THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (PYRUS MALUS L.) SEEDS

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ABSTRACT

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (PYRUS MALUS L.) SEEDS

By

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The main goal of this research was to contribute to an understanding of dormancy. Abscisic acid (ABA) was identified by gas-liquid chromatography and mass spectrometry in extracts of methylene chloride that has been partitioned against acidic methanolic extracts from dormant apple seeds of 'McIntosh'. Both 'free' and hydrolysable ('bound') ABA were found in the water diffusate from the seeds, and in the methanolic extracts of seed coats, cotyledons and embryonic axes. The concentration of 'free' ABA was highest in the embryonic axis, intermediate in the seed coat, and least in the cotyledons.

Levels of both 'free' and 'bound' ABA of seeds declined during stratification at both 5° and 20°C. Increasing the temperature to 25°C for one week after 3 weeks at 5°C induced secondary dormancy, and nullified the effect of prior chilling on germination. This treatment also caused a marked reduction in 'free' and 'bound' ABA in the seeds within 2 days.

During seed development within the fruit, two maxima occurred in the level of 'free' ABA in the embryo, one in July-August coincident with high germination capacity (62%) of excised embryos; the other at maturity in late September, when excised embryo failed to germinate.

However, germination capcity had declined to nil 2 weeks prior to rise in the ABA content. Levels of 'bound' ABA remained relatively low until the approach of maturity and then rose slightly. Hand defoliation of branch units on July 30 - when embryos were nearly full size - reduced the concentration of both 'free' and 'bound' ABA in embryonic axes of seeds collected 5 weeks later, and favored the germination of excised embryos. Lesser effects of defoliation were noted at maturity 11 weeks after treatment, and germination of excised embryos occurred regardless of treatment.

Drying the seeds on removal from the fruit increased their content (nanograms per gram fresh weight) of both 'free' and 'bound' ABA, the magnitude of the effect varying with time of harvest.

Application of succinic acid-2,2-dimethylhydrazide to growing fruit lowered the ABA content of the embryo at harvest and reduced the germination capacity of the seeds slightly. Inclusion of inhibitors of gibberellin synthesis in the stratification and/or germination medium had no effect on stratification requirement of intact seed, provided the concentration used were not toxic.

The results of these studies do not support the view that entrance to and release from dormancy in response to chilling are controlled by endogenous levels of ABA.

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (*PYRUS MALUS* L.) SEEDS

By

Orlando Balboa Zavala

A DISSERTATION

Submitted to
Michigan State University
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DEDICATED TO

MY WIFE, DAUGHTER, AND SON

ACKNOWLEDGMENT

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Guidance Committee:

The Paper-Format was adopted for this dissertation in accordance with Departmental and University regulations. The dissertation body was separated into two sections and one appendix. The first section and the appendix were prepared for publication in HortScience. The second was styled for publication in Physiologia Plantarum.

ABBREVIATIONS

The following abbreviations will be used in this dissertation. Names given in parentheses are additional common or trade names.

ABA	abscisic acid
Alar	succinic acid-2,2-dimethylhydrazide (SADH, B-9, B-995)
AMO-1618	4-hydroxy-5-isopropyl-2-methylphenyltrimethylammonium chloride 1-piperidine carboxylate
BA	N^6-benzyladenine
CCC	(2-chloroethyl) trimethyl ammonium chloride (Cycocel, chlormequat)
GA	denotes the series of gibberellins -- use of a subscript denotes a specific gibberellin, as GA_1
GLC	gas-liquid chromatography
GC-MS	combined gas-liquid chromatography and mass spectrometry
IAA	indoleacetic acid
Zeatin	6-(4-hydroxy-3-methyl-2-butenylamino) purine

INTRODUCTION

INTRODUCTION

The rest period of seeds of temperate woody perennials allows these plants to endure low temperature stress. Knowledge of the mechanism(s) underlying this phenomenon may lead to methods for more rapid seed germination, and the production of fruit crops in regions of insufficient chilling. The main goal of this research was to contribute to an understanding of dormancy.

Apple seeds were chosen for this study because of their availability and the considerable body of existing information on this species. Abscisic acid (ABA) and its glucose ester have been identified in apple seeds and ABA is known to inhibit their germination. Furthermore, recent work has suggested that abscisic acid (ABA), an endogenous growth inhibitor present in a wide range of higher plants, is responsible for the rest period, and that chilling reduces the level of ABA in seed tissue, allowing germination to proceed. One of the advantages in studying ABA is that it can be measured by gas-liquid chromatography without recourse to bioassay.

This study was designed to do the following: (a) to measure the levels and distribution of ABA in apple seeds during their entrance into dormancy and the breaking of dormancy by chilling; (b) to determine the effect of temperature on the decline of ABA during stratification; and (c) to determine the effect of raising the temperature prematurely during chilling, and thereby inducing dormancy, on the level of ABA. Preliminary experiments were conducted with inhibitors of gibberellin synthesis to test the role of gibberellin synthesis in the breaking of dormancy.

LITERATURE REVIEW

LITERATURE REVIEW

Introduction

This review will be concerned primarily with the literature relating to dormancy in apple and pear seeds. The seeds of many trees display some degree of dormancy (Kramer and Kozlowski, 1960), which can be either advantageous or disadvantageous. For the nurseryman it is disadvantageous, however, it may also be an advantage because the nurseryman can keep or store the seed source. However, seed dormancy is advantageous where survival is threatened by adverse conditions.

Dormancy is usually separated into rest (also called constitutive dormancy, primary dormancy, innate dormancy, internal dormancy or endogenous dormancy) and quiescence (also called imposed dormancy, external dormancy, exogenous dormancy or false dormancy).

Definitions:

The following definitions will apply in this dessertation:

Rest is defined as lack of germination capacity of the seed under adequate environmental conditions such as temperature, moisture, light, etc., and quiescence as dormancy imposed by the unfavorable levels of any of these factors. Dormancy is defined as suspended growth of the seed and will include either rest or quiescence. Stratification will refer to the storage of the seeds in a moist environment, regardless of temperature, and chilling to stratification at temperatures between 0 to 10°C. The chilling requirement delays germination until after winter, when conditions again become favorable for growth (Kozlowski, 1971). In hot, dry climates, dormancy permits survival during periods of moisture stress. Thus, dormancy serves a very useful function in nature (Wareing, 1963).

Exogenous Control of Dormancy in Apple and Pear Seeds

The seeds of many Rosaceous species exhibit rest at harvest and must undergo changes, usually called after-ripening, before germination can occur. Seeds of most temperate zone *Pyrus* species which enter a state of dormancy can be made to germinate by continuous exposure to low temperature (1 - 10°C) in a moist medium for at least 6 to 12 weeks. These conditions resemble those which the seeds encounter in nature. If chilling is insufficient, dwarfed and stunted plants are produced (Flemion, 1934).

Light appears to play a minor role in breaking the dormancy of apple seeds, although red light promotes the germination of isolated embryos of dormant seed (Smolenska and Lewak, 1971). Oxygen, on the other hand, may not be necessary, since embryos germinated more rapidly when the rest was broken by N_2 than by chilling (Tissaoui and Come, 1973).

Secondary dormancy, which is as deep or deeper than primary dormancy, may be induced in seeds partially after-ripened by exposure to low temperature and then transferred to temperatures of 25 to 30°C. Abbott (1955) showed that transferring excised embryos of apple to temperatures of 25 to 28°C after 3 weeks at 3°C induced such a secondary dormancy. The critical temperature lies between 18 and 21°C, higher temperature reducing and lower temperature increasing the germination of the seeds (Visser, 1956b).

Exogenous application of several growth promoters stimulates the germination of excised embryos and partially after-ripened seeds of apple and pear. Badizadegan and Carlson (1967) reported that BA alone stimulated germination of excised embryos of dormant apple seeds. Kaminski and Pieniazek (1968) also reported that BA, GA_3, and GA_{4+7}, singly or jointly, greatly enhanced the germination of dormant or partially stratified embryos. Lewak _et al_ (1970) showed that BA, IAA, and GA_3 stimulated the germination of excised embryos, the effect being light independent. The stimulating effect of GA included in the stratification medium varies directly with the concentration and inversely with the duration of cold stratification (Come and Durand, 1970). Westwood and Bjornstad (1968) observed an increase in germination of pear seeds when GA_3 was included in the stratification medium, but not when seeds were treated after stratification.

ABA counteracts the effects of GA_3, GA_{4+7} or BA on both dormant and partially stratified apple embryos (Rudnicki _et al_., 1971). In both dormant and nondormant pear embryos ABA inhibits the incorporation of ^{32}P into several RNA fractions (Khan and Heit, 1969). GA is more effective than BA in counteracting this inhibition, although the pattern of labeling of RNA varies depending on the promoter used.

Endogenous Control of Dormancy in Apple and Pear Seeds

A classification of types of dormancy was proposed by Crocker

(1916). He attributed dormancy to: (a) immaturity of the embryo; (b) impermeability of the seed coat to water, (c) mechanical resistance of the seed coat to embryo growth, (d) low permeability of the seed coat to gases; (e) dormancy resulting from a metabolic block within the embryo itself, (f) a combination of the above, or (g) secondary dormancy. Kozlowski (1971) reclassified these into five groups: (a) impermeable seed coat, (b) mechanically resistant seed coat; (c) immature embryo, (d) rudimentary embryo; (e) morphologically mature but physiologically dormant embryo.

Impermeable seed coat. When the seed coat is removed from dormant apple seeds, some of the embryos germinate but the seedlings are dwarfed (Flemion, 1934). The effects of low temperature stratification appear to be cumulative, since partially stratified seeds give rise to semidwarfed seedlings, whereas fully stratified seeds give rise to normal plants (Flemion, 1934). Visser (1954, 1956b) found that the seed coverings are barriers, not to water, but to oxygen uptake, since in partially stratified apple seeds, the seed coverings, particularly the endosperm, reduced gas exchange to and from the embryo. Removal of a small portion of the endosperm increased the respiratory activity 3-fold. According to Visser, the barrier is more pronounced at higher temperatures. Visser's work was substantiated by the observation of Nikolaeva and Knape (1974) that the seed coat decreased oxygen absorption by 90% and the endosperm alone by 70% in apple seeds, regardless of the state of dormancy. However, Tissaoui and Come (1973) were able to break rest by holding apple embryos in pure nitrogen and concluded that oxygen was not required. From the above, one can conclude that the seed coat in apple is permeable to water but may not be readily permeable to oxygen. However, if oxygen is not required to break rest and if permeability to oxygen does not change during chilling, then one must look elsewhere for the controlling factor in dormancy.

Mechanically resistant seed coat. Removal of the seed coat of non-stratified apple seeds permits a low rate of germination (up to 20%) (Flemion, 1934, Smolenska and Lewak, 1971) suggesting that mechanical resistance of the seed coat may be involved in dormancy. However, radicle elongation is slow and the major site of dormancy must lie within the embryo. Thus the seed coat apparently acts as a physical barrier to the germination of the dormant embryo. Once embryo dormancy has been overcome by chilling, the radicle is capable of penetrating the seed coat and germination occurs.

Rudimentary embryo. *Pyrus* embryos are fully developed and mature and stratification does not result in any gross morphogical change.

Immature embryo or morphogically mature but physiologically dormant embryo. The differences between anatomical and physiological immaturity are subtle. However, several species have embryos which are well differentiated when the seed is dispersed, but which must be imbibed in water to permit further growth before germination can occur (Villiers, 1972). This might be considered as either the final step in seed development or the initial stage of germination. Pyrus seeds typify physiological dormancy, for chilling affects only the ability to germinate, rather than morphological changes.

The Mechanism of the Effect of Chilling on Apple and Pear Seeds.

Several hypotheses have been proposed to explain embryo dormancy, some involving the activity of enzymes, others synthesis and/or degradation of hormones.

Enzymes. Activity of enzymes such as peroxidase, succinic dehydrogenase, lipase and proteases increased during cold stratification of excised embryos of apple, var. Antonowka, but remained unchanged in non-chilled embryos (Nikolaeva and Yankelevich, 1974). 'Free' and 'bound' peroxidase increased during cold stratification in both intact seeds and isolated embryos of apple (Rychter and Szpakowics, 1974), while phosphatase showed two peaks of activity (Rychter et al., 1971). When isolated embryos were held in darkness at 25°C after different periods of low-temperature stratification, GA_3 and GA_7 stimulated phosphatase activity between 10 and 50 days and GA_4 after 30 days of after-ripening. ABA inhibited phosphatase activity but had little effect on its appearance. Changes in enzyme activity during cold stratification may be a result, however, rather than the cause of the breaking of dormancy.

Growth promoters. Several gibberellins have been reported to occur in immature Pyrus seeds, including GA_4 and GA_7 in apple (Dennis and Nitsch, 1966) and GA_{45} in pear (Bearder et al., 1975). GA_4, GA_7 and

GA_9 have been identified in mature apple seeds (Sinska et al., 1973). The participation of these compounds in seed dormancy is far from clear however. GA_4 and GA_7 are present in negligible amounts in dormant seeds (Sinska and Lewak, 1970). However, GA_4 increased 40,000-fold after 30 days of cold stratification only to fall to its initial level before the seeds were capable of germination, while GA_7 remained low during the entire process of stratification (Sinska and Lewak, 1970, 1973). Thus, the observed increase in GA_4 may or may not be a prerequisite for germination. GA_4 also increases when isolated embryos are exposed to red light; in this case a correlation was reported between the increase and germination (Smolenska and Lewak, 1971). Excised embryos of stratified seeds incorporated ^{14}C-mevalonate into GA_4 and into GA_7 if seeds were allowed to dry (Sinska and Lewak, 1974).

Letham and Williams (1969) identified 3 cytokinins in the flesh and seeds of apple fruitlets. One resembled zeatin, a second zeatin riboside and the third zeatin ribotide. Dorman apple seeds also contain 'free' and 'bound' cytokinins with the bound forms reaching the higher levels after 5 weeks of cold stratification. (Borkowska and Rudnicki, 1974).

Luckwill (1948) found a promoter of tomato ovary enlargement in extracts of immature apple seeds but only traces in dry seeds. Following alkaline hydrolysis, or after stratification at 5°C, the presence of an auxin (called auxin 2) could be demonstrated (Luckwill, 1957). Kawase (1958) also reported an increase in a promoter of *Avena* coleoptile elongation in the seed coat, endosperm and embryo of apple seeds during after-ripening.

Growth inhibitors. A marked decrease in endogenous growth inhibitors has been observed during cold stratification of both apple (Luckwill, 1952; Kawase, 1958; Rudnicki, 1969) and pear seeds (Strausz, 1970). Luckwill (1952) reported that dormant seeds contained an inhibitor of *Avena* coleoptile section elongation which declined during after-ripening. The endosperm contained the highest concentration of the inhibitor while the testa and embryo contained progressively less, in the ratio 30:13:1. Later Luckwill (1957) reported the presence of two inhibitors in immature apple seeds. Inhibitor 1 was shown to be toxic and therefore not biologically important. On the other hand, inhibitor 2 was active over a wide concentration range and reached the highest level at stage 3 of seed development (seed fully mature). In the seed coat of dormant apple seed, Kawase (1958) found a strong inhibitor of *Avena* coleoptile elongation which declined to its lowest level during stratification at 5°C.

Phloridzin, a phenolic compound, is abundant in apple tissues (Hutchinson *et al.*, 1959), and retards root growth of apple seedlings in water culture at a concentration of 10^{-4}M (Borner, 1959). Woodcock (1947) isolated phloridzin from apple seeds, and found that it decreased from 8% of the fresh weight to 1% within 8 weeks after petal fall and after 18 weeks it completely disappeared from the seed. However, Pieniazek and Grochowska (1967) demonstrated that phloridzin, phloretin

and chlorogenic acid were the main phenolic compounds present in dormant apple seeds. Phloridzin was at its highest level in the seed coat and dropped to negligible amounts during stratification, while increasing in the embryo. Phloretin remained unchanged in the seed coat during after-ripening, while embryos showed only traces of phloretin after 11 weeks of cold stratification. Chlorogenic acid, on the other hand, completely disappeared from the seed coat and endosperm during stratification regardless of temperature. Phloridzin alone had no effect on germination of apple embryos but when applied with GA and/or BA greatly enhanced the germination capacity of dormant and nondormant embryos (Kaminski and Pieniazek, 1968).

Come and Mittard (1970) reported a negative correlation between percentage germination of seeds stratified within the fruit and the phloridzin content of the seed coat following UV irradiation. Both phloridzin and chlorogenic acid were labile when exposed to UV light. They concluded that these compounds absorb oxygen and thereby reduce its availablity to the embryo.

Rudnicki (1969) identified ABA in dormant apple seeds, and reported that all inhibitory activity disappeared from the water diffusate after 3 weeks of chilling.

In pear seed, Strausz (1970) found a significantly higher concentration of an inhibitor tentatively identified as ABA in the seed surface and seed coat than in the embryo. During chilling, the concentration decreased in the embryo but remained unchanged in the seed coat. Although Okhawa (1974) was not able to identify ABA in immature pear seeds, G. C. Martin and F. G. Dennis (personal communication) have recently done so.

As noted above, GA_4 increased markedly during cold stratification of apple seeds. Inclusion of ABA in the stratification medium completely prevented this increase, and the GA_4 which had accumulated during after-ripening disappeared when ABA was added to the incubating medium at 20°C (Rudnicki et al., 1972). According to these workers, ABA may inhibit GA_4 synthesis and/or promote its breakdown.

Interaction of promoters and inhibitors. Endogenous growth promoters appear to be at their lowest levels in dormant apple seeds (Sinska and Lewak, 1970; Smolenska and Lewak, 1971; Borkowska and Rudnicki, 1974) and progressively increase during chilling, while the reverse is true for ABA (Rudnicki, 1969). These factors together with the results of application of growth regulators to dormant and nondormant seeds suggest an interaction of promoters and inhibitors in controlling seed dormancy. Khan (1971) proposed that germination in some seeds is controlled by interaction between cytokinins, gibberellins and inhibitors with cytokinins playing a permissive role. According to this hypothesis, germination requires GA. However, inhibitors such as ABA can block the action of GA, and cytokinins are necessary to counteract this inhibition. This hypothesis does not hold for apple seeds, since both cytokinins and gibberellins stimulate germination of excised apple embryos (Kaminski and Pieniazek, 1968; Badizadegan and Carlson, 1967), and ABA counteracts the effects of both. Although endogenous ABA decreases dramatically during 3 weeks of cold stratification (Rudnicki, 1969) and at the same time, cytokinins and gibberellins are reaching their highest levels (Sinska and Lewak, 1970; Borkowska and Rudnicki, 1974) apple seeds are incapable of germination at this time.

Metabolism of ABA during chilling. Several workers have reported a decline in the level of ABA or ABA-like inhibitors during cold stratification (Sondheimer *et al.*, 1968; Williams *et al.*, 1973; Rudnicki, 1969; Strausz, 1970), although the same decline occurred during warm stratification in hazel (*Corylus avellana* L.) seeds (Williams *et al.*, 1973). The question thus arises as to the fate of ABA during stratification. Sondheimer *et al* (1974) reported the appearance of ^{14}C-labelled phaseic acid, dihydrophaseic acid, and an unidentified compound in dormant and nondormant ash seed (*Fraxinus americana* L.) after feeding 2-^{14}C-ABA. They suggested that ABA is metabolized to phaseic acid and this in turn is converted to dihydrophaseic acid. They supported this by isolating the labelled phaseic acid and showing that it was converted to dihydrophaseic acid. However, CO_2 was the only identified product of 1-^{14}C-ABA metabolism in apple seed during stratification at 5°C (Rudnicki and Czapski, 1974). These authors suggested that other metabolites could have been formed and remained undetected. One such product may be abscisyl-D-glucoside, reported by Milborrow (1970) to be formed in several tissues, and identified in apple seed by Bulard *et al* (1974)

Summary

The failure of freshly harvested *Pyrus* seeds to germinate is caused primarily by the dormancy of the embryo, which may be overcome by chilling.

Raising the temperature to 25°C or more after dormancy is broken completely negates the effect of previous chilling. Light has little effect on dormancy. Both gibberellins and cytokinins are effective in promoting the germination of excised embryos, particularly when dormancy has been partially broken by chilling. ABA, on the other hand, inhibits the germination of nondormant embryos and counteracts the effect of promoters on dormant embryos.

The available evidence suggests that dormancy of apple and pear seeds is associated with low levels of gibberellins and cytokinins and high levels of ABA. Chilling apparently reduces the content of ABA and promotes the synthesis or release of promoters, as well as certain hydrolytic enzymes.

Several aspects of the role of ABA remain to be established, viz.: (a) its distribution within the seed; (b) its role in the induction of both primary and secondary dormancy; and (c) the effect of temperature on its disappearance during stratification.

SECTION ONE

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (*Pyrus malus* L.) SEEDS. I. ROLE OF ABSCISIC ACID DURING THE ONSET OF DORMANCY

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (*Pyrus malus* L.) SEEDS. I. ROLE OF ABSCISIC ACID DURING THE ONSET OF DORMANCY

Abstract. Abscisic acid (ABA) levels were measured during the growth and development of apple embryos of the cultivar 'McIntosh', using gas-liquid chromatography. Two maxima in 'free' ABA were observed; in mid-August and in late September. The relatively high rate of germination of excised embryos was noted when the first maximum was observed; and there was negligible germination of the embryos when the second maximum was reached. A similar lack of correlation between germination capacity and ABA content was noted in a second cultivar. Branch defoliation in late July, when embryos had reached 75% of their final fresh weight, reduced the ABA content of embryonic axes and favored the germination of embryos sampled 5 weeks after treatment, but did not prevent the induction of dormancy. Drying seeds on removal from the fruit markedly increased the content of both 'free' and 'bound' ABA in the embryonic axes of immature seeds, and in most portions of mature seeds. I conclude that the level of ABA is not the primary factor controlling embryo dormancy during apple seed development.

Luckwill (5) reported the presence of two growth inhibitors in immature apple seeds, but did not measure quantitative changes in their concentration during seed development. Rudnicki (9) identified ABA in mature apple seeds and observed that the level of a diffusible

inhibitor of wheat coleoptile elongation, assumed to be ABA, declined during stratification at 2 - 4°C. I have subsequently shown that the ABA content of the seed decline during stratification, but this decline is temperature-independent, suggesting that ABA concentration does not play a primary role in the breaking of dormancy by chilling (1). A question remains as to its role in the induction of dormancy. Several workers have observed an increase of ABA content during the late stage of seed development such as in peach (2) and in a dormant ground-nut cultivar (10). The primary purpose of this investigation was to determine if ABA levels in the developing seeds are correlated with their germination capacity. Desiccation is known to reduce germination capacity of hazel seeds (8) and other species (3) and to increase the ABA content of several plant tissues (4, 7). Work of Mielke and Dennis (6) on the level of ABA in sour cherry primordia suggested that defoliation might reduce the ABA content of seed tissues and thereby affect their dormancy. I therefore tested the effect of defoliation in late summer on the accumulation of ABA in the seeds.

Samples of fruits, cv. 'McIntosh', were collected from mature trees in a commercial orchard at Leslie, Michigan, those of 'Golden Delicious' from an abandoned orchard at East Lansing, Michigan. Branches approximately 10 cm in diameter at the base were used for defoliation studies, leaves being removed July 30, 1974, when the embryos had reached approximately 75% of their final fresh weight. The seeds were removed within two hours of harvest, 150 to 300 seeds being collected for each treatment. Two replicates of 25

(defoliation and drying studies) or 100 seeds (maturation experiment) were used for extraction of ABA experiment, and 4 samples of 25 seeds each were tested for germination in petri dishes containing moist sand at 20°C and 200 foot-candles of fluorescent light. Two samples were tested with seed coat intact, 2 with seed coat removed (excised embryos). The protrusion of the radicle through the seed coat (intact seed) or geotropic bending (excised embryos) was used as criterion for germination.

Seed retained for ABA determination were dissected into seed coat, cotyledons and embryonic axes immediately on removal from the fruit (maturation and defoliation studies) or after 4 months of storage over anhydrous $CaCl_2$ at 20°C (drying experiment). The seed portions were immersed in absolute methanol, ground in a mortar and pestle and left overnight at 4°C. The supernatant was evaporated under vacuum at 40°C, redissolved in 25 ml of phosphate buffer, fractionated and the crude acidic ('free') and base hydrolyzable ('bound') ABA were methylated and injected in the GC using an electron capture detector as described previously (1).

Effect of seed development on ABA content and germination. 'Free' ABA content of McIntosh embryos (cotyledons plus embryonic axes) rose gradually from July 13 to August 17, dropped in mid-August through mid-September, then rose again at fruit maturity in late September (Fig. 1). Level of 'bound' ABA rose reaching the highest level in early August fell gradually then remained constant until early September. The level then rose slightly at maturity, paralleling the sharp increase

in'free'ABA. Seeds with intact testa failed to germinate regardless of the time of sampling. Germination of excised embryos was negligible on the first sampling date, even though the embryos had reached almost 90% of the final fresh weight. Germination reached a maximum of 62% on August 3, declined to a plateau then fell sharply to nil as maturity approached. There appear to be no relationship between ABA content and the germination capacity of excised embryos until the approach of fruit maturity in late September, when the former rose as the latter fell. However, the decline in germination capacity occurred 2 weeks prior to the rise in the ABA content.

The content of 'free' ABA in 'Golden Delicious' seeds dropped to a level of 1/3 to 1/8 the original content between July 30 and September 5, depending on the tissue sampled (Table 1). Decreases were also apparent in 'bound' ABA levels, except in the embryonic axes, which contained twice as much in September as in July. Between September and October the content of 'free' ABA rose in the seed coat and embryonic axes but fell in the cotyledons. The level of 'bound' ABA paralleled that of 'free' ABA in the seed coat, but declined rather than increase in the embryonic axis. No change occurred in the cotyledons during this period.

Again, no germination occurred in intact seeds. Percentage germination of excised embryos declined steadily from 28% on July 30 to 6% and 0% on September 5 and October 20, respectively. Comparing the ABA content of the embryos only (cotyledons and embryonic axis) with the percentage of germination did not reveal any apparent

Fig. 1. 'Free' and 'bound' ABA during development of apple seeds cv. 'McIntosh', in relation to their germination capacity. Values are means for 2 replicate samples of 100 seeds each.

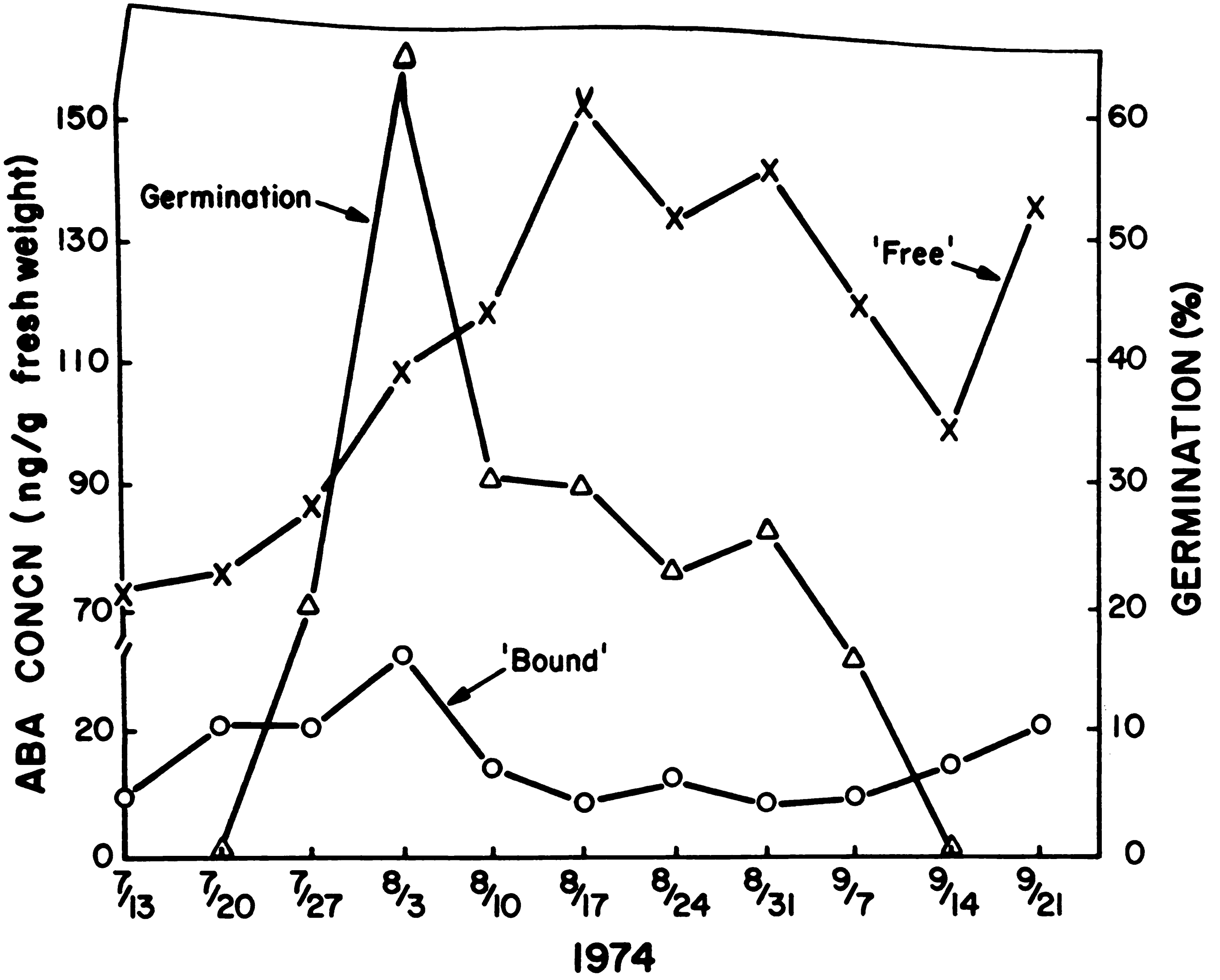
ABA CONCN (ng/g fresh weight)
150
130
110
90
70
20
0
GERMINATION (%)
60
50
40
30
20
10
0
Germination
'Free'
'Bound'
7/13
7/20
7/27
8/3
8/10
8/17
8/24
8/31
9/7
9/14
9/21
1974

Fig. I

Table 1. Effect of defoliation on July 30 on the level of 'free' (F) and 'bound' (H) ABA (ng/g fresh wt.) in apple seed cv. 'Golden Delicious'. Mean for 2 samples of 25 seeds each.

	Collection time (1974)					
	7/30		9/5		10/20	
Tissues	F	H	F	H	F	H
Non-defoliated						
Seed coat	321	109	112	219	459	786
Cotyledons	373	64	44	17	23	23
Embryonic axis	1333	298	298	269	577	485
Defoliated						
Seed coat	-	-	153	171	200	473
Cotyledons	-	-	47	18	46	43
Embryonic axis	-	-	143	194	378	533

correlation between the two.

Effect of defoliation. The most noticeable effect of defoliation was the marked reduction in 'free' ABA in the embryonic axis in September 5 (Table 1). Smaller reduction in the 'bound' ABA were noted in the axes in September but not in October. At maturity, the seed coat contained less and the cotyledons more ABA than did the control. Defoliation increased the germination of excised embryos sampled on September 5 (28% vs. 6% for the control), but no germination took place in embryos sampled on October 20 regardless of treatment. Germination capacity was inversely correlated with ABA content as shown above in the September sample only. Stratification did not affect the germination capacity of the seed sampled on October 20 (96% vs. 90% in the control) after 12 weeks at 5 ±2°C.

Effect of drying seed. Drying increased both 'free' and 'bound' ABA in the embryonic axes of immature seeds (seeds taken 8/24). Although it increased 'bound' ABA in the seed coat and cotyledons (Table 2), it reduced their 'free' ABA content slightly. Similar treatment of mature seed (seed taken 9/21) did not affect 'free' ABA in the embryonic axis, but 'free' and 'bound' ABA were increased 1- to 30-fold in all other cases. No study of the effect of stratification on seed germination was carried out.

In general ABA content does not parallel germination capacity in these comparisons. In 'McIntosh', a relatively early ripening cultivar, no correlation was observed until just prior to harvest. Even then,

Table 2. Effect of drying, on the concentration of 'free' (F) and 'bound' (H) ABA (ng/g fresh wt.) in apple seed, var. 'McIntosh'. Means for two samples of 25 seeds each.

	Sampling date, 1974			
	8/24		9/21	
Tissues	F	H	F	H
		Fresh Seeds		
Seed coat	218	131	936	593
Cotyledon	126	8	120	7
Embryonic axis	409	128	1283	750
		Seed stored 4 months over $CaCl_2$		
Seed coat	189	401	1521	1414
Cotyledon	84	77	671	218
Embryonic axis	1260	1502	1221	2478

'bound' ABA levels paralleled those of 'free' ABA, suggesting that the glucoside was not converted to the 'free' ABA during the seed development. In 'Golden Delicious', a later-ripening cultivar, ABA content was high in July, low in September except for 'bound' ABA in the embryonic axis, and rose slightly in October, yet the germination declined steadily during this period.

Assuming that ABA is synthesized in the leaves and translocated to the seed, defoliation should reduce the content of ABA in the seed. This was true for embryonic axis sampled 5 weeks after defoliation and the germination of excised embryos surpassed that of embryos from control branches. However, no germination occurred in mature embryos regardless of treatment. Drying seed on removal from the fruit generally increased their ABA content. The data obtained do not, in general, support the hypothesis that the inability of apple seed embryos to germinate as maturation proceeds is the result of the accumulation of ABA in the tissues.

LITERATURE CITED

1. Balboa, O., and F. G. Dennis, Jr. 1975. The role of abscisic acid in the dormancy of apple (Pyrus malus L.) seeds. I. Abscisic acid levels during stratification. Physiol. Plant (in preparation).

2. Bausher, M. G., and R. H. Biggs. 1974. Relation to abscisic acid and cytokinin content to developing peach seeds. HortScience 9:296.

3. Crocker, W. 1938. Life-span of seeds. Bot. Rev. 4:235-274.

4. Hiron, R. W. P., and S. T. C. Wright. 1973. Role of endogenous abscisic acid in the response of plants to stress. J. Exp. Bot. 24:769-781.

5. Luckwill, L. C. 1957. Studies of fruit development in relation to plant hormones. IV. Acidic auxins and growth-inhibitors in leaves and fruit of the apple. J. Hort. Sci. 32:18-33.

6. Mielke, E. A., and F. G. Dennis, Jr. 1975. Hormonal control of flower bud dormancy in sour cherry (Prunus cerasus L.) III. Effect of leaves, defoliation and temperature on levels of abscisic acid. Physiol. Plant (in press).

7. Milborrow, B. V., and D. R. Robinson. 1973. Factors affecting the biosynthesis of abscisic acid. J. Exp. Bot. 24:537-548.

8. Ross, J. D., and J. W. Bradbeer. 1971. Studies in seed dormancy. V. The content of endogenous gibberellins in seed in Corylus avellana L. Planta 100:288-302.

9. Rudnicki, R. 1969. Studies on abscisic acid in apple seeds. Planta 86:62-68.

10. Sreeramulu, N., and I. M. Rao. 1971. Physiological studies on dormancy in seed ground-nut (Arachis hypogeae L.) III. Changes in auxin and growth inhibitors content during development of the seeds of a dormant and non-dormant cultivars. Austr. J. Bot. 19:273-280.

SECTION TWO

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (*Pyrus malus* L.) SEEDS II. ABSCISIC ACID LEVELS DURING STRATIFICATION

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (Pyrus malus L.)
II. ABSCISIC ACID LEVELS DURING STRATIFICATION

ABSTRACT

Gas-liquid chromatography (GLC) and mass spectrometry (GC-MS) were used to identify abscisic acid (ABA) in extracts of dormant apple (*Pyrus malus* L.) seeds, and levels of both 'free' and base-hydrolyzable ABA during stratification were quantified by GLC. The concentration of ABA was greatest in the embryonic axis, intermediate in the seed coat, and least in the cotyledons. ABA content, expressed on a whole seed basis, declined to 20 to 25 per cent of the initial level after 7 weeks of stratification, regardless of whether the seeds were held at 5° or 20°C. Induction of secondary dormancy by high temperature was associated with rapid loss of both 'free' and 'bound' ABA. The physiological significance of these observations is discussed.

INTRODUCTION

Many seeds require cold stratification before being able to germinate, and this effect has been attributed to a reduction in the levels of endogenous growth inhibitors, such as, abscisic acid (ABA). ABA or ABA-like compounds have been reported to decline during low temperature stratification of seeds of apple (*Pyrus malus* L.) (Rudnicki, 1969), ash (*Franxinus americana* L.) (Sondheimer *et al.*, 1968), plum (*Prunus*

domestica cv. Italian) (Lin and Boe, 1972), Scots pine (Pinus sylvestris L.) (Kopcewicz and Porazinski, 1973), peach (Prunus persica), (Lipe and Crane, 1966) and hazel (Corylus avellana L.) (Williams et al., 1973). However, only in hazel seeds it has been reported a decline of 60% in ABA level during stratification at both 5 and 20°C, although germination occurred only after chilling.

If during the early phases of cold stratification, apple seeds are transferred to temperatures of 25°C or higher, they usually enter into secondary dormancy (Abbott, 1955; Visser, 1956). To break this dormancy, the seeds require the same amount of cold stratification as non-after-ripened seeds.

Rudnicki (1969) identified ABA in ethanol extract of apple seeds, and used the wheat coleoptile elongation assay to measure the amount of inhibitory activity in water diffusate of whole seeds following various periods of low temperature stratification. Rudnicki and Czapski (1974) followed the degradation of 1-^{14}C-ABA in apple seeds during stratification and determined its distribution in the various tissues. Recently, Bulard et al (1974) have identified the glucose ester of ABA in apple seeds, with retention time of 7.28 min in 1% OVI at 200°C for ABA and 10 min for the β-D-glucopyranose at 180°C.

MATERIALS AND METHODS

Plant material. Apple seeds, cv. 'McIntosh' and 'Red Delicious' were removed from fruit collected in a commercial orchard near

East Lansing, Michigan in 1972, air-dried and stored over anhydrous $CaCl_2$ at room temperature until used. Dry seeds were stratified in darkness beginning 2 months after collection, in moist sand at either 5 ±2° or 20 ±2°C. Four samples of 25 seeds each were removed at every 3 week interval, 2 for extraction and ABA determination, and 2 for testing germination at 20° ±1°C, the protrusion of the radicle through the seed coat was considered as an index of germination.

Extraction and fractionation procedure. Two replicates of 25 dormant, dry seeds, or seed removed from the stratification medium were shaken with 25 ml of distilled water at 5°C for seven days. The water was changed daily, and pooled to give the fraction subsequently called the diffusate. The seeds were then dissected into seed coat (endosperm plus testa), cotyledons, and embryonic axis. The tissues were ground in absolute methanol with a mortar and pestle and extracted overnight at 2°C. The methanolic extract evaporated to dryness and the residue redissolved in 25 ml of phosphate buffer, pH 7.3. This was processed to give an acidic fraction ('free' ABA) and a base-hydrolyzable fraction ('bound' ABA) (Fig. 1). Fractionation of extracts of both stratified and nonstratified seeds labeled with $2\text{-}^{14}C$-ABA resulted in 80% of the radioactivity being recovered in the acidic fraction, and 3% in the bound fraction. For identification of ABA, 100 grams of whole dry seed were ground in absolute methanol and processed as in Fig. 1.

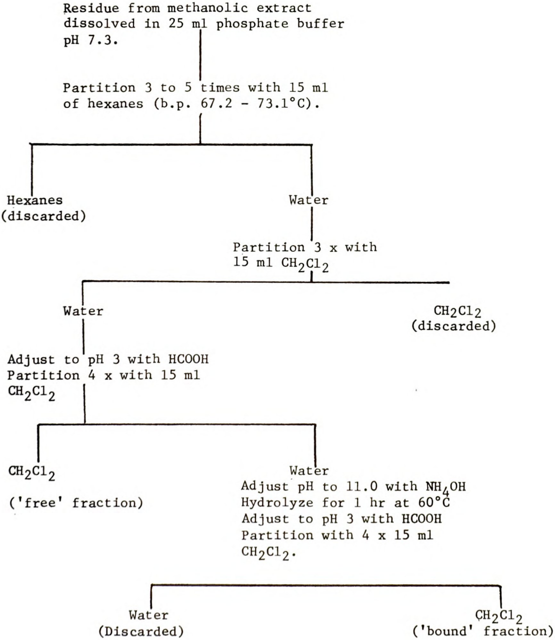

Fig. 1. Flow diagram of procedure used for preparation of fractions containing 'free' and 'bound' ABA.

Gas chromatography. The residues from these two fractions were resuspended in 1 ml of diethyl ether/methanol (9:1 v/v) and methylated with diazomethane following the method of Schlenk and Gellerman (1960) as modified by Powell (1964). The ether-methanol solution was evaporated, the residue was dissolved in ethyl acetate, and aliquots were injected, without further purification into a Packard 7300 gas-liquid chromatograph equipped with an electron capture detector (^{65}Ni foil) and operated either at 5 or 7.5 volts. The column used (2 mm i.d. x 1.83 m) was packed with 3% SE30 (methyl silicone) on 80/100 mesh Gaschrom Q (acid washed, dimethyl chlorosilane). The column temperature was 180° and inlet and detector temperature were 260 and 270°C, respectively. The carrier gas was nitrogen at a flow rate of 40 ml/min at 40 psig. Nitrogen scavenger gas was supplied to the detector at 70 ml/min. The retention time for ABA was 1.5 min. A standard curve based upon peak height was used for quantitative determinations.

Gas chromatography - mass spectrometry. The methylated 'free' acid fraction from 100 grams of dormant seed without further purification were subjected to combined gas-liquid chromatography-mass spectrometry (GC-MS) using an LKB GC-MS, interphased with a POP 8/I computer. The main spectra were determined at 70 eV. For GC a glass column, i.d. 2 mm 6 ft long of 3% SP 2100 (methyl silicone) on supelcoport (acid, washed silane treated diatomite 100/120 mesh) was used with a helium flow rate of 20 ml/min. The column temperature was 200°C and 290°C for the detector. The retention time for ABA was 8 min.

Secondary dormancy. Apple seeds, cv. 'McIntosh', were stratified at 5 ±2°C for 3 weeks. One group of seeds was subsequently transferred to 27 ±1°C for one week, then returned to 5° ±2°C for an additional 12 weeks. The remaining were held continuously at 5 ±2°C. Four samples of 25 seeds each were removed at interval for determination of the germination capacity (both treatments) and ABA content (high temperature only), as described above. Seeds held continuously at 5°C were not analyzed for ABA.

RESULTS

Identification of ABA. GLC traces of methylated 'free' and 'bound' fractions both contained a peak that co-chromatographed with authentic cis,trans-ABA. When nonmethylated samples of the 'free' and 'bound' fractions were injected, no peak was observed. When either methylated extracts or methylated cis-trans ABA were exposed to ultra-violet light (365 nm) for 6 hr, the peak height was markedly reduced. On GC-MS the retention time for both synthetic and for presumed ABA in the extract was ca 8 min. Mass spectrometry showed a molecular ion (M^+) at 278, and a base peak at 190. Major ions with respective intensities as a percent, the base peaks were as follows: c, t-ABA-278 (12), 260 (37), 205 (6), 190 (100), 162 (53), 134 (53), 125 (50), 91 (31), unknown - 278 (11), 260 (31), 205 (5), 190 (100), 162 (33), 134 (34), 125 (31), 91 (17). c, t-ABA was only identified in the 'free' fraction.

Some samples, mostly the 'bound' fraction, showed peaks resembling

t,t-ABA in retention time on GC. However, no attempt was made to identify these compounds.

Effect of temperature on germination and ABA content. Approximately 6 weeks of chilling at 5 ±2°C was necessary for 50% seed germination and 94% germination was obtained after 7 weeks at 5 ±2C. No germination occurred in seed held continuously at 20°C.

The concentration of ABA in the embryonic axis of nonstratified 'McIntosh' seeds was 100 times that found in the cotyledons (Table 1), and 70-fold that in the seed coat (10,250, 120; and 150 ng/g fresh weight). Similar relationships held in the 'Red Delicious' cv. (data not shown). Eighty six to 90% of the 'free' acid was lost from the embryonic axis during 7 weeks at 5°C and similar losses of 'bound' ABA occurred (Table 1). A 30 to 40% loss of 'free' ABA occurred in the seed coat and 36% loss in the cotyledons. Temperature did not markedly affect these losses.

The ABA content of the water diffusate varied considerably among treatments. The highest level of 'free' ABA in the diffusate was observed following stratification at 5 ±2°C for 7 weeks, the concentration being 5 times that found in the diffusate from stratified seeds for 3 weeks. On the other hand, the diffusate from seeds held at 20°C contained less 'free' ABA after stratification. In the case of 'bound' ABA in the diffusate, no change occurred at 20°C, but a 70% decrease occurred at 5°C. Parallel data were obtained with 'Red Delicious' seeds with the exception noted below (data not shown).

Table 1. Effect of stratification time and temperature upon ABA content of 'McIntosh' apple seeds and the water diffusate from the seeds. Means for 2 replicates of 25 seeds each.

Treatment	Diffusate	Seed coat	Cotyledon	Embryonic axis
	Free ABA (ng/g fresh wt.)			
Dormant seeds	400	150	120	10,250
5° for 7 weeks	130	87	75	1,440
20 for 7 weeks	250	170	43	955
	Bound ABA (ng/g fresh wt.)			
Dormant seeds	200	150	35	8,500
5° for 7 weeks	60	130	60	27
20° for 7 weeks	200	82	39	480
	Free ABA (ng/seed)			
Dormant seeds	155	2	3	6
5° for 7 weeks	5	1.1	1.9	0.8
20° for 7 weeks	10.1	2.4	1.1	0.5
	Bound ABA (ng/seeds)			
Dormant seeds	8	1.8	0.9	4.9
5° for 7 weeks	2.2	1.7	1.5	0.1
20° for 7 weeks	5.1	1.1	1.0	0.2

Figure 2. Concentration of 'free' and 'bound' ABA in the water diffusate and in methanol extracts of seed coat cotyledons, and embryonic axis of apple seeds during stratification at 5 and 20°C. Each bar represents the mean of at least 4 replicates of 25 seeds each. Per cent of germination at 20°C is indicated in parenthesis above each treatment.

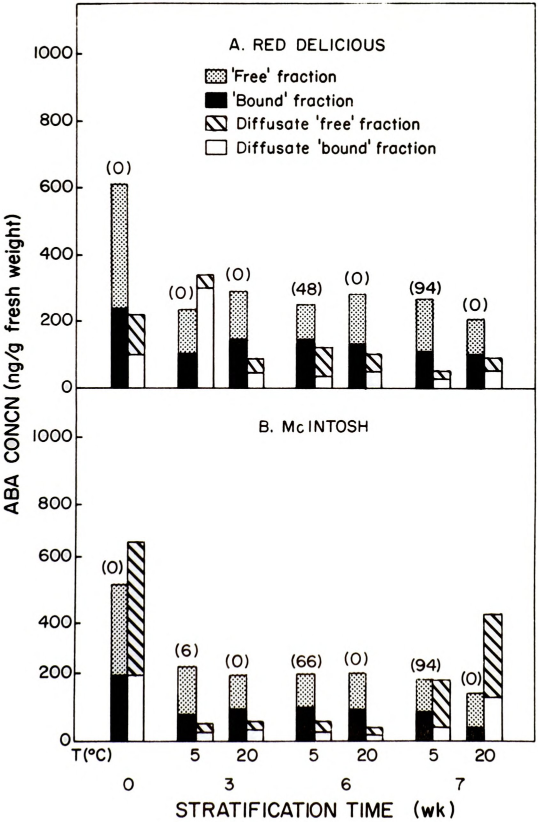
A. RED DELICIOUS
'Free' fraction
'Bound' fraction
Diffusate 'free' fraction
Diffusate 'bound' fraction
(0)
(0)
(0)
(48)
(0)
(94)
(0)
B. McINTOSH
(0)
(6)
(0)
(66)
(0)
(94)
(0)
ABA CONCN (ng/g fresh weight)
0
200
400
600
800
1000
T(°C)
5
20
5
20
5
20
0
3
6
7
STRATIFICATION TIME (wk)

Fig. 2

Figure 3. Effect of inducing secondary dormancy with high temperature upon total ABA content of apple seeds. A. ABA content of seed during one week at 27 ±1°C after 3 weeks at 5°C. B. ABA content of seeds following their return to 5 ±2°C following the treatment in A. In B, 0 represents the day the seeds were returned to 5° as well as the day on which stratification of the control, continuous chilling began. Each value is the mean of at least 4 replicates of 25 seeds each. 'Bound' ABA (o______o); 'free' ABA (x ____x); germination under continuous chilling (x - - - x); germination of seeds returned to 5°C after 1 week at 27 ±1°C (o - , - o).

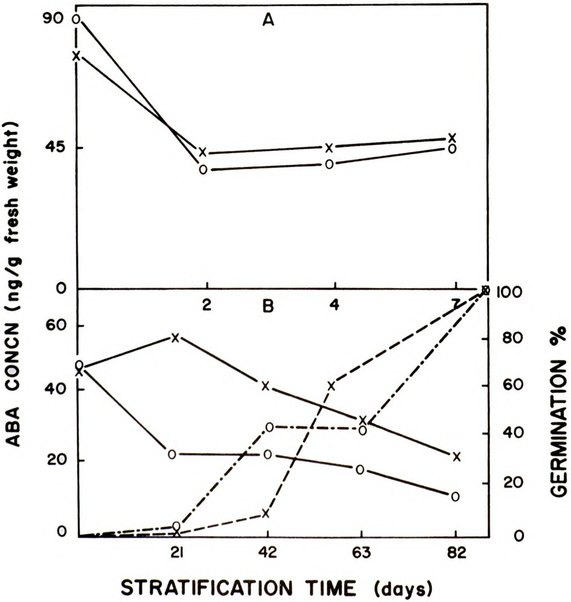
A
B
ABA CONCN (ng/g fresh weight)
90
45
0
2
4
7
60
40
20
0
21
42
63
82
100
80
60
40
20
0
GERMINATION %
STRATIFICATION TIME (days)

Fig. 3

The concentration of ABA (ng/g fresh weight), including that in the diffusate declined markedly within the first 3 weeks, with smaller variations thereafter (Fig. 2). Again, no consistent effect of temperature was evident. In contrast with the result obtained with 'McIntosh', diffusate from 'Red Delicious' seed contained less ABA after stratification, regardless of temperature.

Effect of induction of secondary dormancy. Contrary to expectation both 'free' and 'bound' ABA declined markedly 2 days after transferring seeds to 27 ±1°C after 3 weeks at 5°C (Fig. 3A). The levels changed little during the remainder of the week at 27 ±1°C. On return to 5 ±2°C, 'free' ABA rose to an intermediate level and then declined while 'bound' ABA dropped to a lower level and then remained relatively constant (Fig. 3B). Germination of seeds in which secondary dormancy had been induced paralleled that of the controls, indicating that raising the temperature after 3 weeks had indeed eliminated the effect of prior stratification at 5 ±2°C.

DISCUSSION

ABA was identified in a methanol extract of dormant seeds, confirming previous work (Rudnicki, 1969).

The concentration of 'free' ABA was highest in the embryonic axis, and lowest in the seed coat. Levels declined from 150, 120 and 10,250 ng/g f.w. in seed coat, cotyledons and embryonic axis, respectively, at 0 weeks, to 87, 75 and 1,440 ng/g after 7 weeks of chilling. Therefore, the decline in the embryonic axis was approximately 1/10 the original level and that in the seed coat and cotyledon to a little over 1/2 the original levels. Levels of 'bound' ABA declined in a parallel fashion suggesting that the loss of 'free' ABA was not the result of conjugation. Changes in the concentration of 'free' ABA in the water diffusate were inconsistent. After stratification, higher levels were found in the diffusate from 'McIntosh' seed held at 5°C lower levels in that from seeds held at 20°C. In the case of 'Delicious' seeds, however, less ABA was present in the diffusate after 7 weeks of stratification, regardless of the temperature. This inconsistency is surprising in view of Rudnicki's (1969) observation that the inhibitor content of similar diffusate declined markedly during low temperature stratification.

The decline in ABA is temperature-independent, yet chilling is required to break dormancy. Hence, the changes in germination capacity of the seeds cannot be ascribed to changes in ABA content alone.

Furthermore, 1.5 x 10^{-5}M ABA is required to completely inhibit germination of stratified apple embryos (Rudnicki, 1969), while our data show that endogenous ABA level in fully dormant embryos is approximately 6 x 10^{-7}M assuming a 20% loss during fractionation. Hence, Jacobs (1959) third rule (exact substitution) does not hold for the inhibition of apple seed germination by ABA.

ABA levels fell during the induction of secondary dormancy by high temperature after 3 weeks at 5 ±2°C. Therefore, induction of secondary dormancy cannot be attributed to changes in ABA level.

If ABA plays a role in the dormancy of apple seeds, it may antagonize the germination-promoting effect of hormones synthesized only during chilling (Sinska and Lewak, 1970; Borkowska and Rudnicki, 1974). These may be essential for the synthesis of promoters while ABA removal is temperature independent.

REFERENCES

Abbott, D. L. 1955. Temperature and dormancy of apple seeds. --Proc. XIV Int. Hort. Congress pp. 746-753.

Borkowska, B. & Rudnicki, R. 1974. Changes in the cytokinin level in stratified apple seeds. --Proc. XIX Int. Hort. Congress, Warsaw p. 496.

Bulard, C., P. Barthe, G. Garello and M. T. LePage-Degivry. 1974. Mise en evidence d'acide abscisique lie au β-D-glucopyranose dans les embryons dormants de *Pyrus malus* L. --C. R. Acad Sci. Ser. D 278:2145-2148.

Jacobs, W. P. 1959. What substance normally controls a given biological process? I. Formulation of some rules. --Developmental Biol. 1:527-533.

Kopcewicz, T. & Porazinski, Z. 1974. Influence of low temperature on germination and endogenous regulation in Scots pine (*Pinus sylvestris* L.) --Acta Soc. Bot. Pol. XLII 233-240.

Lin, C. F. & Boe, A. A. 1972. Effect of some endogenous and exogenous growth regulators on plum seed dormancy. --J. Amer. Soc. Hort. Sci. 97:41-44.

Lipe, W. N. & Crane, J. C. 1966. Dormancy regulation in peach seeds. --Science 153:541-542.

Powell, L. E. 1964. Preparation of indole extracts from plants for gas chromatography and spectrophotofluorometry. --Plant Physiol. 39:836-842.

Rudnicki, R. 1969. Study of abscisic acid in apple seeds. --Planta 86:63-68.

________ & Czapski, J. 1974. The uptake and degradation of 1-^{14}C-abscisic acid by apple seed during stratification. --Ann. Bot. 38:189-192.

Schlenk, H. & Gellerman, J. D. 1960. Esterification of fatty acids with diazomethane on a small scale. --Anal. Chem. 32:1412-1414.

Seeley, S. D. 1971. Electron capture gas chromatography of plant hormones with special reference to abscisic acid in apple bud dormancy. --Ph.D. Thesis, Cornell University, 128 pp.

Sinska, I. & Lewak, St. 1970. Apple seed gibberellins. --Physiol. Veg. 8:661-667.

Sondheimer, E., Tzou, D. S. & Galson, E. C. 1968. Abscisic acid levels and seed dormancy. --Plant Physiol. 43:1443-1447.

Visser, T. 1956. Some observations on respiration and secondary dormancy in apple seeds. --Proc. Kon. Ned. Akad. Wetensch C 59:314-324.

Williams, R. W., Ross, J. D. & Bradbeer, J. W. 1973. Studies in seed dormancy. VII. The abscisic acid content of the seed and the fruit of *Corylus avellana* L. --Planta 110:303-310.

APPENDIX

THE ROLE OF ABSCISIC ACID IN THE DORMANCY OF APPLE (*Pyrus malus* L.) SEEDS

III. EFFECT OF GROWTH RETARDANTS ON SEED DORMANCY AND ABA CONTENT

Abstract. Inclusion of AMO-1618 (4 hydroxy-5-isopropyl-2-methylphenyl-trimethylammonium chloride piperidine carboxylate) in the stratification medium did not inhibit the germination of intact apple (*Pyrus malus* L.) seeds or of excised embryos from partially stratified seeds. Spraying trees with SADH (succinic acid-2,2-dimethylhydrazide) at 1000 ppm reduced germination following stratification in some but not in all cases, and reduced the ABA content of embryonic axes but increased that of the cotyledons. These data suggest that the reduction in germination caused by SADH treatment are probably not an effect of increased concentration of endogenous ABA.

During low-temperature after-ripening of apple embryos GA_4 content increases, but falls to the original level before dormancy is broken (2). Red light promotes the germination of excised embryos of dormant seeds, as well as the production of endogenous gibberellin (4) while AMO-1618 inhibits both responses following red light treatment. Sinska and Lewak (3) also reported that AMO-1618 inhibits the synthesis of GA_4 in partially after-ripened apple embryos.

If the rise in GA_4 content observed by Sinska and Lewak (2) plays a role in the breaking of dormancy, prevention of the synthesis during low temperature stratification should reduce the germination capacity of the seed. I therefore tested the effect of AMO-1618 during stratification on the germination of intact apple seeds. AMO-1618 and CCC (2-chloroethyl)trimethyl ammonium chloride) were also tested on germination of embryo of partially stratified seed.

SADH in contrast to AMO-1618 and CCC does not inhibit GA synthesis in *Fusarium monoliforme* Sheld (2). However, SADH appears to inhibit some step(s) in the synthesis of GA precursors in peas (6) and reduce the germination of hazel seeds (3). I therefore compared seeds from sprayed vs. non-sprayed fruits as to their germination capacity and ABA content.

Samples of mature fruits were collected from a commercial orchard at Hartford and at Belding, Michigan. SADH was supplied at 500 or 1000 ppm in late July or early August, and the fruits were harvested in mid or late September. Seeds were removed within 24 hours of collection, or were left in the fruit during storage at 4 ±2°C for 5 months, depending on the experiment. For stratification seeds were held on moist sand for 12 weeks at 5 ±2°C. Germination was induced at 20 ±2°C for 10 days, and values were converted to Σ_{10} (5). ABA content was determined by dissecting seeds, previously soaked in water for 24 hours, and extracting seed coats, cotyledons, and embryonic axes with absolute methanol. The crude methylene chloride fraction ('free' ABA) and the base hydrolyzable fraction ('bound' ABA) were analyzed for ABA, using an electron capture gas chromatography as described previously (1). The leachate was also processed for ABA, and was called the diffusate.

Effect of the presence of growth retardants during stratification on germination. Stratification of seeds in water containing the growth retardants did not affect germination (94% germination in both seeds treated or not treated with the growth retardants).

Effect of tree sprays of SADH on germination and ABA. SADH treatment had no effect on the germination of excised embryos from fruit stored for 5 months at 4 ±2°C (Table 1). Germination of intact seeds was much reduced in comparison with that of embryos, and SADH possibly inhibited their germination in 'Red Delicious', but not in the other cultivars. The germination in 'Jonathan' seeds stratified in moist sand was also reduced by SADH treatment at the 6th and 9th week but not at the 12th week. SADH thus appears to have a small inhibitory effect on seed germination following stratification.

The ABA content of freshly harvested 'Jonathan' seeds was affected by SADH treatment, but the response varied with the seed tissue sampled (Table 2). Both 'free' and 'bound' ABA were higher in the cotyledon but lower in the embryonic axis of seed, from sprayed trees. The amount of 'free' ABA in the diffusate was increased by SADH treatment, but the 'bound' ABA was not appreciably affected.

Assuming that the embryonic axis is the locus of dormancy in these seeds, SADH appears to reduce ABA content concomitantly with germination capacity, suggesting that the two are not casually related.

Table 1. Effect of tree sprays of SADH on germination (Σ_{10}) of intact seeds (I) or excised embryos (R) of apple after chilling either in the fruit or in moist sand. Values are means for 2 samples of 25 seeds each.

		(Σ_{10})					
		Cultivar					
SADH	Time at 4-5°C	McIntosh		Jonathan		Delicious	
(ppm)	(wks)	R	I	R	I	R	I
				In the fruit at 4°C			
0	20	982	110	532	46	742	4
500	20	724	50	588	48	538	28
1000	20	744	12	572	22	526	0
				In moist sand at 5°C			
0	6				30		
	9				558		
	12				592		
1000	6				16		
	9				224		
	12				658		

Table 2. Effect of SADH treatment of trees on the ABA content of apple seed cv. 'Jonathan'. Values are means for 2 samples of 25 seeds each.

	ABA (ng/g Fresh wt.)			
Treatment	Cotyledons	Seed Coat	Embryonic axis	Diffusate
Alar, 1000 ppm				
Free ABA	84	113	214	451
Bound ABA	157	207	336	512
Control				
Free ABA	43	120	1298	121
Bound ABA	30	174	514	587

LITERATURE CITED

1. Balboa, O., and F. G. Dennis. 1975. The role of abscisic acid in the dormancy of apple (Pyrus malus L.) seeds. I. Abscisic acid levels during stratification. Physiol. Plant. (In preparation).

2. Ninnemann, H., J. A. D. Zeevart, H. Kende, and A. Lang. 1964. The plant growth retardant CCC as inhibitor of gibberellin biosynthesis in Fusarium monoliforme. Planta 61:229-235.

3. Ross, J. D., and J. W. Bradbeer. 1971. Studies in seed dormancy. VI. The effect of growth retardant on the gibberellin content and germination of chilled seeds of Corylus avellana L. Planta 100:303-308.

4. Sinska, I., and S. Lewak. 1970. Apple seed gibberellins. Physiol. Veg. 8:661-667.

5. __________, and __________. 1974. Gibberellin biosynthesis in apple seed dormancy. Proc. XIX Int. Hort. Congress, Warsaw. p. 493.

6. Smolenska, G. S., and S. Lewak. 1971. Gibberellins and the photosensitivity of isolated embryos from non-stratified apple seeds. Planta 99:144-153.

7. Timson, J. 1965. New method of recording germination data. Nature 207:216-217.

8. Wylie, A. W., R. Kyugo, and R. M. Sachs. 1970. Effects of growth retardants on biosynthesis of gibberellin precursors in root tips of peas, Pisum sativum L. J. Amer. Soc. Hort. Sci. 95: 627-630.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Abscisic acid was identified in a methanolic extract of dormant seeds by gas chromatography-mass spectrometry. Stratification of the seeds at 5 ±2°C produced the same decline of the ABA level in the 2 cultivars studied. Yet, only chilled seeds germinated. Induction of secondary dormancy by high temperature (27°C) was associated with rapid loss of both 'free' and 'bound' ABA. Thus, the induction of secondary dormancy cannot be ascribed to the rise in the abscisic acid in apple seeds.

The 'free' ABA content of seeds sampled during their development in the fruit showed two peaks, one in mid-August, the other at fruit maturity. 'Bound' ABA remained relatively constant, paralleling the increase of 'free' ABA at fruit maturity. Seeds with intact seed coat failed to germinate regardless of the time of sampling. Excised embryo germination was negligible in the first sampling date, reached a maximum of 62% early August, and then fell to nil as maturity approached. The decline in germination occurred 2 weeks prior to the rise of the ABA level. Thus, there appears to be no relationship between ABA content and germination of isolated embryos until the approach of fruit maturity in late September.

Branch defoliation of 'Golden Delicious' trees in late July affected markedly the 'free' ABA in the embryonic axis 5 weeks after defoliation. Smaller reduction of the 'bound' ABA were noted in the axis in both September and October samplings. Germination of excised embryos was inversely correlated with ABA level in the September sampling only. Chilling requirements of the seeds from defoliated branches was not affected in seeds sampled on October 20. Drying seed on removal from the fruit generally increased their ABA content. The data obtained do not support the hypothesis that the inability of embryos of apple seed to germinate as development proceeds is the result of accumulation of ABA in the seed tissues.

Seeds from fruit of trees sprayed with succinic acid-2,2-dimethylhydrazide showed less ABA content than those of the control. It also reduced the germination capacity of the seeds from 88% in the control to 54% in the seeds from fruit of trees sprayed, after 9 weeks of cold stratification suggesting that the two are not casually related.

In a preliminary experiment, AMO-1618 neither impaired the germination of intact seeds when it was included in the stratification medium nor did it affect the germination of isolated embryos of partially stratified seeds in the fruit.

BIBLIOGRAPHY

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Abbott, D. L. 1955. Temperature and dormancy of apple seeds. Proc. XIV Int. Congr. pp. 746-753.

Badizadegan, M. and R. F. Carlson. 1967. Effect of N^6-bezyladenine on seed germination and seedling growth of apple (*Malus sylvestris* Mill.). Proc. Amer. Soc. Hort. Sci. 91:1-7.

Balboa, O., and F. G. Dennis. 1975. The role of abscisic in the dormancy of apple (*Pyrus malus* L.) seeds. I. Abscisic acid levels during stratification. Physiol. Plant. (in preparation).

Bausher, M. G., and R. H. Biggs. 1974. Relation to abscisic acid and cytokinin content to developing peach seeds. HortScience 9:296.

Bearder, J. R., F. G. Dennis, J. MacMillan, G. C. Martin and B. O. Phinney. 1975. A new gibberellin (A_{45}) from seed of *Pyrus communis* L. Tetrahedron Letters 9:669-670.

Borkowska, B. and R. Rudnicki. 1975. Changes in the cytokinin level in stratified apple seeds. Proc. XIX Int. Hort. Congress. Warsaw p. 496.

Borner, H. 1959. The apple replant problem. I. The excretion of phloridzin from apple root residue. Contr. Boyce Thompson Inst. 20:39-56.

Bulard, C., P. Barthe, G. Garello, and M. T. LePage-Degivry. 1974. Mise en evidence d'acide abscisique lie auβ-D-glucopyronose dans les embryons dormants de *Pirus malus* L. C. R. Acad. Sci. Ser. D 278:2145-2148.

Come, D. and M. Durand. 1971. Influence de l'acide gibberellique sur la levee de dormance des embryons de pommier (*Pirus malus* L.) par la froid. C. R. Acad. Sci. Ser. D. 273:1937-1940.

________, and C. Mittard. 1970. Amelioration de la germination des grans de pommier (var. Golden Delicious) par irradiation ultravioletta. Physiol. Veg. 8:603-612.

Crocker, W. 1916. Mechanism of dormancy in seeds. Amer. J. Bot. 3:99.

________. 1938. Life-span of seeds. Bot. Rev. 4:235-274.

Dennis, F. G., Jr., and J. P. Nitsch. 1966. Identification of gibberellins A_4 and A_7 in immature apple seeds. Nature 211:781-782.

Flemion, F. 1934. Dwarf seedlings from non-after-ripened embryos of peach, apple and hawthorn. Contr. Boyce Thompson Inst. 8:286-293.

Hiron, R. W. P., and S. T. C. Wright. 1973. The role of endogenous abscisic acid in the response of plants to stress. J. Exp. Bot. 24:769-781.

Hutchinson, A., C. D. Taper and G. H. P. Towers. 1959. Studies of phloridzin in Malus. Can. J. Biochem. Physiol. 37:901-910.

Jacobs, W. P. 1959. What substances normally controls a given biological process? I. Formulation of some rules. Develop. Biol. 1:527-533.

Kaminski, W., and J. Pieniazek. 1968. The effect of different growth regulators on the germination of apple cultivar Antonovka dehusked seeds. Bull. Acad. Pol. Sci. (Ser. Sci. Biol.) 16:719-723.

Kawase, M. 1958. The after-ripening of dormant apple seeds in relation to auxin content. J. Hort. Assoc. Japan 27:256-264.

Khan, A. A., and C. E. Heit. 1969. Selective effect of hormones on nucleic acid metabolism during germination of pear embryos. Biochem. J. 113:707-712.

__________. 1971. Cytokinin: permissive role in germination. Science 171:853-859.

Kopcewicz, T. and Z. Porazinski. 1974. Influence of low temperature on germination and endogenous regulation in Scots pine (Pinus sylvestris L.) . Acta Soc. Bot. Pol. XLII:233-240.

Kozlowski, T. T. 1971. Growth and development of trees. I. Seed germination, ontogeny, and shoot growth. Academic Press, New York. 443 pp.

Kramer, P. J., and T. T. Kozlowski. 1960. Physiology of Trees. McGraw-Hill, New York.

Letham, D. S., and M. W. Williams. 1969. Regulators of cell division in plant tissues. VIII. The cytokinins of the apple fruit. Physiol. Plant 22:925-936.

Lewak, S., and G. Smolenska. 1968. Le systeme phytochrome dans la germination de semmences de pommier. Physiol. Veg. 6:403-406.

________, K. Bialek, and I. Sinska. 1970. Sensitivity of apple seed germination to light and some growth regulators. Biol. Plant. 12:291-296.

Lin, C. F., and A. A. Boe. 1972. Effect of some endogenous and exogenous growth regulators on plum seed dormancy. J. Amer. Soc. Hort. Sci. 97:41-44.

Lipe, W. N., and J. C. Crane. 1966. Dormancy regulation in peach seeds. Science 153:541-52.

Luckwill, L. C. 1948. The hormone content of the seed in relation to endosperm development and fruit drop in the apple. J. Hort. Sci. 24:32-44.

________. 1952. Growth-inhibiting and growth-promotor substances in relation to the dormancy and after-ripening of apple seeds. J. Hort. Sci. 27:53-65.

________. 1953. Studies of fruit development in relation to plant hormones. I. Hormone production by developing apple seed in relation to fruit drop. J. Hort. Sci. 28:14-24.

________. 1957. Studies of fruit development in relation to plant hormones IV. Acidic auxins and growth-inhibitors in leaves and fruits of the apple. J. Hort. Sci. 32:18-33.

________, P. Weaver, and J. MacMillan. 1969. Gibberellins and other growth hormones in apple seeds. J. Hort. Sci. 44:413-424.

Mielke, E. A., and F. G. Dennis. 1975. Hormonal control of flower bud dormancy in sour cherry (Prunus cerasus L.). III. Effect of leaves, defoliation and temperature on levels of abscisic acid. Physiol. Plant. (in press)

Milborrow, B. V. 1970. The metabolism of abscisic acid. J. Exp. Bot. 21:17-29.

________, and D. R. Robinson. 1973. Factors affecting the biosynthesis of abscisic acid. J. Exp. Bot. 24:537-548.

Nikolaeva, M. G., and D. A. Knape. 1974. Germination and gas exchange of apple seeds depending on stratification and degree of maturation. Proc. XIX Int. Hort. Congress, Warsaw. p. 497.

________, and B. B. Yankelevich. 1974. The changes in enzyme activity in apple seed during stratification and induction of secondary dormancy. Proc. XIX Int. Hort. Congress, Warsaw p. 502.

Ninneman, H., J. A. D. Zeevart, H. Kende, and A. Lang. 1964. The plant growth retardant CCC as inhibitor of gibberellin biosynthesis in *Fusarium monoliforme*. Planta 61:229-235.

Okhawa, M. 1974. Endogenous growth inhibitors associated with seed in Japanese pear fruit. Proc. XIX Int. Hort. Congress, Warsaw. p. 408.

Pieniazek, J., and R. Rudnicki. 1969. The inhibitory effect of endogenous abscisic acid (ABA) on after-ripening of apple seed in the fruit. Bull. Acad. Pol. Sci. 27:707-711.

________, and M. J. Grochowska. 1967. The role of the natural growth inhibitor (Abscissin II) in apple seed germination and the changes in the content of phenolic substances during stratification. Acta Soc. Bot. Pol. XXXVI:579-587.

Powell, L. E. 1964. Preparation of indole extracts from plants for gas chromatography and spectrophotofluorometry. Plant Physiol. 39:836-842.

Ross, J. D., and J. W. Bradbeer. 1971. Studies in seed dormancy. V The content of endogenous gibberellins in seed of *Corylus avellana* L. Planta 100:288-302.

________, and ________. 1971. Studies in seed dormancy. The effects of growth retardants on the gibberellin content and germination of chilled seeds of *Corylus avellana* L. Planta 100:303-308.

Rudnicki, R. 1969. Studies on abscisic acid in apple seeds. Planta 86:62-68.

________, W. Kaminski, and J. Pieniazek. 1971. The interaction of abscisic acid with growth stimulators in germination of partially after-ripened apple embryos. Biol. Plant. 39:836-842.

________, I Sinska, and S. Lewak. 1972. The influence of abscisic acid on the gibberellin content in apple seed during stratification. Biol. Plant. 14:325-329.

________, and J. Czapski. 1974. The uptake and degradation of 1-C^{14}-abscisic acid by apple seed during stratification. Ann. Bot. 38:189-192.

Rychter, A., R. Rudnicki, and S. Lewak. 1971. Regulation of acid phosphatase activity by abscisic acid and gibberellin in apple seed during stratification. Bull. Acad. Pol. Sci. 19:211-214.

________, and I. Szpakowicz. 1974. Free and bound peroxidase during apple seed stratification. Proc. XIX Int. Hort. Congress, Warsaw. p. 492.

Seeley, S. D. 1971. Electron capture gas chromatography of plant hormones with special reference to abscisic acid in apple bud dormancy. Ph.D. Thesis. Cornell University 128 pp.

Schlenk, H., and J. L. Gellerman. 1960. Esterfication of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-1414.

Sinska, I., and S. Lewak. 1970. Apple seeds gibberellins. Physiol. Veg. 8:661-667.

________, and ________, P. G. Gaskin, and J. Macmillan. 1973. Reinvestigation of apple-seed gibberellins. Planta 114:359-364.

________, and ________. 1974. Gibberellin biosynthesis in apple seed dormancy. Proc. XIX Int. Hort. Congress, Warsaw. p. 493.

Smolenska, G. S., and S. Lewak. 1971. Gibberellins and the photosensitivity of isolated embryos from non-stratified apple seeds. Planta 99:144-153.

Sondheimer, E., D. S. Tzou, and E. C. Galson. 1968. Abscisic acid levels and seed dormancy. Plant Physiol. 43:1443-1447.

________, E. C. Galson, E. Tinelli, and D. C. Walton. 1974. The metabolism of hormones during seed germination and dormancy. IV. The metabolism of (S)-2-^{14}C-abscisic acid in ash seed. Plant Physiol. 54:803-808.

Sreeramulu, N., and I. M. Rao. 1971. Physiological studies on dormancy in seed of ground-nut (Arachis hypogeae L.) III. Changes in auxin and growth inhibitors content during development of the seeds of a dormant and non-dormant cultivars. Aust. J. Bot. 19:273-280.

Strausz, S. D. 1970. A study of the physiology of dormancy in the genus <u>Pyrus</u>. Ph.D. Thesis. Oregon State University, Corvallis, Oregon.

Timson, J. 1965. New method of recording germination data. Nature 207:216-217.

Tissaoui, T. 1970. Importance de l'oxygene et de la position des embryons de pommier (<u>Pirus malus</u> L.) dans la germination et l'induction d'une dormance secondaire. C. R. Acad. Sci. Ser. D. 271:308-310.

________, and D. Come. 1973. Levee de la dormance de l'embryons de pommier (<u>Pirus malus</u> L.) en l'absence d'oxygene et de froid. Planta 111:315-322.

Villiers, T. A. 1972. Seed dormancy. In Seed Biology. T. T. Kozlowski, ed. Academic Press, New York. pp. 220-276.

Visser, T. 1954. After ripening and germination of apple seed in relation to seed-coats. Proc. Kon. Ned. Wetensch C 57:175-185.

________. 1956a. The role of the seed coat and temperature in after-ripening, germination and respiration of apple seed. Proc. Kon. Ned. Wetensch C 59:211-222.

________. 1956b. Some observations on respiration and secondary dormancy in apple seeds. Proc. Kon. Ned Wetensch C 59:314-324.

Wareing, P. F. 1963. The germination of seeds. Vistas Bot. 3:195-227.

Westwood, M. N., and H. D. Bjornstad. 1968. Chilling requirements of dormant seed of 14 pear species as related to their climatic adaptation. Proc. Amer. Soc. Hort. Sci. 92:141-149.

Williams, P. M., J. D. Ross, and J. W. Bradbeer. 1973. Studies in seed dormancy. VII. The abscisic acid content of the seed and fruit of Corylus avellana L. Planta 110:303-310.

Woodcock, D. 1947. Isolation of phloridzin from apple seeds. Nature 159:100.

Wylie, A. W., R. Kyugo, and R. M. Sachs. 1970. Effects of growth retardants on biosynthesis of gibberellin precursors in root tips of peas, Pisum sativum L. J. Amer. Soc. Hort. Sci. 95:627-630.