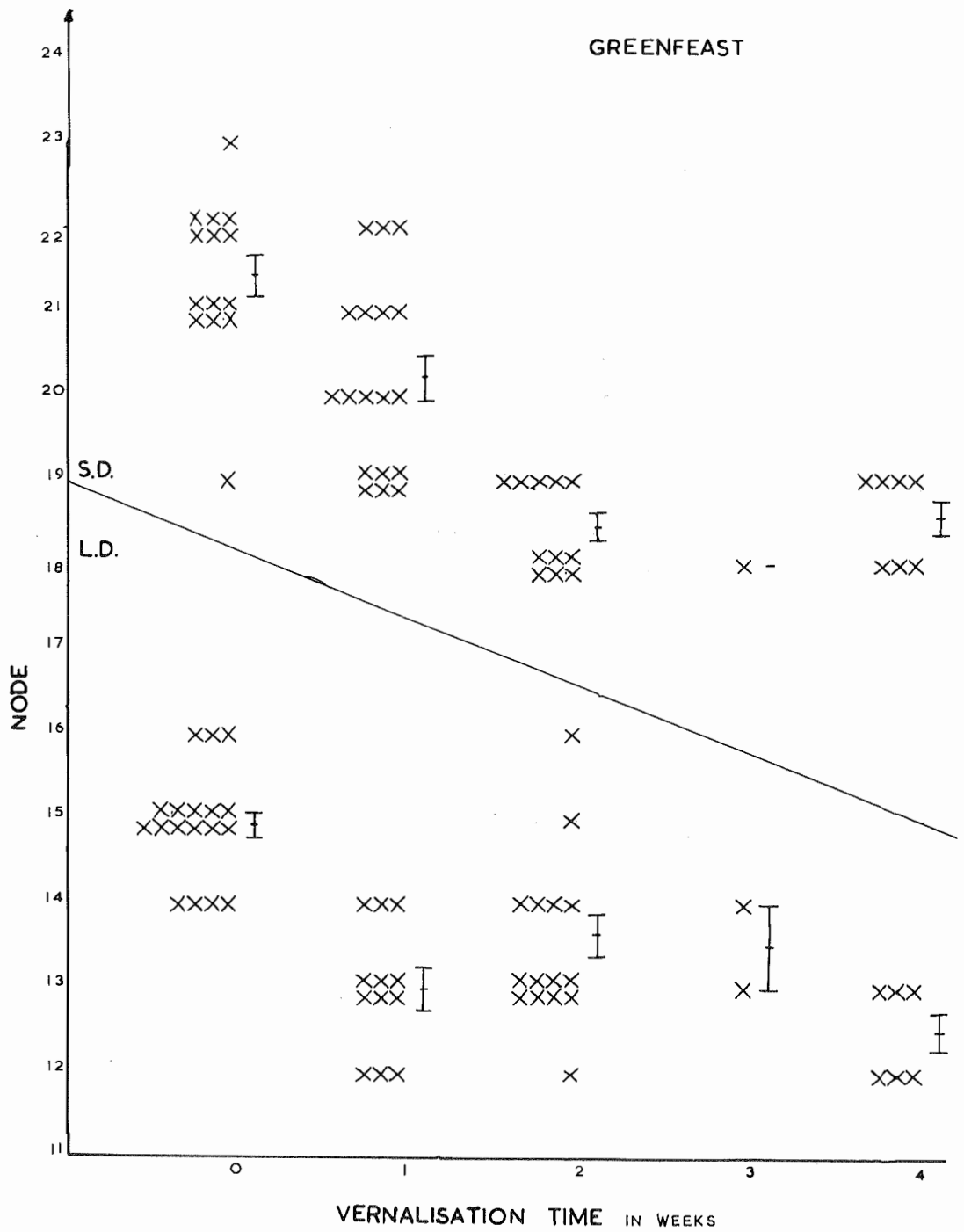


Figure 10.

Figure 11.

The effect of the length of the vernalization treatment and of cotyledon removal on the flowering of 'Greenfeast' plants in both long day and short day conditions is shown by the relative lengths of the columns of the histogram. The data summarized is based on the number of plants indicated at the base of each bar. The standard errors of the means are shown at the tops of each bar.

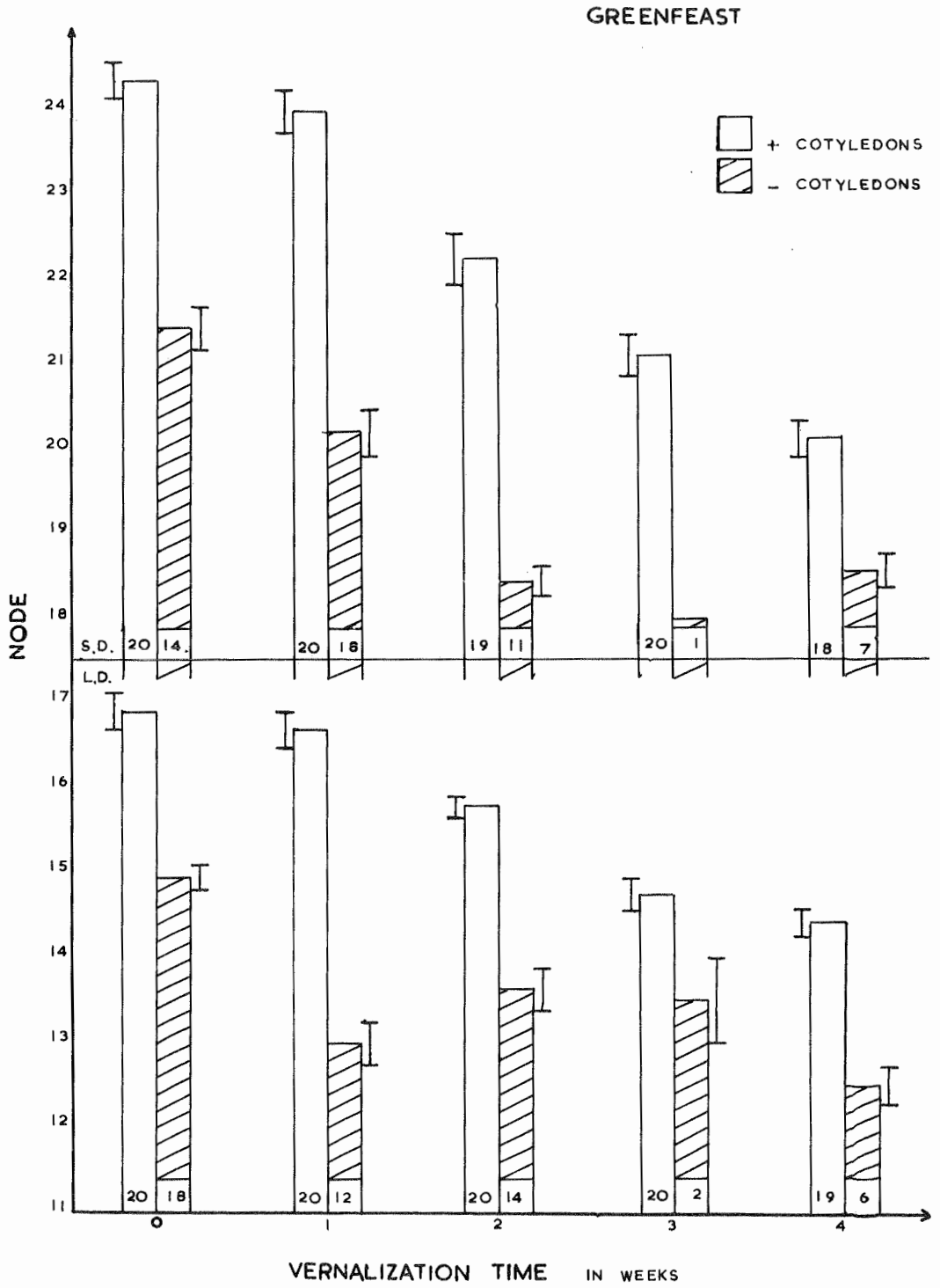


Figure 11.

TABLE 3.5

The analysis of variance for the results obtained in experiment 3.2

Effect	Degrees of freedom	Sum of squares	Variance	Variance ratio	Probability
P	1	3139.50	3139.50	3608.62	$\ll 0.001$
V	4	267.74	66.94	76.94	$\ll 0.001$
C	1	329.91	329.91	379.21	$\ll 0.001$
PV	4	13.20	3.30	3.79	$0.01 < P < 0.05$
PC	1	6.36	6.36	7.31	$0.01 < P < 0.05$
VC	4	168.03	42.01	48.29	$\ll 0.001$
PVC	4	20.25	5.06	5.82	≈ 0.001
Error	279	242.52	0.87		
Total	298	4187.51			

SUMMARY

The presence of a flower inhibitor (colysanthin) in the late variety 'Greenfeast' as proposed by Barber and Paton for late (Sn) varieties of peas has been confirmed. Colysanthin is synthesised in the cotyledons during and after the first four days of germination, whereupon it begins to move into the plumule. Cotyledons of the early variety 'Massey' also contain some temporary inhibitory substance after 3 days of germination.

Peas have a genetically defined node, below which they will not flower. This is probably due to a threshold requirement for inducer substance at the apex before it can become effective, and would be caused (at least in part) by the time taken for the production and mobilization of the stimulus. Colysanthin raises this threshold level, perhaps by making the apex less sensitive to the inducer, the amount of colysanthin arriving at the apex determining the amount by which the threshold level is raised.

An effect of vernalization appears to trigger a reaction at the apex which causes the destruction or inactivation of colysanthin, thereby lowering the threshold level. This vernalization effect increases with time, and reaches a maximum at about 4 weeks.

By increasing the length of the photoperiod, the overall rate of production of the leaf-generated floral stimulus in the leaves is increased, and thus the predetermined threshold level is attained sooner.

APPENDIX 1.

To demonstrate the method whereby the tests of significance for the graft experiment (table 3.3) were calculated.

The formula used was:-
$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S.S_1 + S.S_2}{n_1 + n_2 - 2}}} \times \sqrt{\frac{n_1 \times n_2}{n_1 + n_2}}$$

where \bar{x}_1 and \bar{x}_2 are the means,

SS_1 and SS_2 are the sums of squares,

and n_1 and n_2 are the numbers in each sample.

e.g. S.D.
$$\begin{array}{l} \text{GU} \\ \text{GU} \end{array} \bar{x} = 23.13, \quad SS = 2.9, \quad n = 8.$$

$$\begin{array}{l} \text{GV} \\ \text{GU} \end{array} \bar{x} = 20.67, \quad SS = 12.0, \quad n = 18.$$

$$\begin{aligned} t &= \frac{23.13 - 20.67}{\sqrt{\frac{2.9 + 12.0}{18 + 8 - 2}}} \times \sqrt{\frac{18 \times 8}{18 + 8}} \\ &= \frac{2.46}{\sqrt{\frac{14.9}{24}}} \times \sqrt{\frac{144}{26}} \\ &= 2.46 \times \sqrt{\frac{144 \times 24}{14.9 \times 26}} \\ &= 2.46 \sqrt{8.9210} \\ &= 2.46 \times 2.99 \\ &= 7.36 \end{aligned}$$

$$\text{number of degrees of freedom} = 18 + 8 - 2 = 24.$$

$$P < 0.001.$$

x from Fisher R.A. - Statistical Methods for Research Workers (10th ed.)
(Oliver and Boyd (1946)). p.122.

APPENDIX 2.

Full analysis of variance for Experiment 3.2
Table A.1.

	L.D.					S.D.					V. Totals		
	+ cots.					- cots.							
	n	Σx	Σx^2	n	Σx	Σx^2	n	Σx	Σx^2	n	Σx	Σx^2	
V ₀	20	338	5730	18	269	4027	20	487	11875	14	300	6440	28072
V ₁	20	334	5594	12	156	2034	20	480	11548	18	364	7382	26558
V ₂	20	316	4998	14	191	2617	19	423	9447	11	203	3749	20811
V ₃	20	295	4363	2	27	365	20	422	8926	1	18	324	13978
V ₄	19	274	3960	6	75	939	18	362	7304	7	130	2416	14619
Total	99	1557	24645	52	718	9982	97	2174	49100	51	1015	20311	104038

Figures are for:- (1) the number of plants scored in each treatment (n); (2) the sum of the values of the flowering node obtained in each treatment (Σx); (3) the sum of the squares of the values of the flowering node obtained in each treatment (Σx^2).

2. Combine the figures for cotyledon treatment - Table A2, for photo-period treatment-Table A3, and for vernalization treatment - Table A4.

Table A2

	L.D.		S.D.	
	n	Σx	n	Σx
V ₀	38	607	34	787
V ₁	32	490	38	844
V ₂	34	507	30	626
V ₃	22	322	21	440
V ₄	25	349	25	492

Table A3

	L.+cots.		S.cots.	
	n	Σx	n	Σx
V ₀	40	825	32	569
V ₁	40	814	30	520
V ₂	39	739	25	394
V ₃	40	717	3	45
V ₄	37	636	13	205

Table A4

	L.D.		S.D.	
	n	Σx	n	Σx
+ cots.	99	1557	97	2174
- cots.	52	718	51	1015

3. Combine the figures for cotyledon treatment and vernalization treatment - Table A5; for photoperiod and vernalization treatment - Table A6; and for photoperiod treatment and cotyledon treatment - Table A7.

Table A5

L.D.		S.D.	
n	Σx	n	Σx
151	2275	148	3189

Table A6

+cots.		-cots.	
n	Σx	n	Σx
196	3731	103	1733

Table A7

V_0		V_1		V_2		V_3		V_4	
n	Σx	n	Σx	n	Σx	n	Σx	n	Σx
72	1394	70	1334	64	1133	43	762	50	841

It can be seen that for all tables, the total sum of $n = 299$, and the total sum of $x = 5464$.

The correction factor (C.F) for all calculations is therefore:-

$$\frac{(5464)^2}{299} = 99850.49$$

Total S.S. (sum of squares) - from Table A1.

$$\begin{aligned}
 &= 104038 - \frac{(5464)^2}{299} \\
 &= 104038 - 99850.49 \\
 &= 4187.51.
 \end{aligned}$$

degrees of freedom = 298.

--oOo--

S.S. for P - from Table A5.

$$\begin{aligned}
 &= \frac{(2275)^2}{151} + \frac{(3189)^2}{148} - \text{C.F.} \\
 &= 34275.66 + 68714.33 - 99850.49 \\
 &= 3139.50
 \end{aligned}$$

degrees of freedom = 1.

--oOo--

S.S. C - from Table A6.

$$\begin{aligned}
 &= \frac{(3731)^2}{196} + \frac{(1733)^2}{103} - \text{C.F.} \\
 &= 71022.25 + 29158.15 - 99850.49 \\
 &= 329.91
 \end{aligned}$$

degrees of freedom = 1.

--oOo--

S.S. V - from Table A7.

$$\begin{aligned}
 &= \frac{(1394)^2}{72} + \frac{(1334)^2}{70} + \frac{(1133)^2}{64} + \frac{(762)^2}{43} + \frac{(841)^2}{50} - \text{C.F.} \\
 &= 26989.39 + 25422.23 + 20057.64 + 13503.35 + 14145.62 - \\
 &\quad - 99850.49. \\
 &= 267.74.
 \end{aligned}$$

degrees of freedom = 4.

--oOo--

S.S. PC - from Table A4.

$$\begin{aligned}
&= \frac{(1557)^2}{99} + \frac{(718)^2}{52} + \frac{(2174)^2}{97} + \frac{(1015)^2}{51} - \text{C.F.} - (\text{S.S. P} + \text{S.S. C}) \\
&= 24487.36 + 9913.92 + 48724.49 + 20200.49 - 99850.49 - \\
&\quad - (\text{S.S. P} + \text{S.S. C}). \\
&= 3475.77 - (3139.50 + 329.91) \\
&= 6.36
\end{aligned}$$

degrees of freedom = 1.

---000---

S.S. PV - from Table A2.

$$\begin{aligned}
&= \frac{(607)^2}{38} + \frac{(490)^2}{32} + \frac{(507)^2}{34} + \frac{(322)^2}{22} + \frac{(349)^2}{25} + \frac{(787)^2}{34} \\
&\quad + \frac{(844)^2}{38} + \frac{(626)^2}{30} + \frac{(440)^2}{21} + \frac{(492)^2}{25} - \text{C.F.} - (\text{S.S. P} + \text{SS. V}) \\
&= 9696.03 + 7503.13 + 7560.26 + 4712.91 + 4872.04 + 18216.74 \\
&\quad + 18745.68 + 13062.53 + 9219.05 + 9682.56 - 99850.49 - \\
&\quad (\text{S.S. P} + \text{S.S. V}). \\
&= 3420.44 - (3139.50 + 267.74) \\
&= 13.20
\end{aligned}$$

degrees of freedom = 4.

---000---

S.S. VC - from Table A3.

$$\begin{aligned}
&= \frac{(825)^2}{40} + \frac{(814)^2}{40} + \frac{(739)^2}{39} + \frac{(717)^2}{40} + \frac{(636)^2}{37} + \frac{(569)^2}{32} \\
&\quad + \frac{(520)^2}{30} + \frac{(394)^2}{25} + \frac{(45)^2}{3} + \frac{(205)^2}{13} - \text{C.F.} - (\text{SS V} + \text{SS C}). \\
&= 17015.63 + 16564.90 + 14003.10 + 12852.23 + 10932.32 + \\
&\quad 10117.53 + 9013.33 + 6209.44 + 675.00 + 3232.60 - 99850.49 - \\
&\quad (\text{S.S. V} + \text{S.S. C}). \\
&= 765.68 - (267.74 + 329.91) \\
&= 168.03
\end{aligned}$$

degrees of freedom = 4.

---000---

S.S. PVC - from Table A1.

$$\begin{aligned}
 & \dots + \\
 & = \frac{(338)^2}{20} + \frac{(334)^2}{20} + \frac{(130)^2}{7} - \text{C.F.} - \left\{ \text{SS P} + \text{SS V} + \text{SS C} \right\} \\
 & \quad \left\{ + \text{SS PV} + \text{SS PC} + \text{SS VC} \right\} \\
 & = 103795.48 - 99850.49 - (\text{S.S.}^2) \\
 & = 3944.99 - (3139.50 + 329.91 + 267.74 + 6.36 + 13.20 + 168.03) \\
 & = 20.25
 \end{aligned}$$

degrees of freedom = 4.

---000---

$$\text{Total S.S.} = 4187.51$$

$$\begin{aligned} \text{Sum of 1st order and 3rd order} \\ \text{interactions} \end{aligned} = 3944.99$$

$$\begin{aligned} \text{Error S.S.} & = 242.52 && \text{This figure agrees} \\ & && \text{with the figure} \\ & && \text{obtained from Table A1. if each} \\ & && \text{individual treatment was summed} \\ & && \text{for :- } \sum x^2 - \frac{\sum^2 x}{n} \end{aligned}$$

$$\text{Total degrees of freedom} = 291-1 = 298$$

$$\text{Sum 1st order, 2nd order and 3rd order} = 19$$

$$\text{Error of freedom} = 279$$

---000---

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