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Levels of Acidic Inhibitors in Peach Seeds
During Stratification

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ABSTRACT

LEVELS OF ACIDIC INHIBITORS IN PEACH SEEDS DURING STRATIFICATION

By

Melvin Keawe Wong

All fractions of an 80% aqueous methanol extract of dormant 'Halford' peach seeds were bioassayed for inhibitors and tested for the presence of naringenin and naringin. Except for the acid fraction only the butanol-soluble fraction had significant quantities of inhibitors and none of the fractions contained naringenin or naringin in quantities sufficient to cause significant inhibition.

One major inhibitory zone was noted on thin-layer chromatograms of the acid fraction of seed coats, cotyledons, and embryonic axes of 'Halford' and 'Okinawa' peach seeds. In 'Halford' seeds the inhibitor concentrations were approximately the same for seed coats and embryonic axes and about ten times less in the cotyledons. The inhibitor did not decrease during cold temperature stratification in the seed coats and cotyledons, but decreases were noted in the embryonic axes at both 3 and 20°C. Similar results were obtained using either 80% aqueous methanol or water as the extracting solvent.

The inhibitor had the same chromatographic properties as abscisic acid on silica gel thin-layer plates and paper. Spectropolarimetry, ultraviolet spectrophotometry, gas-liquid chromatography and gas-liquid chromatography-mass spectrometry all showed abscisic acid to be present in the acid fraction. Quantitative data from bioassay and gas-liquid chromatography indicated that abscisic acid was the compound responsible for most of the inhibition observed in this fraction.

Gas-liquid chromatography-mass spectrometry revealed that the largest peak occurring in eluates of the biologically active zone of thin-layer chromatograms could be a compound similar to abscisic acid with the addition of one oxygen molecule. This compound could be a precursor or a metabolite of abscisic acid.

The seed coat did not weaken structurally during cold temperature stratification.

LEVELS OF ACIDIC INHIBITORS IN PEACH SEEDS DURING STRATIFICATION

By

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A THESIS

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Dedicated

to

my beloved wife and parents

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INTRODUCTION

One of our main objectives is to be able to understand and regulate dormancy. Frost damage to fruit crops is one of the most serious problems of the Michigan fruit industry. Horticulturists attempting to solve the problem of frost damage soon find that little is known about the actual mechanism of dormancy. In this dissertation the physiology of the dormancy of peach (Prunus persica) seeds during stratification was studied with major emphasis on the relationship of abscisic acid to this process.

Seed dormancy is similar to bud dormancy in peach. Seeds were chosen for this study because of their availability and ease of manipulation. Peach seeds are sufficiently large to provide ample quantity of tissue for extraction and to separate easily into seed coats, cotyledons, and embryonic axes. Abscisic acid was investigated in relation to peach seed stratification for several reasons. Abscisic acid is now generally considered a growth regulator. It has been identified in peach seeds and it inhibits the germination of many seeds. A decrease in abscisic acid has been correlated with an increase in

germination of several species during stratification by several investigators.

The following objectives were the basis of this research: (1) to check the validity of the correlation between a decrease in abscisic acid and an increase in germination in peach seeds during stratification; and (2) to study the role of the seed coat in dormancy.

LITERATURE

Definitions

Dormancy is often separated into constitutive dormancy (also called rest, true dormancy, or endogenous dormancy) and exogenous dormancy (also called quiescence). Sussman and Halvorson (127) considered constitutive dormancy to be ". . . a condition in which development is delayed due to an innate property of the dormant state such as a barrier to the penetration of nutrients, a metabolic block, or the production of a self-inhibitor." Suspended growth due to some environmental factors is usually considered exogenous dormancy. In this dissertation dormancy is used in the sense of constitutive dormancy unless otherwise defined.

Stratification is defined by Janick (69) as the storage of seeds in a moist environment. But many horticulturists use the word stratification to mean storage of seeds in a moist and cold environment, and this is how it is used in this dissertation.

Germination is defined by Mayer and Poljakoff-Mayber (92) as ". . . that consecutive number of steps which causes a quiescent seed, with a low water content to show a rise in its general metabolic activity and to initiate the formation of a seedling from the embryo." Externally the end

of germination is usually considered to be the protrusion of some part of the embryo from the seed coat, although this is already considered a part of growth by some investigators (92). In this study peach seeds were considered to have germinated when the radicle protruded from the seed coat.

Bud Dormancy

Among several causes proposed to account for bud dormancy are photoperiod, temperature, and growth regulators. In several studies (40, 41) long photoperiod was found to enhance bud opening from dormant deciduous trees. Erez, et al. (41) recently found that light was obligatory for leaf bud opening in dormant peach shoots. Chilling has long been known to be necessary for the natural breaking of rest (115). But photoperiod and temperature are environmental factors that are not practical to regulate with fruit tree crops on a commercial basis.

Recently studies have turned toward growth regulators. Auxin was the first growth regulator proposed to control dormancy. In 1937 Avery, et al. (5) found an increase in auxin in apple buds at the commencement of growth. However, subsequent exogenous applications of auxin have not generally been effective in breaking dormancy (87).

Gibberellin, another acidic growth regulator, was shown to break dormancy in some species but not in others.

Donoho and Walker (33) and Walker and Donoho (134) were able to stimulate dormant Elberta peach buds into growth with gibberellic acid. With basswood (Tilia americana L.) Ashby (3) induced bud break with gibberellic acid. Similarly, Stuart (126) could induce dormant buds of Hydrangea macrophylla to grow with application of gibberellic acid. Brown, et al. (21) concluded that ". . . the gibberellin effect seen on pear buds was not one of breaking or reducing the rest influence but only one of growth stimulus which is expressed by the development of buds which have emerged at least partly from rest." Brian, et al. (20) delayed bud break in the spring by one to three weeks with gibberellic acid applied weekly between mid-August and late November in Acer pseudoplatanus, Betula verrucosa, Fagus sylvatica, Fraxinus excelsior and Sorbus aucuparia but these same treatments had little effect on Acer rubrum, Castania sativa, Parthenocissus tricuspidata, a Rhododendron x molle cultivar, Taxodium disticum, and Ulmus procera. With Prunus avium gibberellic acid had little effect on vegetative buds but caused complete dormancy or severely damaged flower buds. Similar autumn applications of gibberellin by Weaver (135) prolonged dormancy of Vitis vinifera shoots in the spring. Several investigators have shown that exogenous applications of gibberellic acid were able to overcome apical dominance (19, 67). There is, therefore, some evidence that exogenous applications of

gibberellin can break dormancy in certain species. On the other hand other investigators have shown no effect with exogenous gibberellins. Some of these negative results may have been the result of lack of penetration.

Several investigations have studied levels of endogenous gibberellin during chilling and the release from dormancy. Cajlahjan, et al. (23) detected more gibberellin-like substances and less naringenin in Prunus persica buds after rest. With Acer, Eagles and Wareing (36) correlated an increase in one gibberellin-like substance with the release from rest but failed to do so with another less polar gibberellin-like substance. A substantial increase in gibberellin-like activity just prior to the end of rest in buds of apricot (Prunus armeniaca 'Royal') was reported by Ramsay and Martin (109). Smith and Rappaport (122) showed an association between an increase in endogenous gibberellin-like substance and sprouting of potato tuber buds. The results were not clear as to whether this increase preceded or was a consequence of sprouting.

Cytokinins have also been tested for the breaking of bud dormancy. Weaver (136) was successful in breaking the rest of dormant Vitis vinifera buds with 6-benzyladenine. Pieniazek and Jankiewicz (103) and Benes, et al. (8) were similarly successful with apple buds using the same cytokinin. On the other hand, Weinberger (137) could

not stimulate dormant peach buds with cytokinins until their chilling requirement was nearly satisfied. Cytokinins have also been shown to counteract apical dominance in apple (140), Alaska peas (114), Scabiosa, Coleus, Helianthus annuus, and Helianthus tuberosus (114). Pieniazek and Jankiewicz (103) stimulated the development of apple collateral buds with benzyladenine. These buds are normally inhibited by the main bud. Another chemical used to break bud dormancy was ethylene chlorohydrin (60).

Levels of endogenous inhibitors were first related to dormancy of buds by Hemberg (59, 60, 63). He measured high levels of acidic inhibitors in Fraxinus bud scales in October and lower levels in February when buds started to grow on cuttings brought indoors. Work by Hemberg (60, 61, 62, 64) with potato buds also showed higher levels of acidic inhibitors in dormant buds than in non-dormant buds. Ethylene chlorohydrin treatment decreased the endogenous inhibitors. Since the work of Hemberg many investigators have reported a decline in inhibitors in buds as dormancy was broken (12, 34, 75). Burton (22), however, did not find a correlation between inhibitors and dormancy in potato buds.

Blommaert (12) found that the auxin level was low in peach buds during dormancy and increased during spring growth. The inhibitor level decreased during dormancy even though the level was still high at the start of spring

growth. Similar results were obtained by Dörffling (34) with Acer pseudoplatanus L. Kawase (75) demonstrated a decrease in inhibitory activity during rest in leaf buds of four woody species, Diospyros virginiana L., Malus sylvestris Mill., Prunus persica Batch var. 'Eclipse', and Ulmus americana L.

In a later study Blommaert (13) suggested that the inhibitors may have been one or more phenolic acids. The Rf of Blommaert's inhibitor was similar to that of the β inhibitor described by Bennet-Clark and Kefford (9). Although Hendershott and Walker (66) observed a close relationship in peach buds between the end of rest and the disappearance of the inhibitor, analysis of the data showed a rather high level of inhibitor up to and even at the end of rest. These same authors (65) identified a growth inhibitor in dormant peach flower buds as naringenin (5, 7, 4'-trihydroxyflavanone). El-Mansy and Walker (38) measured the flavanone content of 'Elberta' peach flower buds on a per bud basis during and after rest and concluded that flavanone content was much higher during rest than after the rest period was completed. Again, the level of flavanones in flower buds just before and just after rest was still high when compared with three out of four samples taken before rest. The sample dates leading up to the end of rest are of most interest in the study of dormancy release.

Smith and Kefford (120) cautioned that care must be taken to distinguish between effects upon dormancy release and spring bud burst. Phillips (100) found that naringenin antagonized competitively the breaking of peach bud dormancy by gibberellic acid. Dennis and Edgerton (32) could not find a correlation between inhibitory activity of peach bud extracts and the status of buds with regard to rest. Most of the inhibitor in buds was confined to the scales. Therefore, if data are expressed on a fresh weight basis while buds are expanding, an apparent decrease in inhibitor could be due primarily to dilution. Corgan (25) found that the naringenin levels in peach flower buds were high throughout the dormant season and remained high for more than 30 days after rest was terminated. Erez and Lavee (39) identified prunin (naringenin 7-glucoside) in dormant peach buds and found that the prunin levels on a per bud basis remained rather constant from December to March except for a slight decrease at the end of December. Pieniazek (102) identified phloridzin, another phenolic glucoside, in dormant leaf and flower buds of the apple variety 'Antonovka'. This was the only major inhibitor she was able to detect. She found no significant correlation between the content of phloridzin and the rest status of buds. No correlation between rest and inhibitor levels was found by Strausz (125) with three Pyrus types when corrections were made for growth. Only one inhibitor, which

was tentatively identified as abscisic acid, was noted in the buds, and the level of this inhibitor remained relatively stable throughout rest.

The onset of dormancy has been correlated by several investigators with short photoperiods and an increase of inhibitors (36, 74, 101). Phillips and Wareing (101) detected higher inhibitor levels in mature leaves and stem apices of sycamore (Acer pseudoplatanus) trees two to five days after they were transferred from long days to short days. This inhibitor increased during late summer and early autumn and was found to inhibit growth of sycamore tissue. Similar data were found by Eagles and Wareing (36) and Kawase (74) with Betula. The inhibitor in sycamore has since been identified as abscisic acid (27, 30). On the other hand Saunders and Lenton (116), using gas-liquid chromatography, found higher levels of abscisic acid in Betula plants grown under long days than in those grown under short days.

Seed Dormancy

Flemion demonstrated in the early 1930's that catalase, peroxidase and lipase activity progressively increased during stratification of Sorbus (42) and Rhodotypos (43). Furthermore, the moisture content, titrable acidity, soluble nitrogen, and sucrose increased and the crude fat content decreased before germination.

The role of the seed coat in dormancy has been clearly demonstrated in some of the early work of Flemion (42, 44, 45). If the outer and inner coats of non-stratified apple, peach, Crataegus, and Rhodotypos seeds were removed, the embryos germinated and developed hypocotyls and epicotyls within a week, but the resultant plants were much less vigorous and had short internodes and a stunted appearance in comparison with plants resulting from stratified embryos. The changes that occurred at low temperatures with rosaceous seeds seemed cumulative since the dormant seeds produced dwarfs, partially stratified seeds produced semi-dwarfs, and fully stratified seeds produced normal plants (46). Similarly, shoots developing from partially chilled buds were stunted in their growth. The morphological and histological characteristics of physiologically dwarfed seedlings of peach were carefully studied by Flemion and Beardow (48). Physiologically dwarfed seedlings had enlarged petioles, distorted midribs, and crinkled blades with malformed areas that were thickened and chlorotic (48). Flemion and Beardow (49) maintained a peach seedling as a dormant, physiological dwarf for ten years. These dwarfs were made to grow normally by adequate chilling (49). Both long photoperiods and gibberellic acid lengthened the internodes but resulted in spindly stems (47). When gibberellic acid was combined with supplementary light the etiolation effects were

gradually overcome with peach and Rhodotypos kerrioides Sieb. and Zucc. When the seed coats and cotyledons were removed from peach seeds and the embryonic axes were grown in nutrient culture at room temperature the resulting plants were normal (51). Grafting experiments by Flemion and Waterbury (52) demonstrated that dwarf plants made ample root growth and could support growth of normal scions, but when dwarf tops were grafted on normal roots they retained their dwarf characteristics. Therefore, they concluded that the locus of dwarfing was in the shoot, which made poor growth due to lack of essential constituents or the presence of inhibitors. The work of Flemion and her co-workers in the area of dwarfing is pertinent to dormancy since the same conditions and chemical changes which are necessary for normal germination may also be necessary for overcoming the dwarf characteristics. Flemion and de Silva (50) also studied extracts of peach seeds in various stages of dormancy but found no correlation between the breaking of rest and the levels of promoters and inhibitors.

Early studies with seed dormancy involved measuring increases in acidity, water-holding power, catalase and peroxidase activity, and sugars, and decreases in fat content (37, 118). Changes in respiration were also studied. Roberts (110) recently reviewed the relationship of seed dormancy and oxidation and concluded that there was little evidence in most cases for the view that covering structures

imposed dormancy by limiting oxygen uptake and therefore prevented germination by restriction of respiration. But in some species where inhibitors had been implicated in dormancy there was evidence that inhibitors increased the requirement for oxygen or that oxygen was necessary to oxidize inhibitors to an inactive form (110). In some species in which specific germination inhibitors were not clearly involved, dormancy was partially broken by a number of electron acceptors such as oxygen and nitrate but not by reduced nitrogenous compounds. Furthermore, respiratory inhibitors often partially broke dormancy in these species. Roberts (110) advanced the hypothesis that a number of dormancy-breaking agents may stimulate germination by increasing the operation of the pentose phosphate pathway.

Although light is required in a wide range of species for germination, few cases are known where light is actually necessary during stratification to overcome constitutive dormancy (11).

Effect of Exogenous Growth Regulators

Growth regulators play an important role in the dormancy of peach seeds. Although auxins occur in seeds and increase at the time of germination, there is little evidence that they are directly involved in dormancy (87).

Ever since the discovery of gibberellins, investigators have considered the possibility that they might function in overcoming dormancy. Frankland (54) stimulated

dormant hazel (Corylus avellana) and beech (Fagus sylvatica) seeds and embryos to germinate with gibberellic acid, but failed to stimulate germination of the intact nuts even at high concentrations. Apple (Malus sp.) and rowan (Sorbus aucuparia) embryos responded to gibberellic acid only after removal of the endosperm and testa. Further experiments were carried out by Frankland and Wareing (56) to investigate the failure of intact nuts of hazel and beech to respond to exogenous gibberellic acid. Hazel nuts failed to germinate even when holes 2 mm in diameter were bored in the sides of the pericarp. When gibberellic acid was added the nuts germinated only if the hole was made at the apex where the radicle could emerge. These results suggested that gibberellin treatment cannot overcome the mechanical resistance of the pericarp. On the other hand, beech nuts germinated if the pericarp was perforated anywhere and treated with gibberellic acid, suggesting that the pericarp inhibited diffusion of gibberellic acid to the embryo.

Bradbeer and Pinfield (18) found that gibberellin treatment of dormant hazel seeds could break rest and increase incorporation of [8-^{14}C] adenine into nucleotides, while decreasing incorporation of [2-^{14}C] acetate into lipids in the cotyledons. Inhibition of hazel seeds in either water or gibberellin solution increased incorporation of radioactivity into RNA and protein in both cotyledons and embryonic axes.

Bradbeer and Colman (16) and Bradbeer and Floyd (17) found that stratification decreased incorporation of labeled acetate into total lipids in both cotyledons and embryonic axes, and increased incorporation of labeled adenine into nucleotides. Because gibberellins caused the above effects only in the cotyledons and not in the embryonic axes, Bradbeer and Pinfield (18) suggested that the primary effect of gibberellin action may occur in the cotyledons.

Gray (58) was able to break dormancy of peach seeds with gibberellic acid in agar. Mes (93) repeated the work of Gray and found only a slight increase in germination, and all the seedlings which developed remained dwarf. Numerous attempts have been made by Mes to induce germination of 'Kakamos' peach seeds and normal growth of unstratified embryos excised from the seeds. None of the methods tried by Mes, including gibberellic acid, proved successful in obtaining growth comparable with that of seedlings from stratified seeds. Donoho and Walker (33) found that gibberellic acid increased the germination of peach seeds, but the treated seeds had already received 35 days of stratification and the untreated seeds germinated to the extent of 30 per cent. Therefore, only a part of the stratification requirement was eliminated.

Bachelard (6) found that gibberellic acid could replace the stratification and/or light treatment required

for germination of some dormant Eucalyptus species but not of others.

Jackson and Blundell (68) increased the germination of Rosa arvensis with gibberellic acid but at least half of the control seeds germinated after being soaked for six hours in water. Fogle (53) reported a 39 per cent germination of dormant cherry seeds after treatment with gibberellins but, like peach, the seedlings were dwarfed.

The stratification requirements of seeds of several pear species and interspecific crosses were studied by Westwood and Bjornstad (138) in relation to climatic adaptation. Pretreatment with gibberellic acid enhanced germination only in species requiring long periods of stratification. The stratification requirements of seeds from interspecific crosses were intermediate between those of the parents. The same workers (139) observed that seeds obtained from apple fruits that resulted from flowers sprayed with gibberellic acid prior to pollination gave higher germination than control seeds. They suggested that a shift in promoter-inhibitor balance had occurred during seed development.

Cytokinins also break seed dormancy of many species. Jackson and Blundell (68) increased the germination of dormant Rosa arvensis seeds with 6'-benzylaminopurine. However, soaking the control seeds in water for an additional six hours increased germination from ten to

fifty per cent. They postulated that this might be the result of leaching of inhibitors.

Frankland (54) stimulated germination of dormant embryos of beech, hazel and rowan with kinetin but was unsuccessful with intact seeds and nuts of these species. Bachelard (6) found that kinetin either had no effect or inhibited germination of dormant Eucalyptus pauciflora Sieb. seeds, while thiourea, potassium nitrate and gibberellic acid promoted germination. Badizadegan and Carlson (7) increased the germination of mature 'McIntosh' embryos with N^6benzyladenine, but the resulting seedlings were dwarfed with rosetted leaves and short internodes. N^6benzyladenine also improved the germination and subsequent seedling growth of peach seeds (24).

Khan (79) and Khan and Downing (80) noted that kinetin reversed to a large extent the abscisic acid inhibition of α-amylase synthesis in whole barley seeds. Kinetin and gibberellic acid together could not completely overcome the effects of abscisic acid in inhibiting coleoptile griwth, but caused nearly a complete reversal of the abscisic acid inhibition of α-amylase synthesis (80). Khan and Downing therefore suggested that the factors controlling α-amylase synthesis may not have a dominant role in all growth responses of the seed. Abscisic acid, kinetin, or benzyladenine alone inhibited root growth of oat, but abscisic acid together with benzyladenine was even more

effective (79, 80). Antagonisms between cytokinins and abscisic acid have been shown in germination of lettuce (78) and Xanthium (77).

Coumarin is a natural inhibitor of seed germination (92). This inhibition however can be reversed by kinetin in kale (81) and in lettuce seeds (76, 77) in the presence of red light. Kinetin plus red light has also been found to reverse the inhibition of lettuce seed germination by xanthatin (76).

Thiourea has long been known to influence germination of many seeds. Tukey and Carlson (129) in 1945 found that thiourea aided in the breaking of peach seed dormancy. Frankland (56) stimulated germination of dormant beech and hazel seeds with thiourea. This was of interest since kinetin was unable to break dormancy of these same seeds even though it did have a stimulatory effect on the excised embryos. Villiers and Wareing (132) showed that thiourea could overcome the natural inhibitor of germination in Fraxinus seeds. Thiourea also increases the germination of dormant seeds of many other tree species (70), and of lettuce (128).

Other compounds which have increased germination are potassium nitrate (70), ethylene chlorohydrin (84), and ethylene (130).

Although many substances inhibit germination, only those compounds whose effects can be reversed by dormancy

breaking treatments may be pertinent. A type of inhibition that is common in nature is osmotic inhibition. Such a situation probably exists in many fruits. Coumarin inhibits the germination of seeds of a wide variety of plants, but is seldom found in seeds in inhibitory concentrations (92).

Abscisic acid, considered by many to be a plant hormone, strongly inhibits germination. It is widely distributed in the plant kingdom and occurs in most tissues of higher plants (94). Relatively high amounts of abscisic acid have been found in fruits and seeds (94, 104). Apple juice is rich in abscisic acid and inhibits germination of stratified apple embryos (104). Kaminski (71) showed that apple embryos taken from seeds treated either with apple juice or with an abscisic acid-like inhibitor isolated from apple juice did not germinate as well as the controls. The effect of apple juice on cherry seeds was similar to the effect on apple seeds. Kaminski and Pieniazek (72) showed that abscisic acid greatly reduced the germination of unstratified apple embryos treated with gibberellins and/or benzyladenine. Rudnicki (112) recently identified abscisic acid in unstratified apple seeds. More abscisic acid was necessary to inhibit germination of apple seeds as stratification was prolonged. On the other hand, high amounts of abscisic acid have also been found in Acer saccharinum seeds, a non-dormant species, and synthetic abscisic acid

at concentrations up to 10 µg/ml did not inhibit growth of embryos (113).

Sondheimer and Galson (122) studied the effect of abscisic acid and other growth regulators on embryos of Fraxinus ornus, a non-dormant species, and Fraxinus americana, a dormant species. Abscisic acid inhibited germination, leaf development, and chlorophyll synthesis in both species. Gibberellins reversed the abscisic acid-induced inhibition of root development but not the inhibition of leaf growth and chlorophyll synthesis.

Bradbeer (13) found that abscisic acid decreased the germination of unstratified and stratified hazel (Corylus avellana L.) embryos. But when hazel seeds were stratified with abscisic acid, rinsed with water, and germinated at 20°C there was no effect upon germination (15). Lipe and Crane (88) inhibited germination of excised embryos of peach with abscisic acid or with a peach seed extract containing an abscisic acid-like inhibitor.

Endogenous Growth Regulators

Although gibberellins are commonly accepted as playing a key role in overcoming dormancy, the investigations of endogenous levels of gibberellins are neither extensive nor completely in agreement with this concept.

Frankland and Wareing (55, 56) found a small increase of gibberellin-like activity in hazel seeds after twelve weeks of stratification. In beech the

gibberellin-like activity differed qualitatively but not quantitatively between unstratified and stratified seeds. They found no differences in auxins or inhibitors.

Work by Ross and Bradbeer (111) attributed the small increase of gibberellin activity found by Frankland and Wareing in hazel seeds during stratification to increased gibberellin synthesis resulting from increased temperatures. They further found that the embryonic axes contained 100 times more gibberellin-like activity than the cotyledons. A growth and germination promoter which increased during stratification of hazel seeds was reported by Villiers and Wareing (133).

In contrast Strausz (125) was not able to find growth promoters consistently during stratification in Pyrus seeds, and Proctor and Dennis (108) were unable to detect either qualitative or quantitative changes in gibberellin-like substances in cherry seeds during low or high temperature stratification. Martin, et al. (91) studied auxins, cytokinins, and gibberellins in kernels of walnuts before, during, and after stratification and found no gibberellins or kinins and only small and variable amounts of auxins.

Luckwill (89) and Kawase (73) found that apple seed coats contained a growth-inhibiting substance which decreased to an undetectable level during low temperature stratification. Many subsequent investigators have

attempted to establish the role of inhibitors in seed dormancy.

Biggs (10) reported higher amounts of growth inhibitors in unstratified peach embryos than in stratified embryos. Flemion and de Silva (50) could find no correlation in peach seeds between rest and growth promoting or inhibiting substances. Villiers and Wareing (132, 133) found that an inhibitor in Fraxinus seeds did not decrease during stratification, but that a promoter was produced. This study indicated that rest may be induced by an inhibitor, but a promoter may be necessary to break rest. Although leaching increased germination, growth inhibiting substances present in the embryo and endosperm did not decrease during leaching.

Abscisic acid is a natural growth inhibitor found in numerous plants and in almost all parts of higher plants (95). It affects a wide range of physiological processes in minute concentrations similar to other plant hormones (1). Several lines of evidence, including the detection of abscisic acid in phloem and xylem sap, lend support to the translocatability of this compound (14, 84, 98). One of the physiological processes in which abscisic acid has been shown to have a pronounced effect is seed germination (1).

In 1966 Lipe and Crane (88) correlated the termination of rest in peach seeds with the disappearance of an inhibitor tentatively identified as abscisic acid.

Identification was based on chromatographic analysis, ultraviolet absorption spectra, and effects on plant growth. The inhibitor was located primarily in the outer and inner integuments. It had disappeared after 6 weeks of stratification, but even after twelve weeks seeds with intact seed coats germinated more slowly than excised embryos. Seedlings produced from excised embryos, which would otherwise produce normal seedlings, were induced to behave like those from unstratified seeds with integuments by application of either seed extract or synthetic abscisic acid.

Sondheimer and Galson (123) found that abscisic acid inhibited germination of excised non-dormant Fraxinus embryos and that a combination of gibberellic acid and kinetin could reverse this inhibition. With this knowledge Sondheimer, et al. (124) used Milborrow's (94) "racemate dilution" method to measure abscisic acid quantitatively in Fraxinus americana, a dormant species, before and after stratification, and in Fraxinus ornus, a non-dormant species. They found that abscisic acid decreased during stratification of dormant seeds by 68 per cent to a level similar to that found in non-dormant species.

Studies by Martin, et al. (91) and Rudnicki (112) strengthened the support for a negative correlation between abscisic acid levels and germination capacity. Martin, et al. (91) found that abscisic acid decreased rapidly in

walnut seeds during two weeks of stratification and remained relatively constant for the next six weeks. They based their identification of abscisic acid on ultraviolet absorption spectra, Rf values established by co-chromatography on paper and silica gel plates, and derivatives analyzed by gas liquid chromatography.

With the previous knowledge that abscisic acid occurred in high quantities in apple fruits and leaves and that this inhibitor retarded germination of stratified apple embryos (104), Rudnicki (112) investigated endogenous levels of abscisic acid during stratification of apple seeds. Based on chromatographic behavior, fluorescence, ultraviolet spectra, and growth inhibition in the wheat coleoptile straight growth test abscisic acid was identified in unstratified apple seeds. Using the wheat coleoptile test Rudnicki detected a rapid decrease of abscisic acid during stratification of these seeds. After three weeks of stratification he could no longer detect abscisic acid.

There are some conflicting reports on the role of abscisic acid in dormancy. Bradbeer (15) found high inhibitor levels in dormant hazel pericarp plus testa and lower amounts in the embryos. Abscisic acid, if present, accounted for less than two per cent of the inhibition.

Strausz (125) was not able to detect significant changes in levels of an inhibitor tentatively identified

as abscisic acid in Pyrus fruit and seed coats after stratification. Identification was based on chromatography and ultraviolet spectra. However, there was a significant decrease of this inhibitor in the embryo.

Even though abscisic acid is generally considered a growth inhibitor, relatively high quantities have been found in growing tissues (108). Also, high levels of abscisic acid were found by Rudnicki and Suszka (113) in seeds of Acer saccharinum L., a non-dormant species, which did not decrease until eight days after germination.

Detection of Abscisic Acid

Abscisic acid was first shown to have an intense Cotton effect in its optical rotatory dispersion (ORD) curve by Cornforth, et al. (28). Since then several investigators have used ORD as a means of identification and as a quantitative method of analysis (26, 28, 29, 19, 124). The major limitation is the relative scarcity of spectropolarimeters available for use.

Gas-liquid chromatography using flame ionization (31, 57, 85) and electron capture (117) detectors has been used to identify and quantify abscisic acid. Lenton, et al. (85) were able to detect abscisic acid in phloem and xylem sap of willow (Salix viminalis L.) using gas-liquid chromatography with a flame ionization detector. They estimated concentrations of 10 µg of abscisic acid per 100 ml of xylem sap. Davis, et al. (31) also used a flame ionization

detector to detect trimethylsilyl derivatives of abscisic acid. Preliminary purification of abscisic acid from cotton fruit extracts included column chromatography with carbon-celite and elution with 60 per cent acetone. Seeley and Powell (117) used the electron capture detector to detect as little as 10 picograms of methyl abscisic acid.

For identification of abscisic acid Lenton, et al. (86) suggested ultraviolet irradiation to convert the methyl ester of the naturally occurring cis-trans abscisic acid to an equal mixture of cis-trans and trans-trans abscisic acid. Gas-liquid chromatography was used for both separation and measurement of the two isomers. Asmundson, et al. (4) were also able to separate cis-trans and trans-trans abscisic acid by using dry column chromatography.

Abscisic acid was identified in apple cider by Gaskin and MacMillan (57) and in immature fruits of Ceratonia siliqua by Most, et al. (98) by utilizing gas-liquid chromatography-mass-spectrometry.

A spectrofluorometric method for the quantitative determination of abscisic acid in thin-layer chromatograms was described by Antoszewski and Rudnicki (2). They were able to detect as little as 0.5 μg of abscisic acid. Powell (106) described a method for determining the infrared spectra of microgram quantities of indole acetic acid, several gibberellins, and abscisic acid.

When sufficient quantities of relatively pure abscisic acid can be isolated, other standard chemical and

physical methods such as melting point determination, nuclear magnetic resonance, infrared spectrometry, etc., can be more easily utilized.

Many bioassays can be used to detect abscisic acid but only a few have been utilized to any extent. Some of the more frequently used bioassays for abscisic acid are the wheat and oat coleoptile straight growth bioassay (88, 99, 112), dissected wheat embryo bioassay (94), rice seedling bioassay (82), cotton explant abscission test (121), and the growth of Lemna minor (131).

MATERIALS AND METHODS

I. Inhibitors in the Acid Fraction of 'Halford' Peach Seeds During Stratification--First Year

Experiments were done to determine if a correlation exists between a decrease of inhibitor levels in the acid fraction and an increase of germination during stratification at 3°C, and to compare the levels of inhibitors in the different parts of the seeds. As a control, additional seeds were stratified at 20°C. The general procedure is described in Figure 1.

'Halford' peach pits were soaked in distilled water for 48 hours and stratified at 3 and 20°C in moist peat moss in perforated metal containers. Samples were removed at intervals during stratification. To test the germination capacity of seeds, the pits were washed and cracked, and the seeds placed on moist filter paper in petri dishes in the light at 20°C. Additional seeds were separated into seed coats, cotyledons, and embryonic axes. These samples were extracted and the acid fraction prepared (Figure 2) according to a modified method of Milborrow (94).

The acid fraction was dissolved in either 90% ethanol or 100% methanol and streaked on silica gel thin-layer chromatograms 100 microns thick with a fluorescent indicator (Eastman Kodak Co., Rochester, N. Y.). An

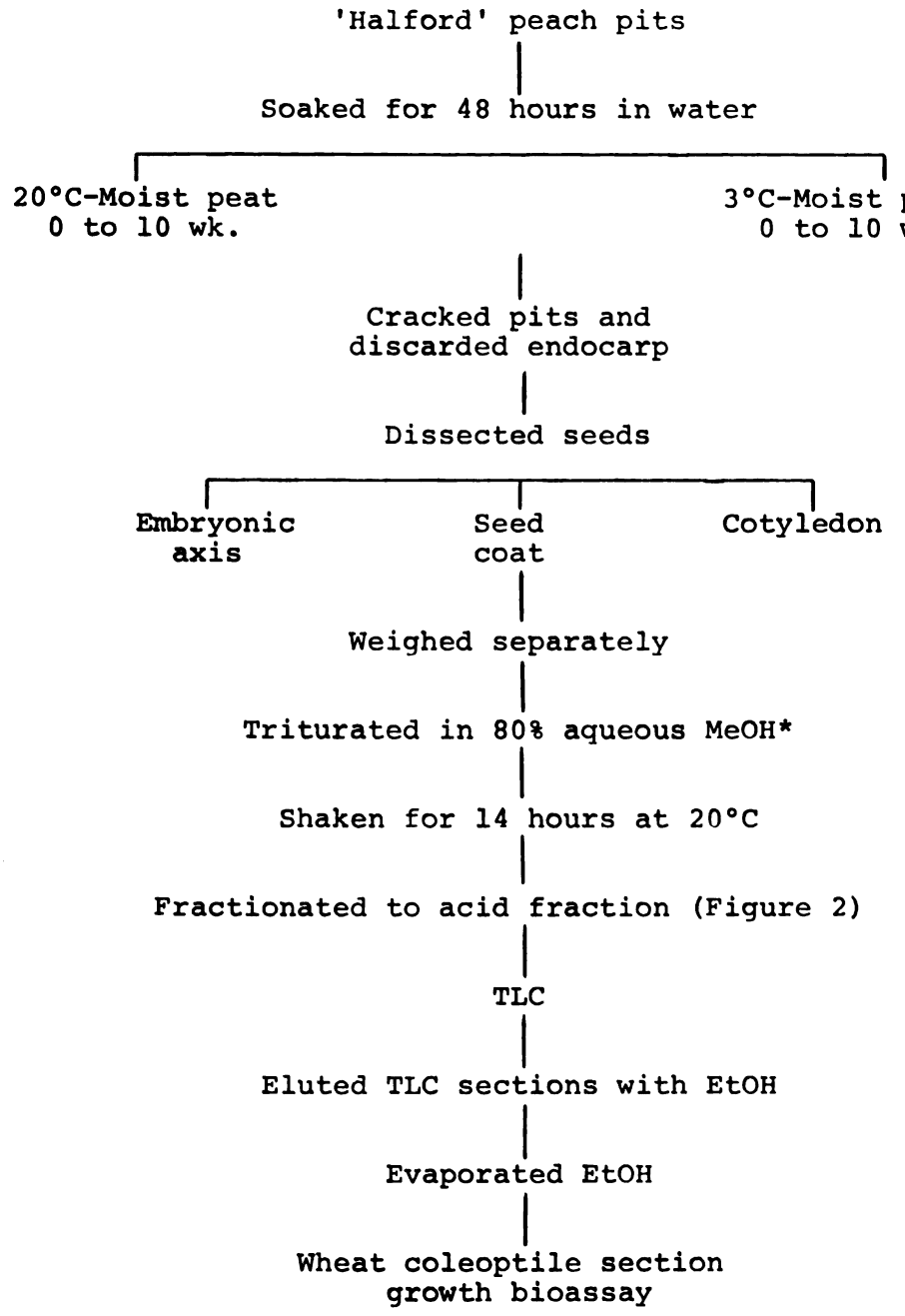

Figure 1. General procedure for bioassay of acid inhibitors in peach seeds during stratification.

*Water used in some experiments.

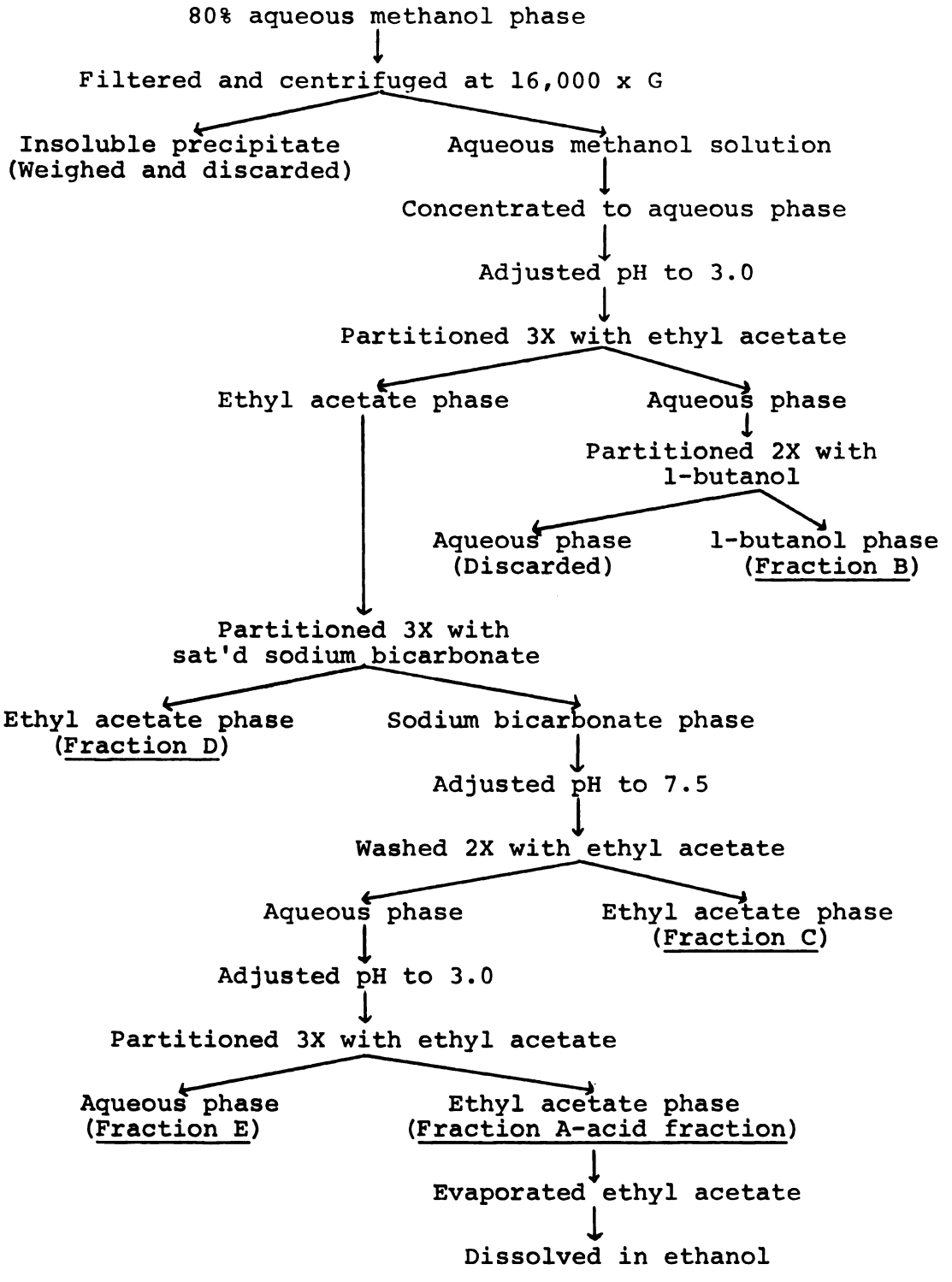

Figure 2. Flow chart for fractionation of peach seed extracts.

abscistic acid standard was also streaked on each chromatogram. The chromatograms were developed in 10:1:1 isopropanol:ammonium hydroxide:water. The developing tank was usually lined with filter paper, the solvent system added 24 hours before development, and the tank sealed. A standard 24 hours was used because the Rf of abscisic acid, as determined by its absorption of ultraviolet light, varied with the length of the equilibration period. After drying for 24 hours the chromatograms were divided into ten Rf sections and eluted with 90% ethanol. Aliquots were taken for bioassay or physical methods of detection.

Many of the initial studies with the wheat coleoptile section bioassay were conducted with the 'Genesee' variety but later bioassays were made with the 'Yorkstar' variety. The general procedure was as follows. (1) Wheat seeds were planted in vermiculite in perforated pans and sub-irrigated for 2 hours. The pans were drained for 10 minutes. (2) The seeds were germinated in the dark at 20°C for 72 hours. (3) Coleoptiles approximately 25 mm long were chosen and 4 mm sections were cut 2 mm from the tip under red light. (4) Five sections were placed in each test tube containing the sample in 0.3 ml of 2 per cent sucrose and 10^{-2} M phosphate-5 X 10^{-3} M citrate buffer (pH 5.3) according to Nitsch and Nitsch (99) for 22 hours in the dark. Samples were prepared by drying the sample in a test tube under vacuum, and adding buffer just prior to the

bioassay. A clinostat was used to prevent geotropic curvature. (5) The images of the coleoptile sections were enlarged six times with a photographic enlarger and measured with a ruler to the nearest millimeter.

II. Inhibitors in the Acid Fraction of 'Halford' Peach Seeds During Stratification--Second Year

The first year's data indicated that no substantial decrease in the inhibitor occurred during stratification. One of the purposes of this experiment was to repeat the work of the first year. In addition, since the results of the first year differed from those of Lipe and Crane (88), possible reasons for this difference were explored. The major difference between the first year's study and the work of Lipe and Crane (88) was the method of extraction. Lipe and Crane used water whereas I used 80 per cent aqueous methanol. Therefore, water extraction was compared with aqueous methanol extraction in this study. Samples were extracted with water for 48 hours at 5 to 8°C. Methanol extraction was repeated as in the first year. Otherwise, the general procedure was the same.

III. Inhibitors in the Acid Fraction of 'Okinawa' Peach Seeds During Stratification

The purpose of this experiment was to see if the results obtained with 'Halford' seeds (a variety with a medium stratification requirement) could be repeated with

'Okinawa' seeds (a variety with a short stratification requirement). The same procedure was used for 'Okinawa' seeds as for the 'Halford' seeds except that the stratification period was much shorter. Pits were stratified at 3 and at 20°C, and samples taken at 0 and 4 weeks. The seeds were again separated into seed coats, cotyledons, and embryonic axes and these samples were extracted with aqueous methanol or water. The acid fraction was chromachromatographed on silica gel thin-layer plates. Inhibitors were detected with the wheat coleoptile section bioassay.

IV. Bioassay of Inhibitors in All Fractions of Dormant 'Halford' Peach Seeds and the Color Tests for the Presence of Naringenin and Naringin

As a preliminary study a means of separating abscisic acid and naringenin was sought since both of these compounds have been implicated in bud dormancy. The Rf's of abscisic acid and naringenin on Eastman silica gel thin-layer plates 100 μ thick and on Whatman no. 1 paper were determined for several solvent systems (Table 2). The effects of naringenin and naringin, the glycoside of naringenin, on wheat coleoptile section elongation were also determined.

Dormant 'Halford' peach seeds were extracted with 80 per cent aqueous methanol and partitioned into various fractions as shown in Figure 2. Ten seed equivalents of

these fractions were then spotted on Eastman silica gel plates 100 μ thick and on Whatman no. 1 paper, and developed in 10:1:1 isopropanol:ammonium hydroxide:water. These chromatograms were then separated into ten Rf sections and bioassayed with wheat coleoptile sections.

To detect naringenin and/or naringin, parallel chromatograms were sprayed with 2 N sodium hydroxide followed by diazotized p-nitroaniline (DPNA), 2% aluminum chloride in ethanol, or sodium borohydride followed by hydrochloric acid fumes.

V. Identification of the Inhibitor in the Acid Fraction

Initial Studies

One major inhibitor was detected in the acid fraction at the Rf of abscisic acid. Its activity was consistent with levels of abscisic acid reported to occur in plant tissues (94). Several methods have been reported to identify small quantities of abscisic acid in plant samples. One method is the measurement of the optical rotatory dispersion (ORD) of abscisic acid (94), which has an unusually strong "Cotton Effect" with a maximum at 289 mμ and a minimum at 246 mμ. The "Cotton Effect" is the strong increase of rotatory power as approached from the long wavelength side followed by a drop and a change in sign. This method is also useful as a quantitative tool.

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To prepare samples for measurement of the ORD with the spectropolarimeter the acid fraction was chromatographed on silica gel thin-layer plates, which had been pre-washed three times with 90 per cent ethanol (redistilled) and two times with redistilled chloroform. The solvent system used was 10:1:1 isopropanol:ammonium hydroxide:water. The plates were cut into ten Rf sections and eluted with 90 per cent ethanol. The ethanol was evaporated, and the residue redissolved in distilled water acidified to pH 3.0 with concentrated sulfuric acid. This was extracted with ethyl acetate, the ethyl acetate evaporated, and the residue dissolved in 0.005 N sulfuric acid in ethanol. These samples were placed in a cell with a 10 mm light path and the ORD measured on a Jasco ORD/UV-5 Optical Rotatory Dispersion Recorder (Japan Spectroscopic Co., Ltd.).

The ultraviolet (UV) absorption spectrum of abscisic acid has also been used for identification (94). Samples were prepared as for ORD. A blank thin-layer plate was eluted similarly and used as a reference. Samples were scanned from 310 to 210 mμ on a Beckman DB-G spectrophotometer.

Sample preparation for gas-liquid chromatography was the same as for preparation of the ORD samples up to and including the elution of the thin-layer plates. The samples were then methylated with diazomethane according to

Schlenk and Gellerman (119). The procedure involved mixing 0.7 ml carbitol (diethylene glycol), 0.7 ml ether, 1.0 ml 60% potassium hydroxide, and a small quantity (ca. 2 mg) of diazald (N-methyl-N-nitroso-p-toluene sulfonamide) dissolved in 0.5 ml ether, and bubbling nitrogen through the solution. The diazomethane evolved passed into the sample tube, which contained the sample residue, 1.0 ml absolute ethanol, 3.0 ml ether, and 0.3 ml absolute methanol. The sample was considered methylated after ten minutes or when a yellow tinge appeared. The sample was then evaporated and taken up in absolute chloroform for gas-liquid chromatography analysis.

A Packard Gas Chromatograph Series 7300 equipped with a hydrogen flame ionization detector and a 6 ft x 2 mm i.d. U-shaped glass column packed with 2% QF-1 on chromosorb W (100/120 mesh) was used. The solid support was acid washed and dimethylchlorosilanized. The temperatures for inlet, column, and detector were 250, 210, and 250°C respectively. The flow rates (ml per min. as measured by gauge) for hydrogen, nitrogen and air were 40, 40, and 350 ml/min respectively. The attenuation was 1×10^{-10}.

Sample preparation for gas-liquid chromatography-mass spectrometry was the same as for the preparation of the gas-liquid chromatography samples. Samples were first dissolved in chloroform and then analyzed with an LKB 9000

Gas Chromatograph-Mass Spectrometer (LKB Instruments, Inc., Rockville, Md.). The gas-liquid chromatograph conditions are given in Table 5 in the column listing 3% SE-30 on Supelo-port. The ionization voltage used for all analyses was 70 eV.

Further Studies

To clarify some of the results of the initial study, solvent blanks and standard abscisic acid were treated as follows: (1) no treatment; (2) chromatographed on Eastman silica gel plates 100 μ thick with binder and fluorescent indicator; (3) chromatographed on plates 250 μ thick made with EM Reagents (Division of Brinkmann Instruments, Inc., Westbury, N. Y.) silica gel H without binder or fluorescent indicator; and (4) processed through the partitioning procedure to the acid fraction as shown in Figure 2. Samples were dissolved in 0.05 N formic acid in ethanol for obtaining ultraviolet spectra.

Samples analyzed via gas-liquid chromatography were methylated by the method of Schlenk and Gellerman (119) procedure as modified by Powell (105) with the exception that ether was substituted for methylene chloride. This procedure involved: (1) dissolving the sample in a 1:9 methanol:ether solution; (2) generating diazomethane from a test tube containing 1.5 ml of carbitol (2-(2-ethoxy-ethoxy) ethanol), 1.0 ml of 60% potassium hydroxide, and 1.5 ml of a saturated solution of diazald

(N-methyl-N-nitroso-p-toluenesulfonamide) in ether into the sample tube in a stream of nitrogen saturated with ether; (3) stopping the reaction upon appearance of a yellow color in the sample tube; and (4) evaporating the methanol:ether solution of the sample tube under a stream of nitrogen. Methylated samples were dissolved in chloroform before injection into a Packard 7300 series gas-liquid chromatograph with a flame ionization detector. Gas chromatograph conditions are given in Table 5 in the column listing 2% QF-1 on Gas Chrom Q.

Dormant 'Halford' peach seed samples were processed to the acid fraction and thin-layered on Eastman silica gel plates 100 μ thick containing binder and fluorescent indicator or EM Reagents silica gel H 250 μ thick without binder or fluorescent indicator. These samples were bioassayed and analyzed with a gas-liquid chromatograph as just described.

The most biologically active section (Rf 0.4-0.5) of the acid fraction thin-layered on EM Reagents silica gel H was methylated as above and gas chromatographed on 2% DC 200 on Chromosorb W 80/100 mesh (detected with electron capture), 3% SE-30 on Supeleo-port 80/100 mesh (detected with total ion current), and 2% QF-1 on Gas Chrom Q 60/80 mesh (detected with flame ionization). Details of conditions are given in Table 5.

The same sample was dissolved in chloroform and analyzed with an LKB 9000 Gas Chromatograph-Mass

Spectrometer (LKB Instruments, Inc., Rockville, Md.). The gas-liquid chromatograph conditions are given in Table 5 in the column listing 3% SE-30 on Supeleo-port. The ionization voltage was 70eV.

VI. Seed Coat Studies

At the beginning of this study it was not known if the seed coat prevented seed germination by merely acting as a barrier which weakened during stratification. An experiment was therefore designed to test this possibility. A very simple apparatus (Figure 3) was put together to do this. It consisted of a medium point pen refill to simulate the radicle of the seed, a board with a groove in it to cradle the pen refill, and a Hunter Spring Mechanical Force Gauge (Model No.-L-1000M) to measure the pressure applied. The radicle half of the seed coat was removed intact ten hours after removal from the stratification medium. The force required to penetrate the seed coat at the radicle end was measured by pushing the inner radicle end against the pen refill until the point protruded. Samples of 'Halford' and 'Okinawa' seeds were taken at various times during stratification to test the seed coat strength.

Figure 3. Apparatus for testing seed coat strength.

RESULTS

I. Inhibitors in the Acid Fraction of 'Halford' Peach Seeds During Stratification--First Year

In preliminary experiments standard curves for synthetic abscisic acid were obtained with both 'Genesee' and 'Yorkstar' wheat varieties (Figures 4 and 5). Each treatment was replicated three times and the range is indicated by vertical bars. The linearity between 0.01 and 1.0 ppm abscisic acid was found to be consistent.

The effects of coleoptile length and bioassay medium were tested. The length of the coleoptiles was originally chosen to be 25 mm, but time would be saved by using coleoptiles 25 mm to 30 mm in length. The results (Figure 6) indicated little difference in response between 25 mm and 30 mm coleoptiles. The use of water instead of the standard sucrose, phosphate-citrate buffer as the bioassay medium did not alter the response of coleoptile sections to abscisic acid on a %-inhibition basis (Figure 7). However, coleoptile sections grew more in the sucrose, phosphate-citrate buffer mixture than in water alone, and the buffer solution was therefore used for all subsequent experiments.

The possible inhibitory effects of compounds eluted from thin-layer plates with either ethanol or methanol

Figure 4. Elongation response of 'Genesee' wheat coleoptile sections to various concentrations of abscisic acid. Each point represents the average of three replications with the range indicated by vertical bars.

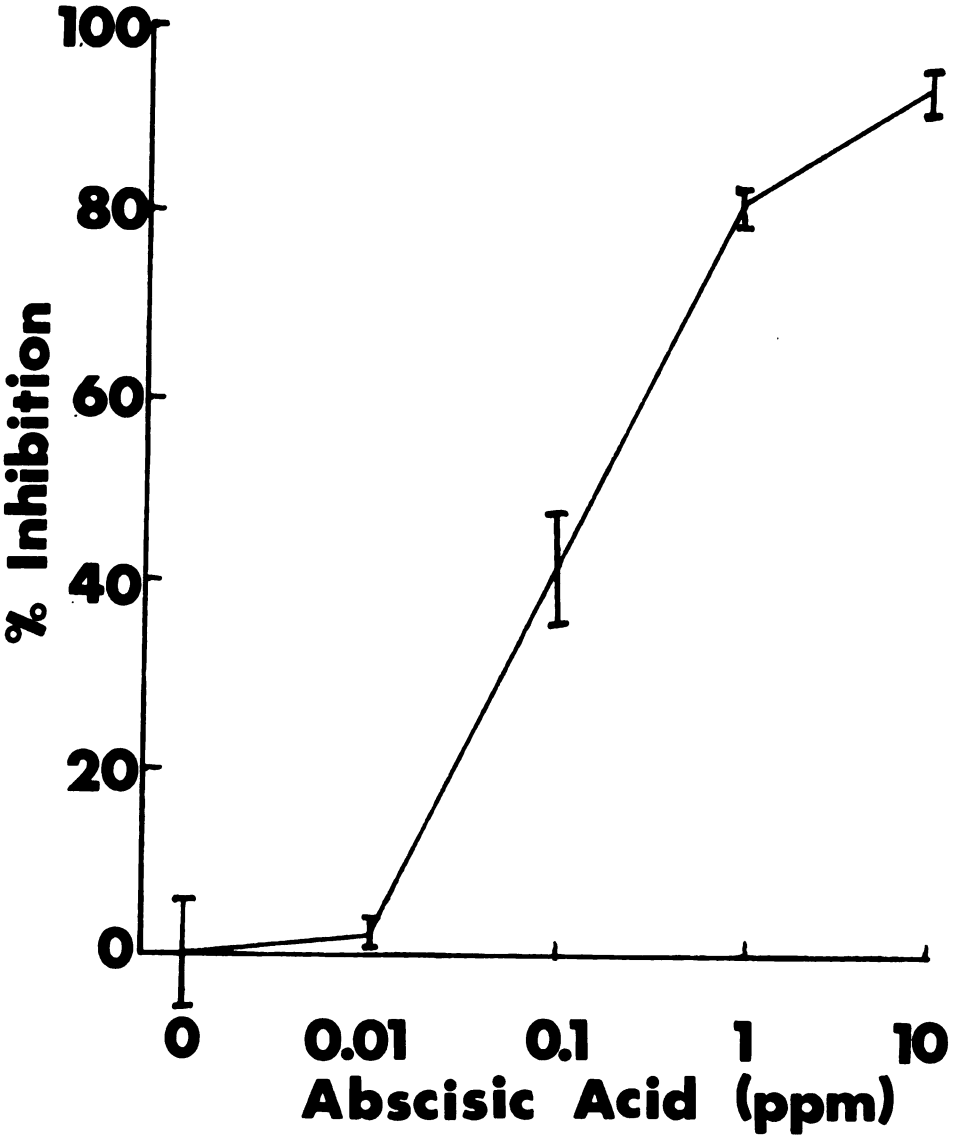

100
80
60
40
20
0
% Inhibition
0
0.01
0.1
1
10
Abscisic Acid (ppm)

Figure 5. Elongation response of 'Yorkstar' wheat coleoptile sections to various concentrations of abscisic acid. Each point represents the average of three replications with the range indicated by vertical bars.

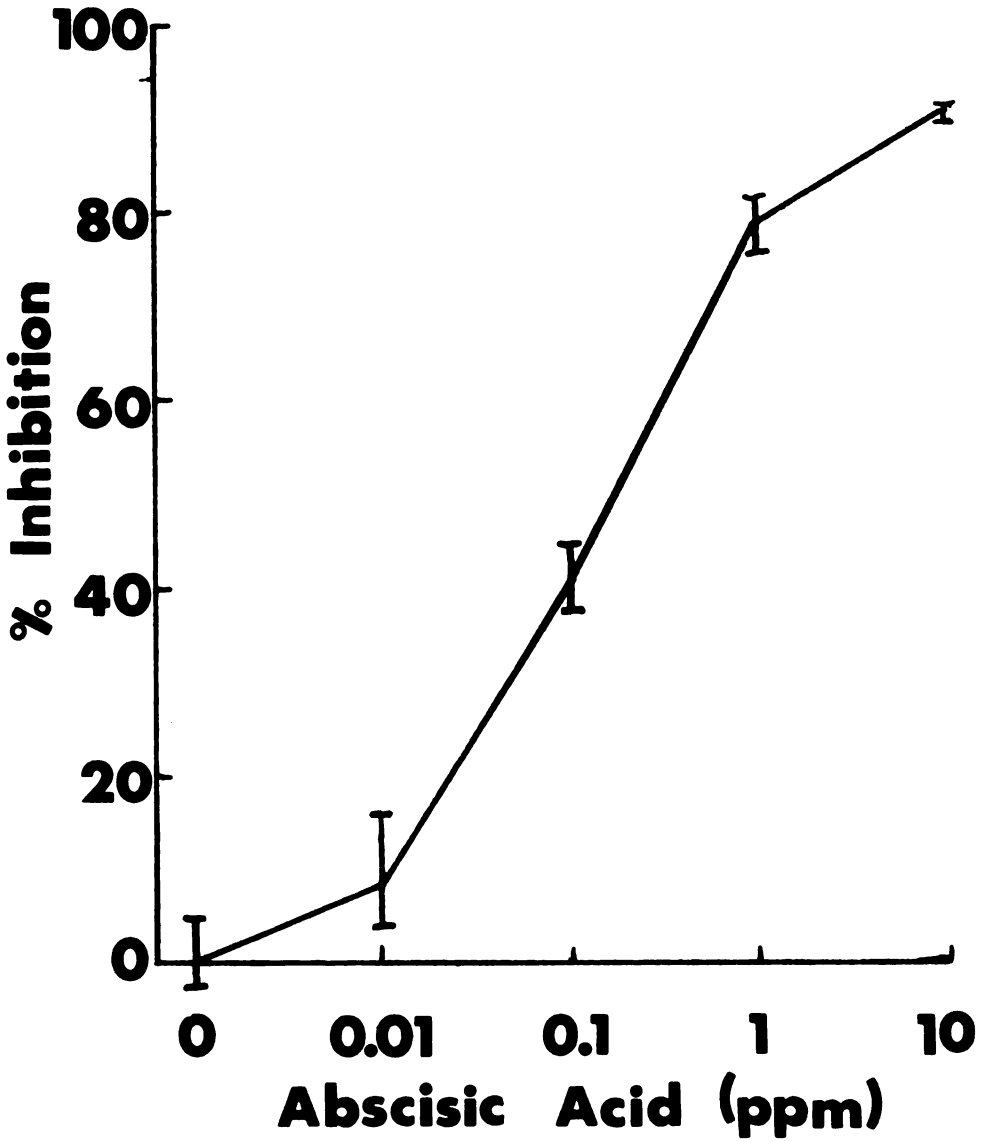

100
80
60
40
20
0
% Inhibition
0
0.01
0.1
1
10
Abscisic Acid (ppm)

Figure 6. Effect of 'Genesee' wheat coleoptile length at the time of cutting upon elongation response to abscisic acid. Each point is the mean for 2 replications.

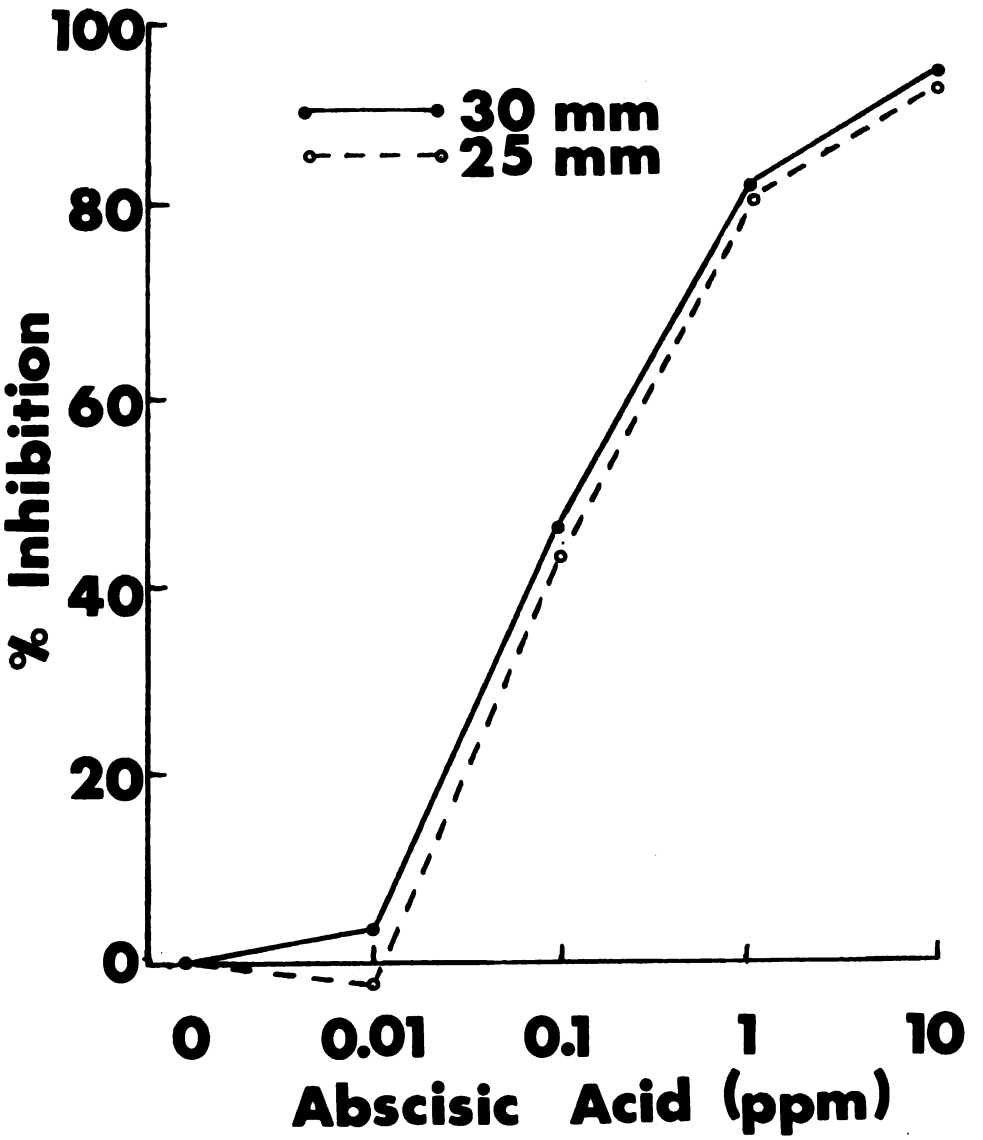
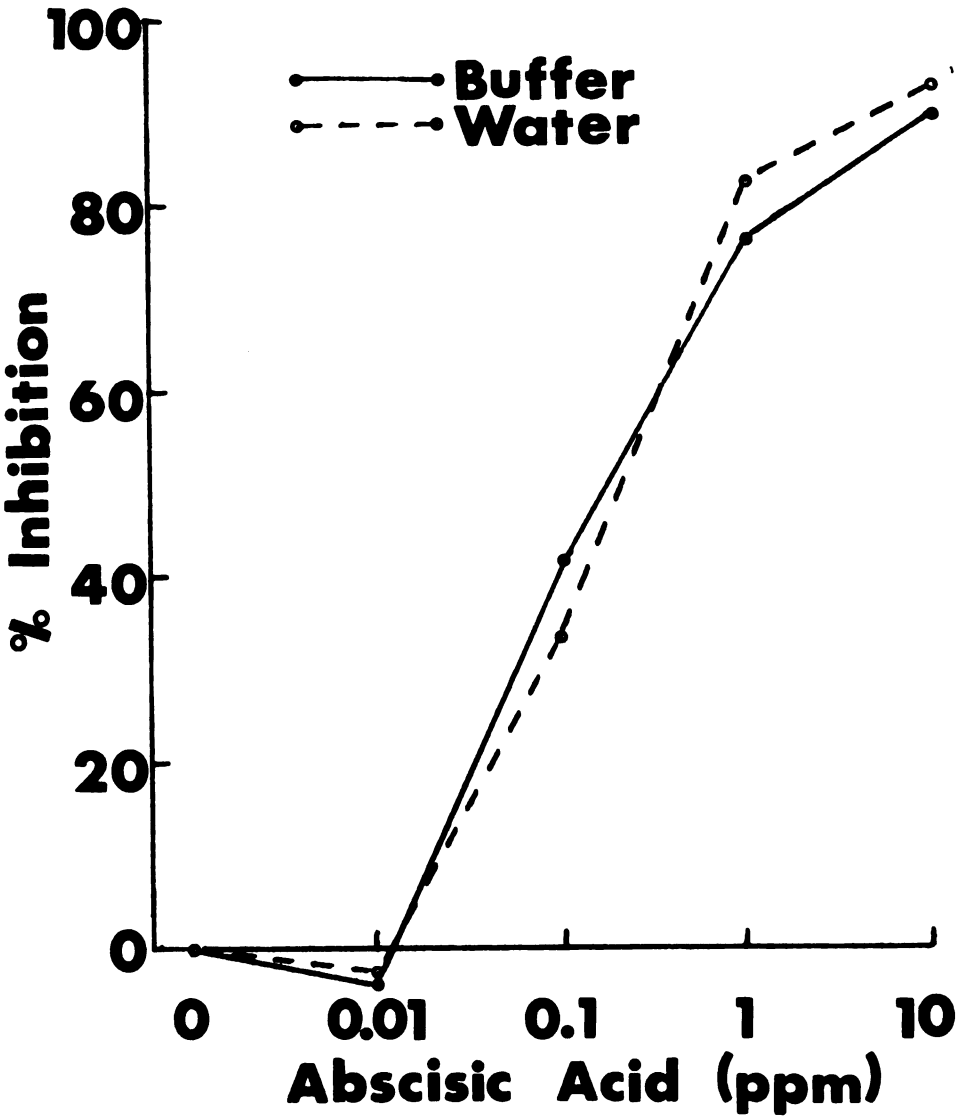

100
80
60
40
20
0
% Inhibition
30 mm
25 mm
0
0.01
0.1
1
10
Abscisic Acid (ppm)

Figure 7. Elongation response of 'Genesee' wheat coleoptile sections to abscisic acid in sucrose, buffer medium versus water alone. Each point is the mean for 2 replications.


100
80
60
40
20
0
% Inhibition
Buffer
Water
0
0.01
0.1
1
10
Abscisic Acid (ppm)

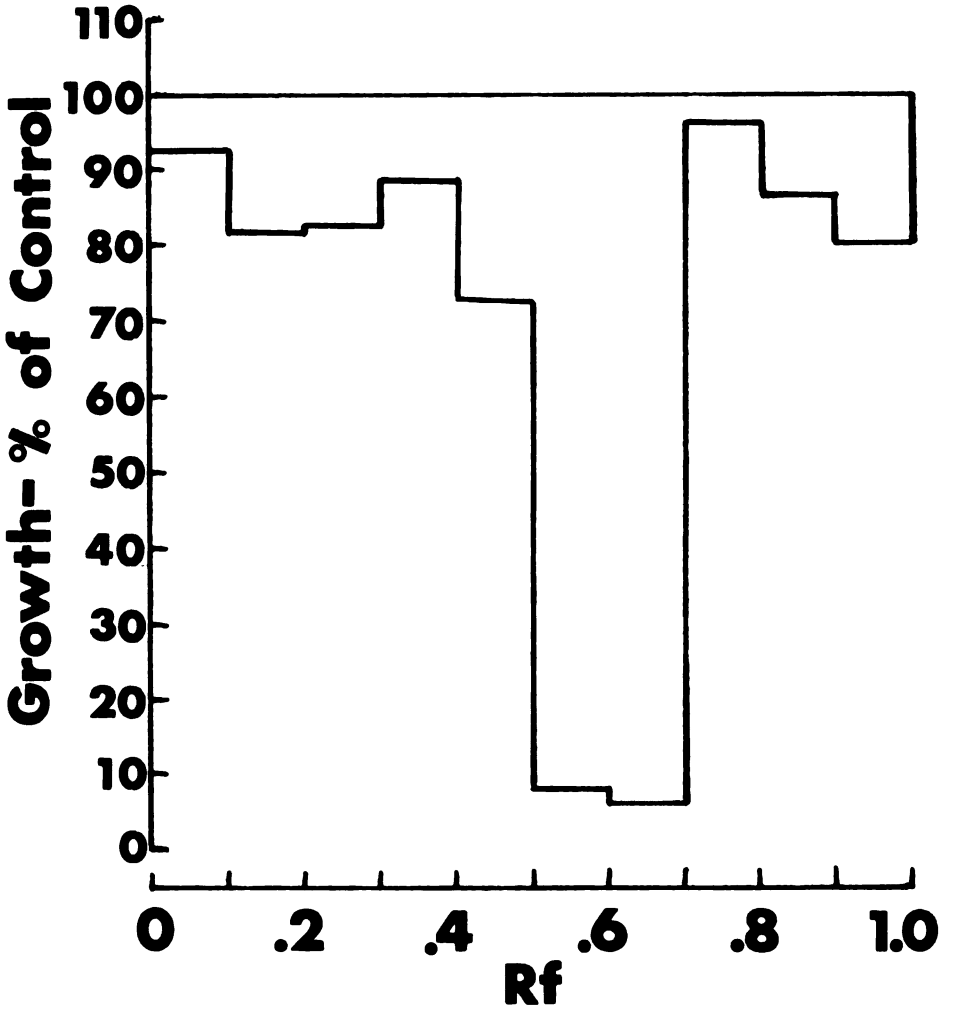
necessitated a preliminary study to test this possibility (Table 1). Elution with methanol resulted in higher levels of inhibitory compounds when compared with elution with ethanol. Even though ethanol eluates inhibited growth slightly, this solvent was used for elution in all other experiments.

TABLE 1.--The effects of ethanol or methanol eluates of sections of unused thin-layer chromatograms on 'Yorkstar' wheat coleoptile section elongation.

Size of TLC (cm^2) section eluted	Eluting solvent and volume (ml)	Average coleoptile length (mm x 6)
0	0	48.9
0	EtOH 5	46.0
0	10	45.6
2	5	47.8
4	5	44.0
6	5	43.6
0	MeOH 5	46.0
2	5	38.6
4	5	36.4

Several seed extracts were tested to evaluate the overall effectiveness of the procedure, which included fractionation to the acid fraction, separation on thin-layer silica gel plates and bioassay. The results of one of these tests involving whole, unstratified 'Halford' peach seeds are shown in Figure 8. This histogram showed

Figure 8. Elongation response of 'Genesee' wheat coleoptile sections to eluates from thin-layer plates of the acid fraction of the equivalent of 2.5 whole 'Halford' peach seeds (1.3 g fr. wt. or 0.65 g extracted dry wt.). Thin-layer plates were developed in 10:1:1 isopropanol: NH_4OH:water.


Growth- % of Control
110
100
90
80
70
60
50
40
30
20
10
0
0
.2
.4
.6
.8
1.0
Rf

one major inhibitory zone with an Rf value and a concentration similar to what would be expected of abscisic acid. The equivalent of 2.5 seeds (1.3 g fr. wt. or 0.65 g extracted dry wt.) was necessary to produce the response shown on this histogram. Assuming the inhibitor to be abscisic acid, the concentration was approximately 0.2 µg/g or 0.2 ppm on an extracted dry weight basis (oven dry weight of tissue after extraction).

The germination of 'Halford' peach seeds approached the maximum (80%) by the sixth week of cold stratification (Figure 9). Seeds did not germinate following warm stratification.

Inhibitor levels in the acid fractions of seed coats and cotyledons of 'Halford' peach seeds stratified at 3°C for 0, 6, and 8 weeks are illustrated in Figure 10. One major inhibitory zone occurred in extracts of both the seed coats and the cotyledons at the Rf of abscisic acid spotted on the same thin-layer plates. The inhibitor did not disappear during cold stratification. The Rf value of the inhibitor was not always constant due probably to differences in the equilibration of the developing tank. However, its Rf corresponded with that of abscisic acid. Each histogram represents four seeds. Since the dry weight of the cotyledons was ten times the dry weight of the seed coats, the concentration of the inhibitor was much higher in the latter.

Figure 9. Germination of 'Halford' peach seeds following stratification at 3°C for various periods of time. Endocarps removed and seeds germinated at 20°C.

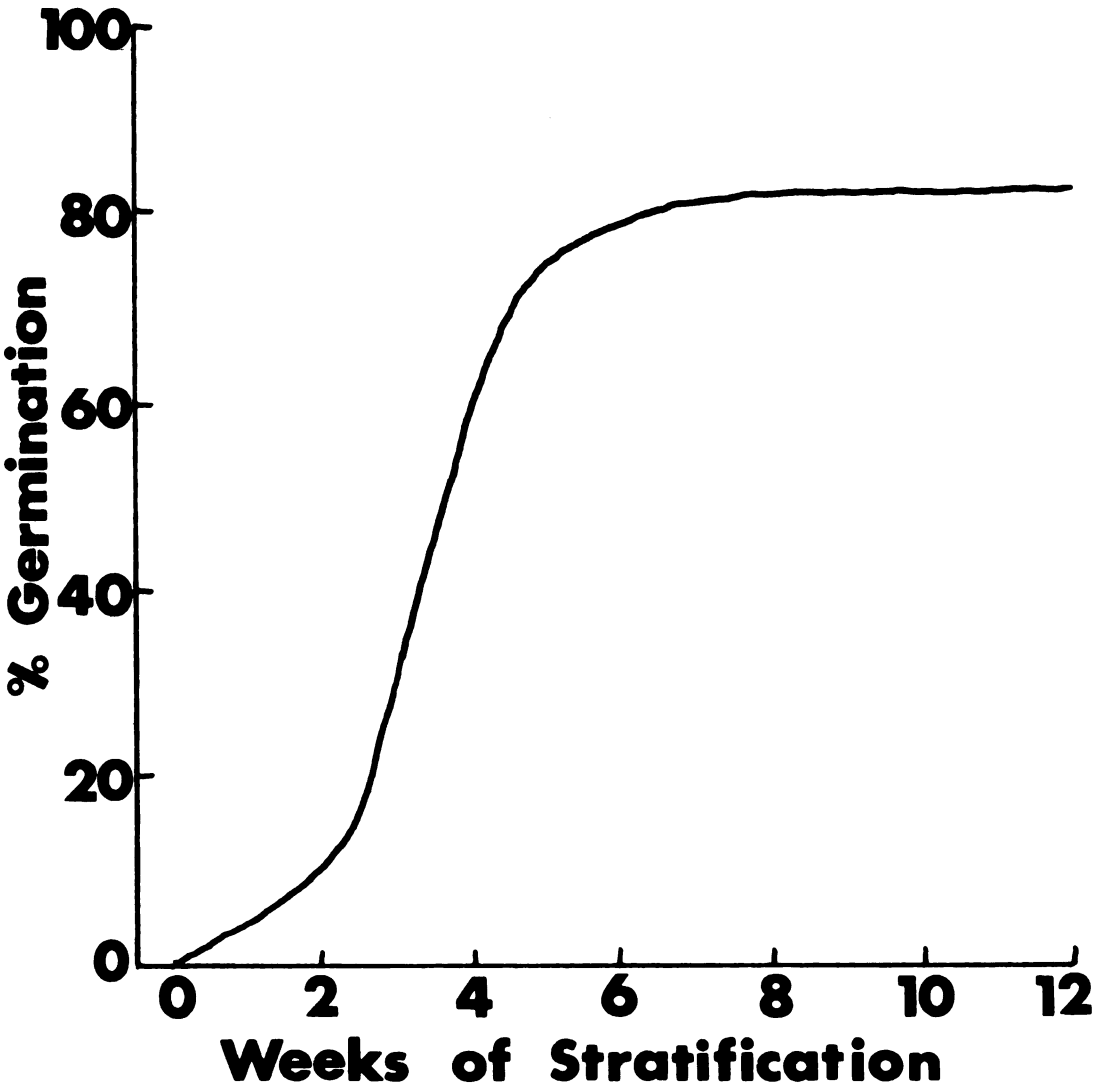

100
80
60
40
20
0
% Germination
0
2
4
6
8
10
12
Weeks of Stratification

Figure 10. Histograms of the elongation response of 'Genesee' wheat coleoptile sections to thin-layer eluates of the acid fraction of seed coats or cotyledons of 'Halford' peach seeds stratified at 3°C for 0, 6, and 8 weeks. Each histogram represents tissue from 4 seeds.

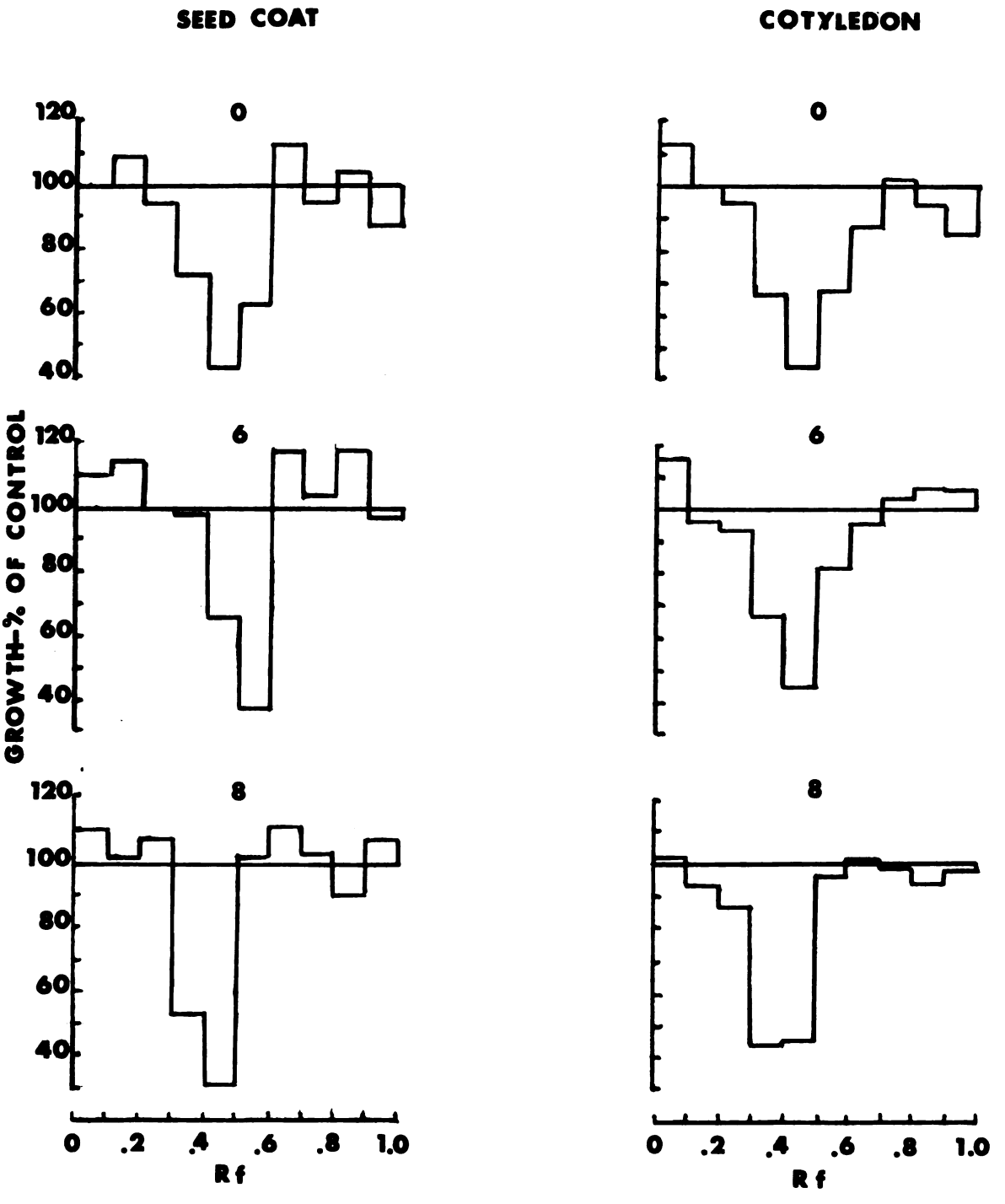

SEED COAT
COTYLEDON
0
6
8
120
100
80
60
40
GROWTH-% OF CONTROL
0 .2 .4 .6 .8 1.0
Rf

Figure 11. Histograms of the elongation response of 'Genesee' wheat coleoptile sections to thin-layer eluates of the acid fraction of embryonic axes of 'Halford' peach seeds stratified at 3 or 20°C for 0, 2, and 8 weeks. Each histogram represents 50 embryonic axes (ca. 45 mg fr. wt.).

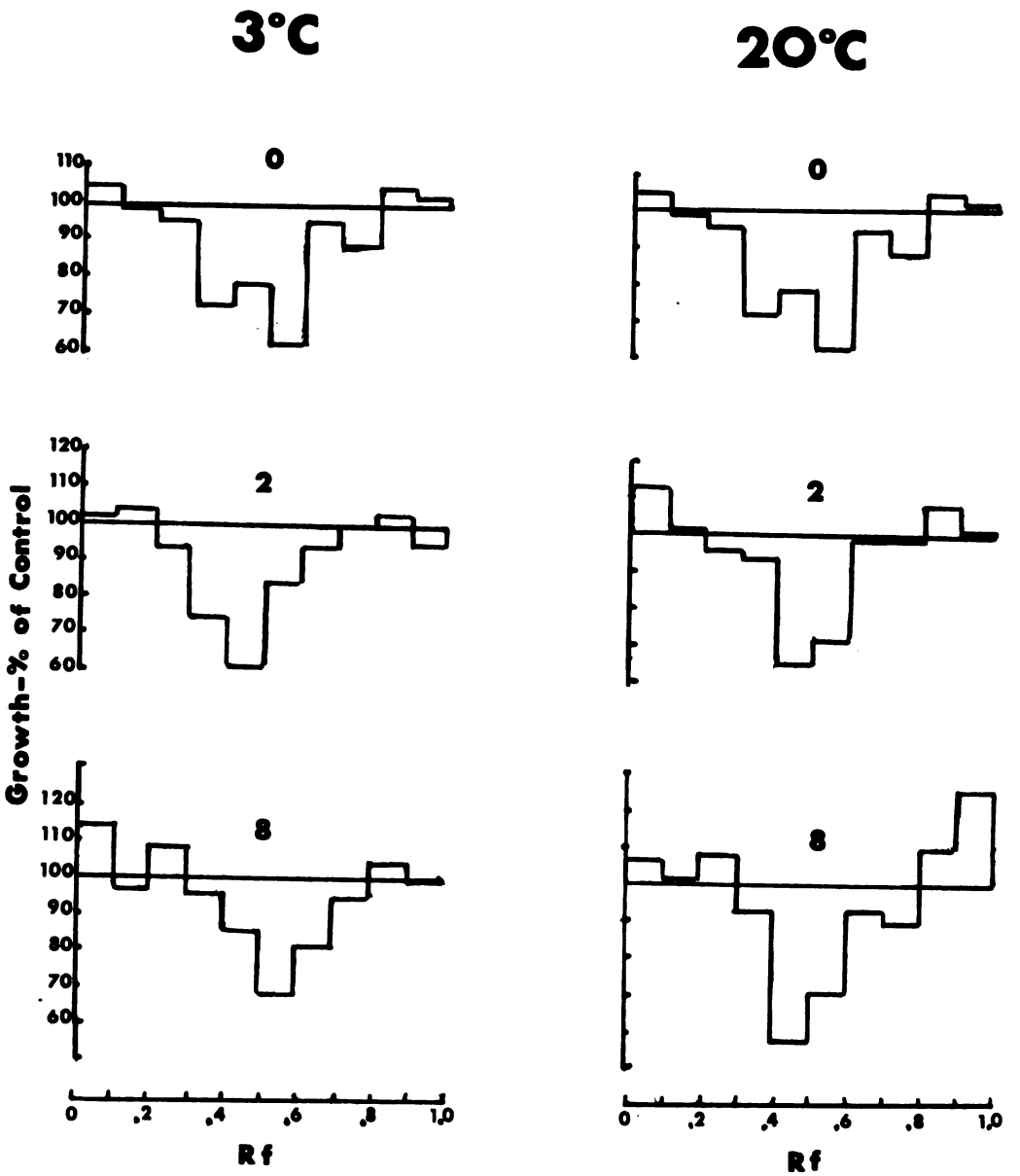

3°C
20°C
0
2
8
Growth-% of Control
110
100
90
80
70
60
120
0
.2
.4
.6
.8
1.0
Rf

Figure 12. Inhibitor levels in the acid fraction of 'Halford seed coats during stratification at 3 or 20°C. Each point is the average of 4 replications of 4 seeds each. The wheat coleoptile bioassay was used to detect inhibitors. Points with no letters in common are significantly different from one another at the 5% level using Duncan's Multiple Range Test (35).

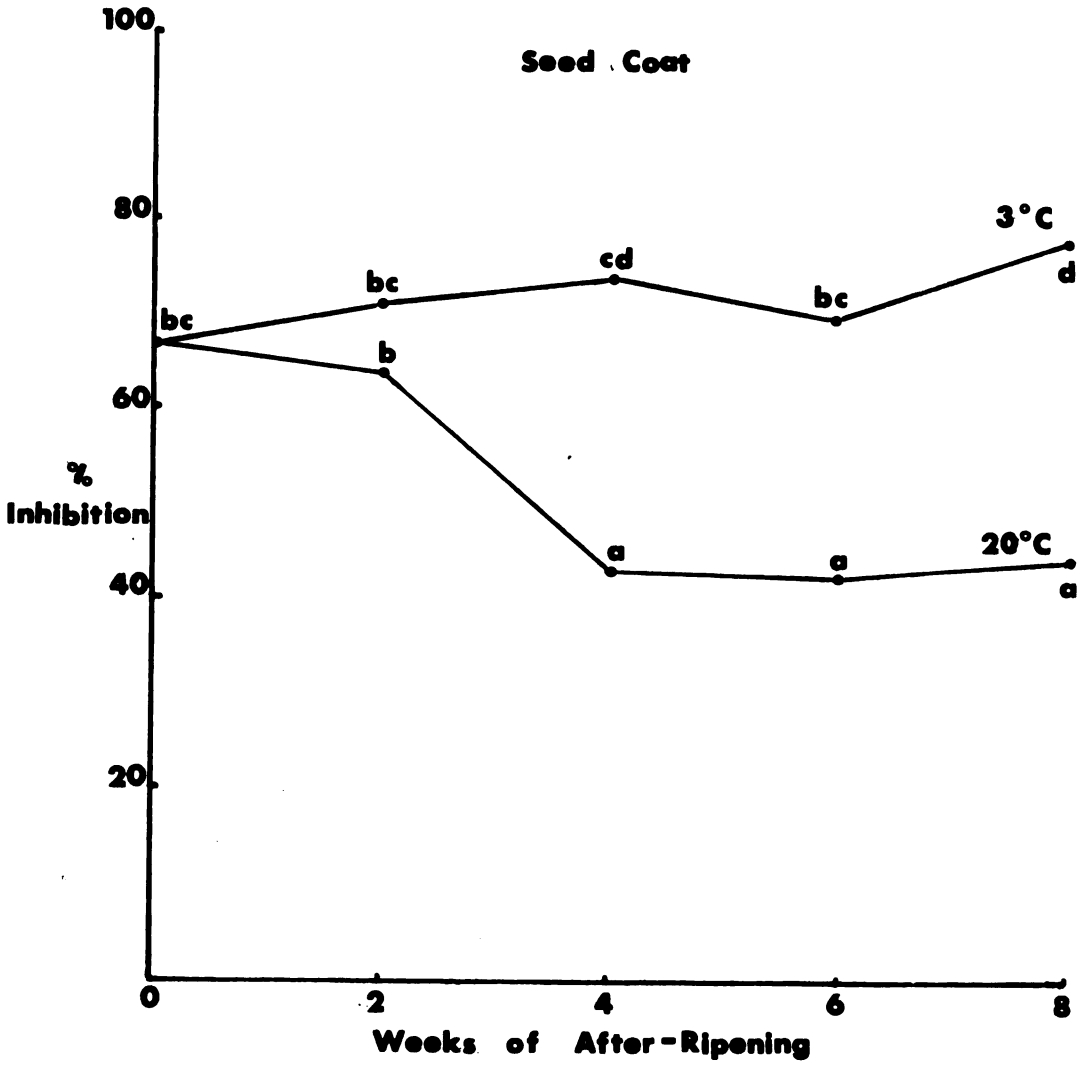
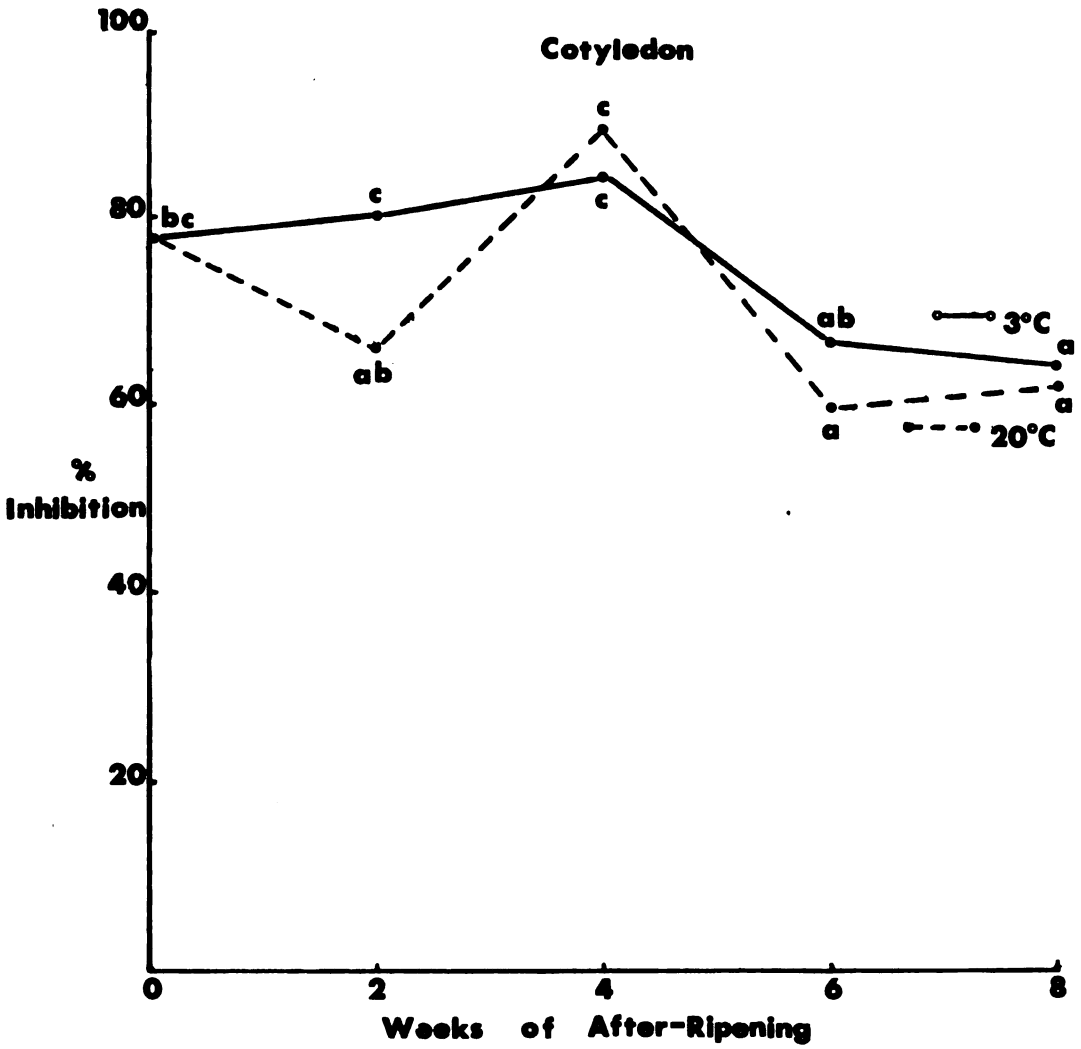

Seed Coat
100
80
60
40
20
% Inhibition
3°C
20°C
bc
bc
cd
bc
d
bc
b
a
a
a
0
2
4
6
8
Weeks of After-Ripening

Figure 13. Inhibitor levels in the acid fraction of 'Halford' cotyledons during stratification at 3 or 20°C. Each point is the average of 4 replications of 4 seeds each. The wheat coleoptile bioassay was used to detect inhibitors. Points with no letters in common are significantly different from one another at the 5% level using Duncan's Multiple Range Test (35).


Cotyledon
100
80
60
40
20
% Inhibition
bc
c
c
c
ab
ab
a
a
3°C
20°C
0
2
4
6
8
Weeks of After-Ripening

Histograms representing inhibitor levels in the acid fraction of embryonic axes of 'Halford' peach seeds stratified at 3 or 20°C for 0, 2, and 8 weeks are illustrated in Figure 11. Each histogram represents 50 embryonic axes (approximately 45 mg fr. wt.). In most cases the inhibitor had the same Rf as abscisic acid. The concentration was similar to that found in the seed coats. However, the results were somewhat variable, and an insufficient quantity of tissue prevented replication.

To obtain more quantitative data chromatogram sections in the inhibitory zone in subsequent comparisons were combined to give one value for each sample (Figures 12 and 13). Each point represents an average of four replications. In the seed coats (Figure 12) the levels of inhibitor decreased during stratification at 20°C but not at 3°C. In the cotyledons (Figure 13) a small decrease occurred during both cold and warm temperature stratification.

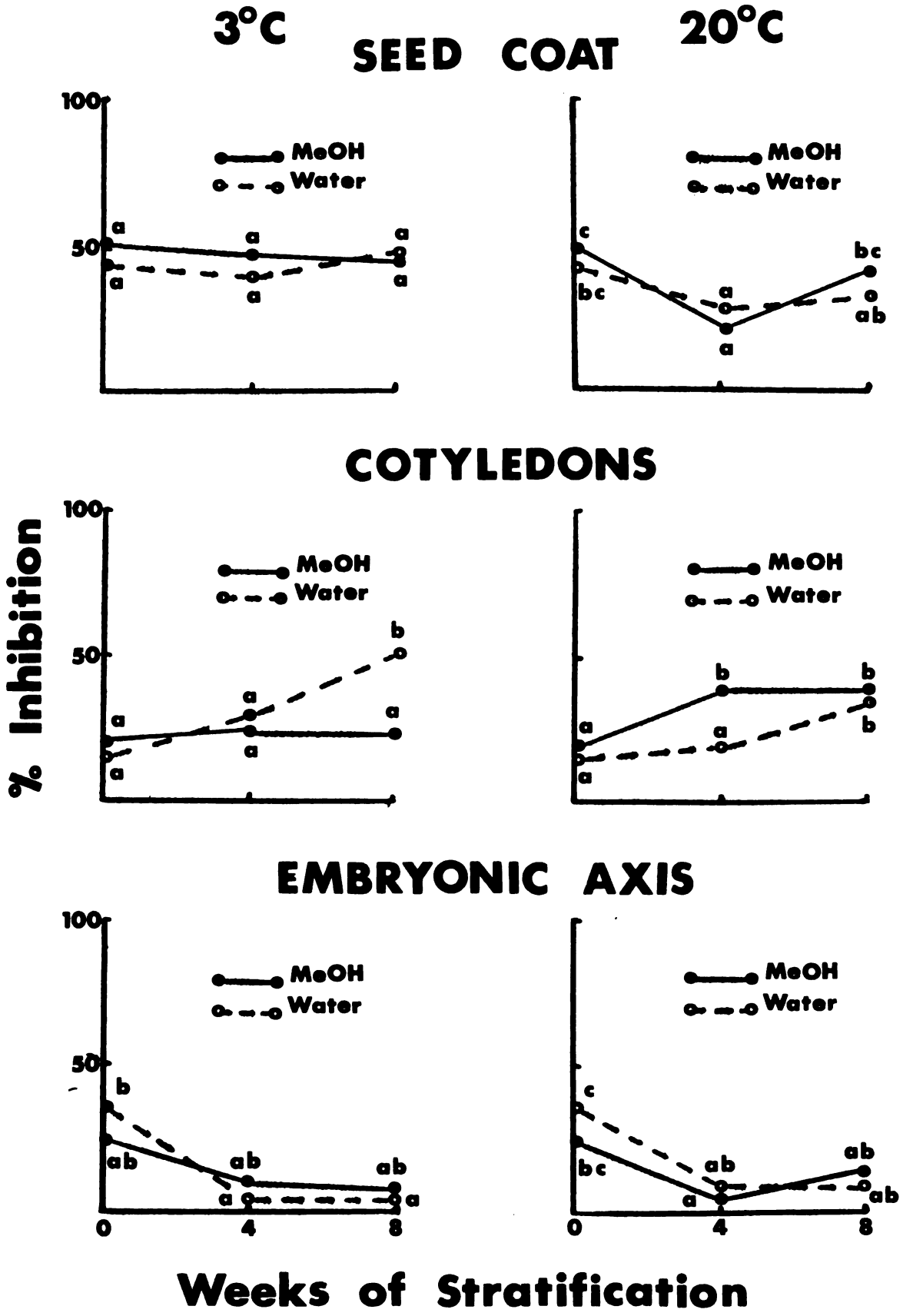
II. Inhibitors in the Acid Fraction of 'Halford' Peach Seeds During Stratification--Second Year

The data just discussed indicated that there seemed to be one major inhibitor with the same Rf as abscisic acid in the acid fraction of 'Halford' peach seeds which did not decrease substantially during cold temperature stratification in either the cotyledons or seed coats.

For the second study, both water alone and 80% aqueous methanol were used for extraction. Water was used to test the possibility that cold stratification caused a "binding" of the inhibitor, thus, making it insoluble in water. This could explain the difference between the results obtained in the first year and those of Lipe and Crane (88), who used water for extraction.

Samples were taken at 0, 4, and 8 weeks during cold and warm temperature stratification and were separated into seed coats, cotyledons, and embryonic axes. Germination was similar to that of the first year study. Except for two points the activity of water extracts did not differ from that of 80% aqueous methanol extracts (Figure 14). The levels of inhibitors in the cotyledons and seed coats did not fall substantially during cold temperature stratification. In contrast, inhibitor levels in the embryonic axes exhibited a marked drop irrespective of the stratification temperature. Possibly a decrease of inhibitors can occur at a wide range of temperatures in the embryonic axes, but other changes, such as an increase in promoters, may also be necessary during cold temperature stratification. The data suggest that more detailed studies with embryonic axes are needed.

Figure 14. Levels of acidic inhibitors, extracted with either water or aqueous methanol, in 'Halford' seed coats, cotyledons, or embryonic axes during stratification at 3 or 20°C. Each point represents the average of 4 replications of 5 seeds each (seed coat and cotyledon) or 2 replications of 50 seeds each (embryonic axis). The wheat coleoptile bioassay was used to detect inhibitors. Within graphs points with no letters in common are significantly different from one another at the 5% level using Duncan's Multiple Range Test (35).

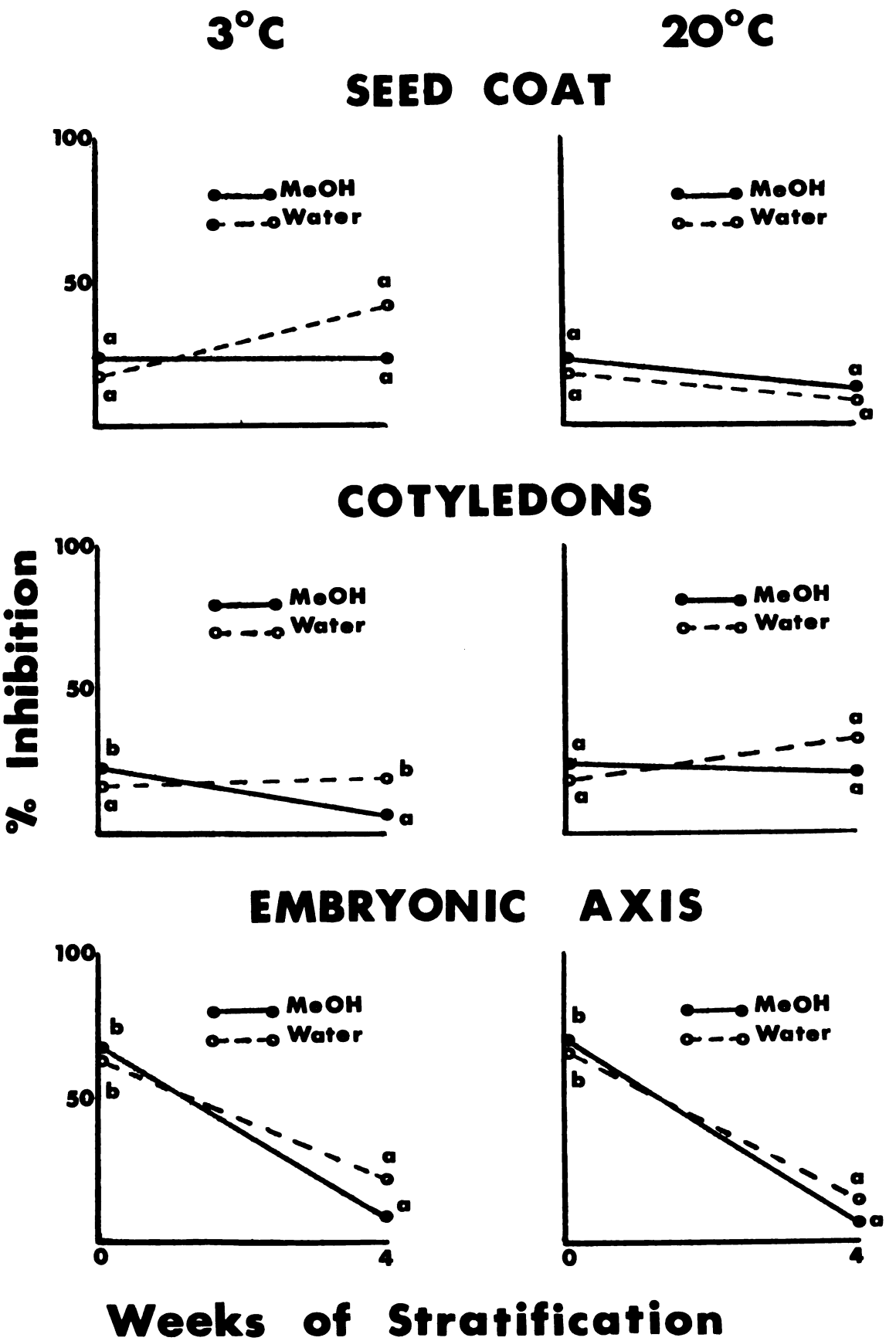

3°C
20°C
SEED COAT
COTYLEDONS
EMBRYONIC AXIS
% Inhibition
100
50
0
4
8
MeOH
Water
Weeks of Stratification

III. Inhibitors in the Acid Fraction of 'Okinawa' Peach Seeds During Stratification

To determine if the trend found with 'Halford' peach seeds (a variety with a medium stratification requirement) would also hold for 'Okinawa' seeds (a variety with a short stratification requirement), samples were taken at 0 and 4 weeks after the start of stratification.

The results were similar to those for 'Halford' seeds but were not as consistent (Figure 15). Germination at 0 and 4 weeks during cold stratification was 1.0 and 84.6% respectively. No increase in germination occurred during warm temperature stratification. In the seed coats no significant decrease in the levels of inhibitors occurred during stratification at either temperature. With the cotyledons the data were not as clear-cut. No decrease occurred at 20°C regardless of extraction solvent. At 3°C a decrease was observed in aqueous methanol extracts but not in water extracts. With the embryonic axes, however, the same trend was noted as in 'Halford' seeds. A marked decrease occurred at 3°C but a similar decrease also occurred at 20°C. The same explanation given for 'Halford' peach seeds concerning this decrease in the embryonic axes can also be applied to these results. Even though a decrease occurred in the warm temperature controls without a concurrent increase in germination, the decrease in the cold stratified seeds may still be necessary for

Figure 15. Levels of acidic inhibitors, extracted with either water or aqueous methanol, in 'Okinawa' seed coats, cotyledons, or embryonic axes during stratification at 3 or 20°C. Each point represents the average of 4 replications of 10 seeds each (seed coat and cotyledon) or 2 replications of 100 seeds each (embryonic axis). The wheat coleoptile bioassay was used to detect inhibitors. Within graphs points with no letters in common are significantly different from one another at the 5% level using Duncan's Multiple Range Test (35).


3°C
20°C
SEED COAT
COTYLEDONS
EMBRYONIC AXIS
% Inhibition
100
50
0
4
MeOH
Water
a
b
Weeks of Stratification

germination. Other reactions that occur during cold stratification may also be necessary. Again, the data suggest that more detailed studies with embryonic axes are needed.

IV. Bioassay of Inhibitors in All Fractions of Dormant 'Halford' Peach Seeds and the Color Tests for the Presence of Naringenin and Naringin

A means of separating abscisic acid and naringenin was sought since both of these growth inhibitors have been reported to affect dormancy. Abscisic acid and naringenin had similar Rf values on silica gel thin-layer plates developed in several solvent systems (Table 2). However, separation occurred on thin-layer plates developed in the upper phase of 8:3:5 benzene:acetic acid:water. Although no separation could be obtained on thin-layer plates developed in 10:1:1 isopropanol:ammonium hydroxide:water, slight separation was effected on paper in the same solvent system.

Naringenin and naringin were assayed with wheat coleoptile sections. A linear curve was obtained for naringenin between 0 and 30 µg (Figure 16). Growth was completely inhibited beyond this level. Naringin gave a weak response even at 1000 µg.

In order to bring the work on the acid fraction into the broad perspective of the whole seed all fractions underlined in Figure 2 were bioassayed with wheat

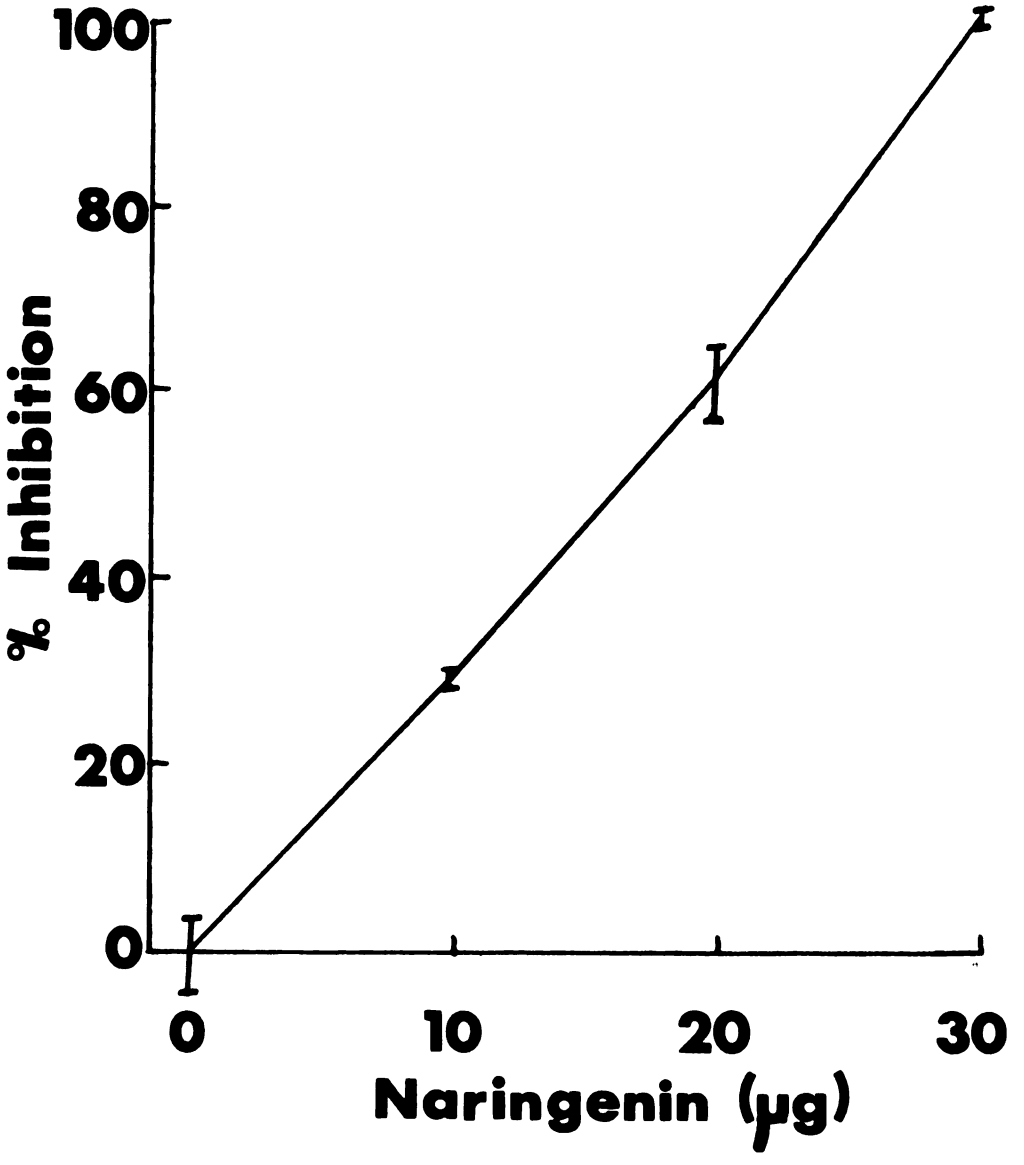
TABLE 2.--Effects of developing solvent and time and method of equilibration on Rf values of abscisic acid and naringenin on thin-layer and paper chromatograms. Each Rf value represents the average of at least two replications.

Developing solvent[a]	Method	Equilibration time (hours)	Tank lined	Rf values	
				Naringenin	ABA[b]
1	(Thin-layer)	30	No	0.70	0.71
		12	Yes	0.42	0.43
1	(Paper)	12	No	0.45	0.72
2	(Thin-layer)	30	No	0.78	0.77
		12	Yes	0.54	0.54
3	(Thin-layer)	30	No	0.0	0.04
					0.07
		12	Yes	0.1	0.02
					0.04
4	(Thin-layer)	12	Yes	0.83	0.74
4	(Paper)	12	No	0.95	0.95
5	(Thin-layer)	12	No	0.09	0.49
					0.56

[a]Solvent systems: (1) 10:1:1: isopropanol:NH_4OH:water; (2) 2:6:1:2 n-butanol:n-propanol:NH_4OH:water; (3) 100:100:1 $CHCl_3$:benzene:acetic acid; (4) 40:11:29 n-butanol:acetic acid:water; (5) 8:3:5 benzene:acetic acid:water (upper phase).

[b]Double values indicate separation of *cis*, *trans*-from *trans*, *trans*-abscisic acid.

Figure 16. Elongation response of 'Yorkstar' wheat coleoptile sections to various concentrations of naringenin. Each point represents the average of 2 replications whose values are indicated by the extremes.


100
80
60
40
20
0
% Inhibition
0
10
20
30
Naringenin (µg)

coleoptile sections after chromatography on both silica gel thin-layer plates and Whatman no. 1 paper in 10:1:1 isopropanol:ammonium hydroxide:water. Strong inhibition occurred only in the acid fraction (Figure 17-A), and the butanol-soluble fraction (Figure 17-B). The neutral-basic fraction (Figure 17-D) promoted growth of wheat coleoptile sections. The other two fractions (Figure 17-C, E) exhibited little biological activity.

The residue of the butanol-soluble fraction was viscous and could not be completely dried. This made chromatography difficult. The Rf of the inhibitor in this fraction may therefore be lower than would be the case for the pure compound(s). However, it is probably not abscisic acid because extensive washing with ethyl acetate should have removed most of this relatively non-polar acid. It could be the glucoside of abscisic acid or a phenolic glycoside, as these compounds would generally partition into this fraction.

Aliquots of the fractions above were chromatographed on thin-layer plates and paper together with abscisic acid, naringenin, and naringin. The chromatograms were sprayed with three reagents commonly used to detect phenolic compounds (Table 3). None of the fractions gave strong positive color reactions for naringenin or naringin. However, a very weak pink color was detected in the neutral-basic fraction (Figure 2-fraction D) at Rf 0.32 on paper and

Figure 17. Elongation response of wheat coleoptile sections to various fractions of an 80% aqueous methanol extract of unstratified 'Halford' peach seeds. Letters refer to fractions in Figure 2. All fractions were chromatographed on silica gel thin-layer plates and Whatman no. 1 paper in 10:1:1 isopropanol: ammonium hydroxide:water. The Rf of abscisic acid is indicated at the top.

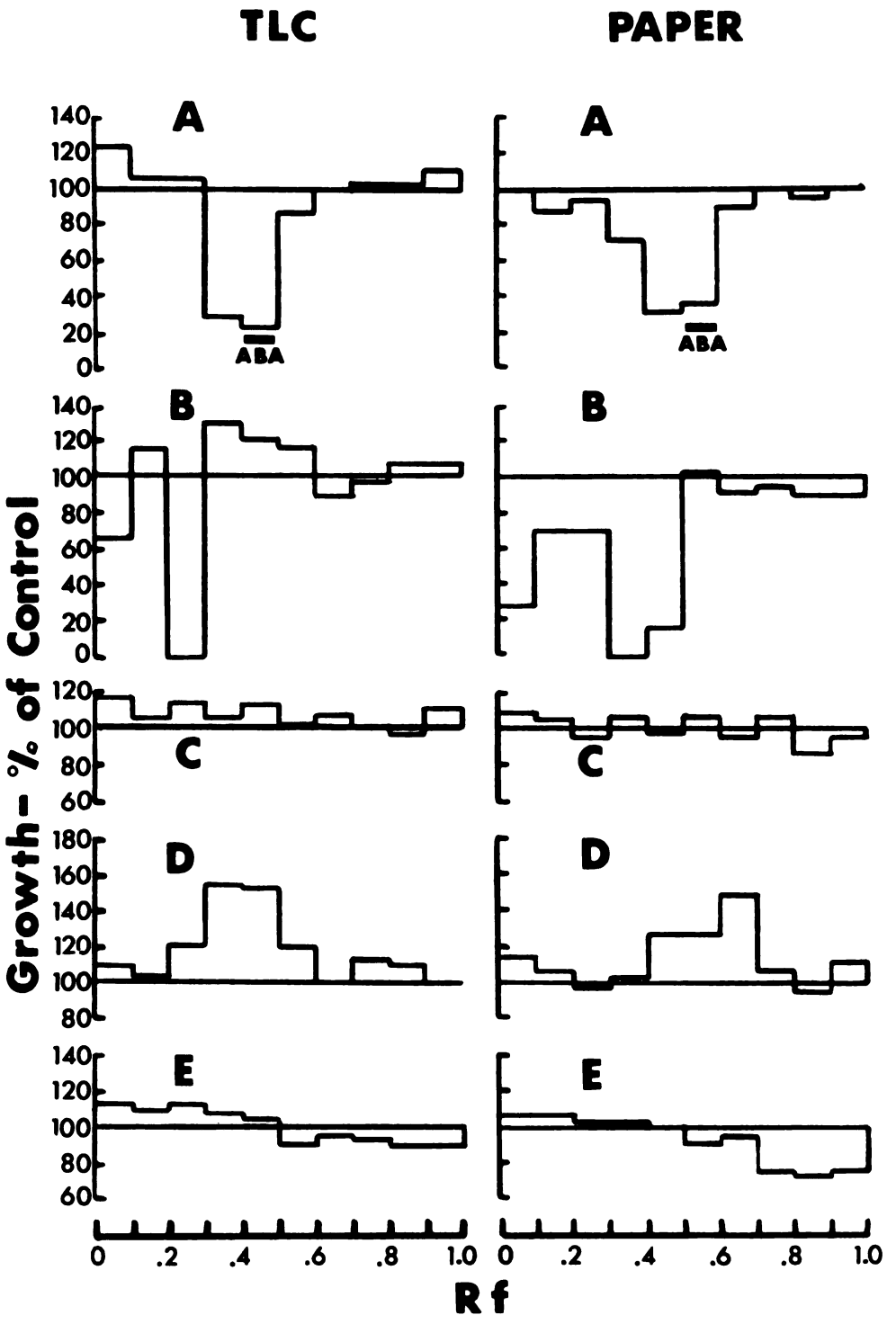

TLC
PAPER
A
B
C
D
E
ABA
Growth- % of Control
Rf
0
.2
.4
.6
.8
1.0

TABLE 3.--Color reactions[a] of naringin, naringenin, and abscisic acid with phenolic reagents following chromatography in 10:1:1 isopropanol:ammonium hydroxide: water.

Compound and Rf	No treatment			$AlCl_3$			NaOH followed by DPNA[c]			$NaBH_4$ followed by HCl
	V[b]	UV-S	UV-L	V	UV-S	UV-L	V	UV-S	UV-L	V
Paper chromatography										
Naringin (Rf .1-.3)	--	YGr	YGr	--	Gr	Gr	YBr	Y	--	Pi
Naringenin (Rf .4-.5)	--	--	--	--	Gr	Gr	YBr	YBr	YBr	Pi
ABA (Rf .5-.6)	--	B	--	--	--	--	--	--	--	--
Thin-layer chromatography										
Naringin (Rf .1-.2)	--	Y	Gr	Y	BlGr	YGr	YO	Gr	Y	Pi
Naringenin (Rf .4-.5)	--	Y	Gr	Y	BlGr	Gr	YO	B	Br	Pi
ABA (Rf .4-.5)	--	B	--	--	B	--	--	B	--	--

[a]Color: B-black, Bl-blue, Br-brown, Gr-green, O-orange, Pi-pink, and Y-yellow.

[b]Light source: V-visible, UV-S-short ultraviolet, and UV-L-long ultraviolet.

[c]DPNA-diazotized p-nitroaniline.

Rf 0.2 on thin-layer plates when sprayed with sodium borohydride followed by hydrochloric acid fumes. The intensity of this pink color, if assumed to be naringenin or naringin, was approximated to be less than 3 µg of naringenin or naringin. This would not account for more than ten per cent inhibition of wheat coleoptile sections if the compound was naringenin and no inhibition if the compound was naringin. In addition, the color reaction occurred at an Rf different from that of naringenin and this fraction promoted instead of inhibited growth of wheat coleoptile sections. The absence of a positive reaction in the acidic and butanol fractions indicated that the inhibitory activity observed in these fractions could not be due to naringenin or naringin.

V. Identification of the Inhibitor in the Acid Fraction

Initial Studies

Since the inhibitor had the same Rf value as abscisic acid in 10:1:1 isopropanol:ammonium hydroxide: water and since the relative concentrations were similar to the expected levels of abscisic acid, further work was designed to determine if this inhibitory compound was indeed abscisic acid.

In the first study the two methods used were spectropolarimetry and gas-liquid chromatography. An extract of unstratified seed coats was prepared as described in

the materials and methods. The acidic fraction was chromatographed and bioassayed, and the highest inhibitory activity was found at Rf 0.4-0.5 followed by Rf 0.5-0.6 and low activity was found at Rf 0.3-0.4. Optical rotatory dispersion (ORD) curves of eluates of these zones are shown in Figure 18. The maxima and the minima are similar to those reported for abscisic acid (91) at Rf 0.4-0.5 and 0.5-0.6. The greatest dispersion was noted at Rf 0.4-0.5. Interfering compounds at Rf 0.3-0.4 prevented meaningful interpretation. This interference was not surprising since high concentrations of fluorescent compounds were noted at that Rf.

These same samples were methylated and analyzed for abscisic acid using gas-liquid chromatography (Figure 19). A peak with a retention time similar to that of cis-trans abscisic acid was observed in all three samples. However the quantities were much higher than were expected from bioassay data. In addition the mass spectra did not correspond to that of abscisic acid.

Whole 'Halford' peach seeds were next extracted and the acidic fractions chromatographed and bioassayed. Samples were taken at 0 and 6 weeks after the start of stratification at 3°C. The germination of seeds after endocarp removal was 3% at 0 weeks and 82% at 6 weeks. As was expected, the level of the inhibitor, as measured by

Figure 18. ORD spectra of eluates from thin-layer chromatograms of the acid fraction of unstratified 'Halford' seed coats. The chromatogram was developed in 10:1:1 isopropanol:ammonium hydroxide:water.

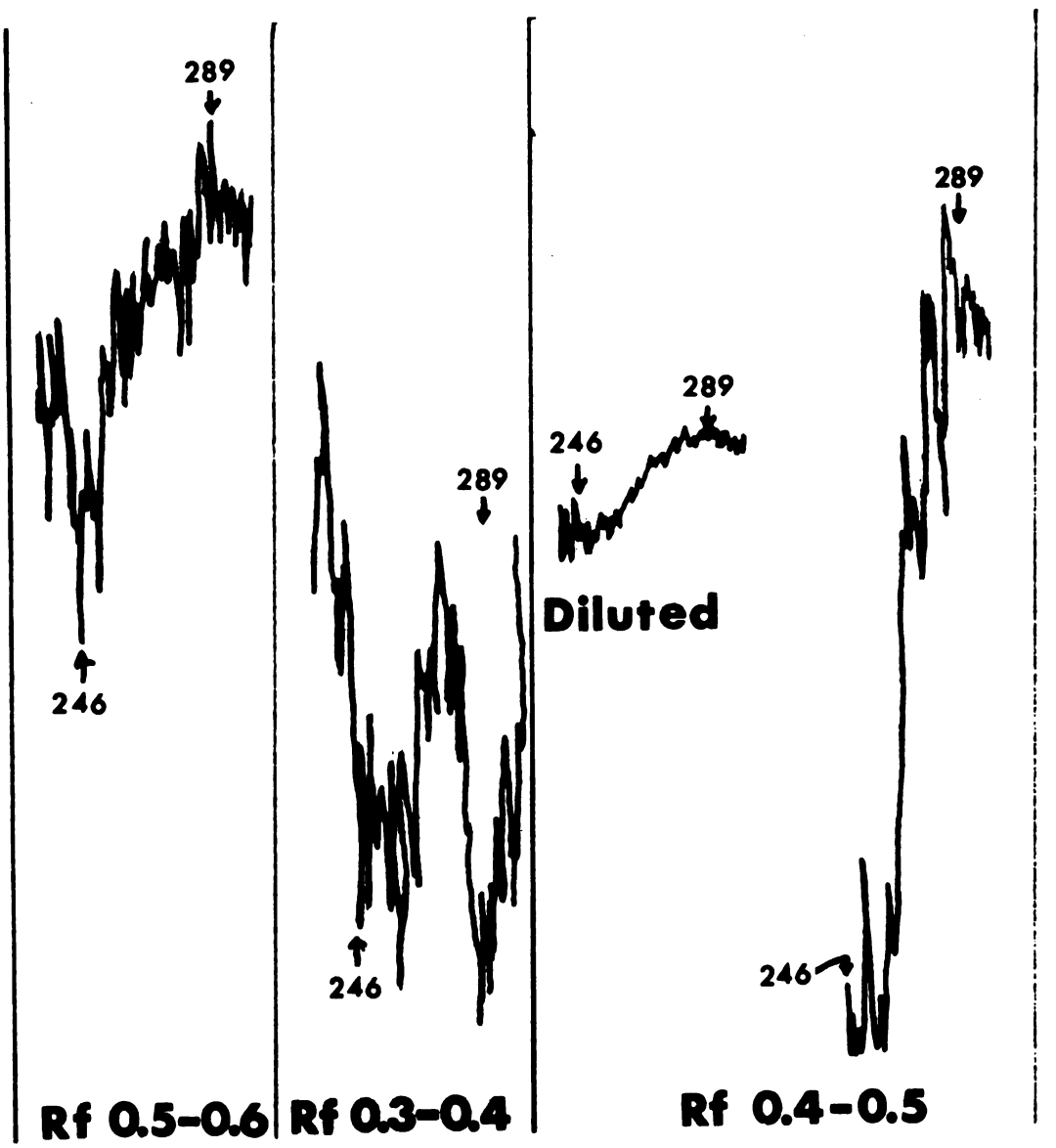

289
246
Rf 0.5-0.6
289
246
Rf 0.3-0.4
289
246
Diluted
289
246
Rf 0.4-0.5

Figure 19. Gas-liquid chromatograms of methylated samples of abscisic acid and of eluates from sections of a thin-layer chromatogram of the acid fraction of unstratified 'Halford' peach seed coats. The chromatogram was developed in 10:1:1 isopropanol:ammonium hydroxide:water. See materials and methods for parameters of the gas-liquid chromatograph. Cis-trans abscisic acid has a retention time of 1.37 minutes.

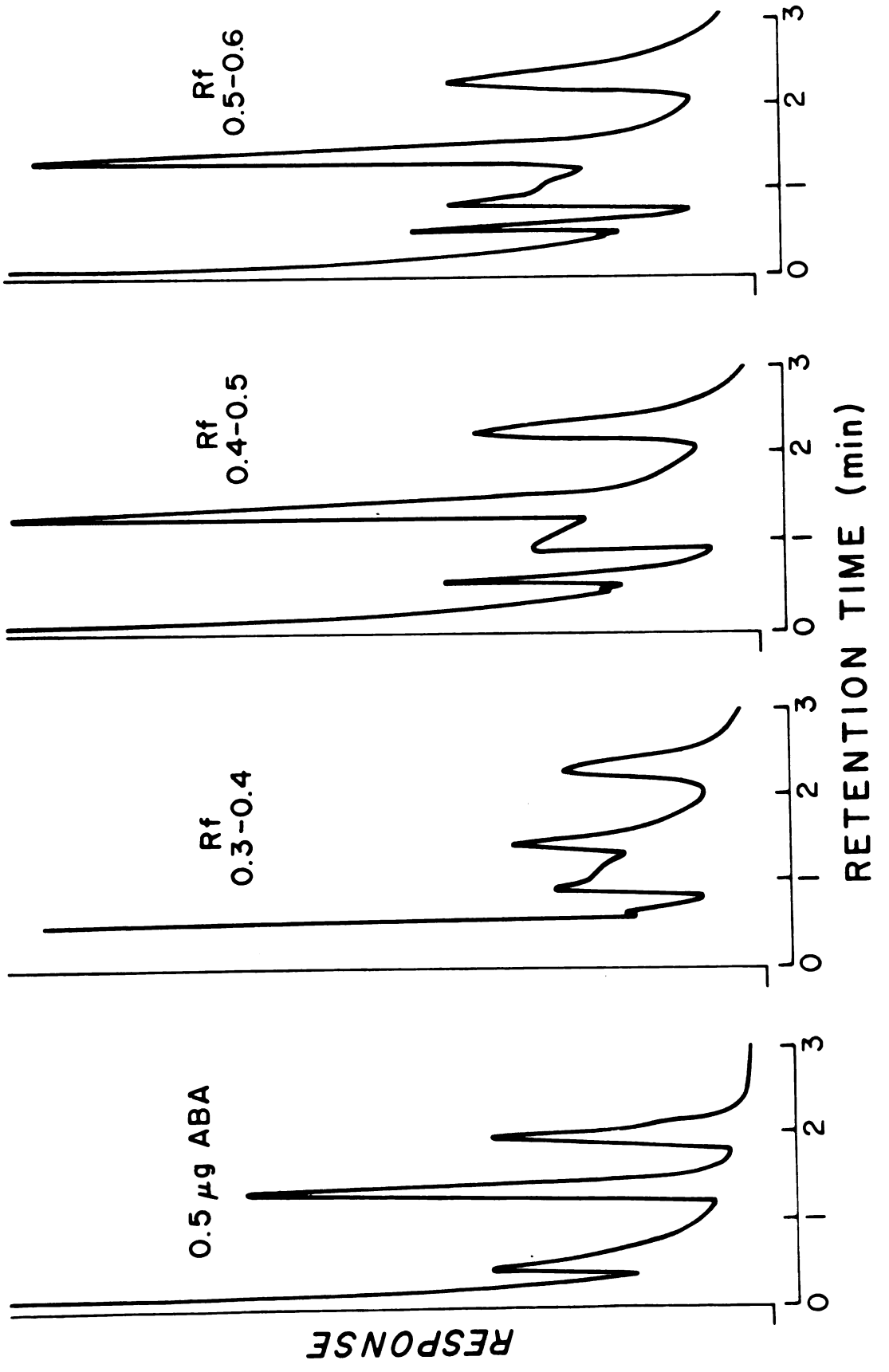

0.5 µg ABA
Rf 0.3-0.4
Rf 0.4-0.5
Rf 0.5-0.6
RESPONSE
RETENTION TIME (min)
0
1
2
3

Figure 20. Elongation response of 'Yorkstar' wheat coleoptile sections to eluates from thin-layer chromatograms of the acid fraction of whole 'Halford' seeds stratified at 3°C for 0 and 6 weeks. The chromatograms were developed in 10:1:1 isopropanol:ammonium hydroxide:water. There was no difference between 0 and 6 weeks at the 5% level of significance.

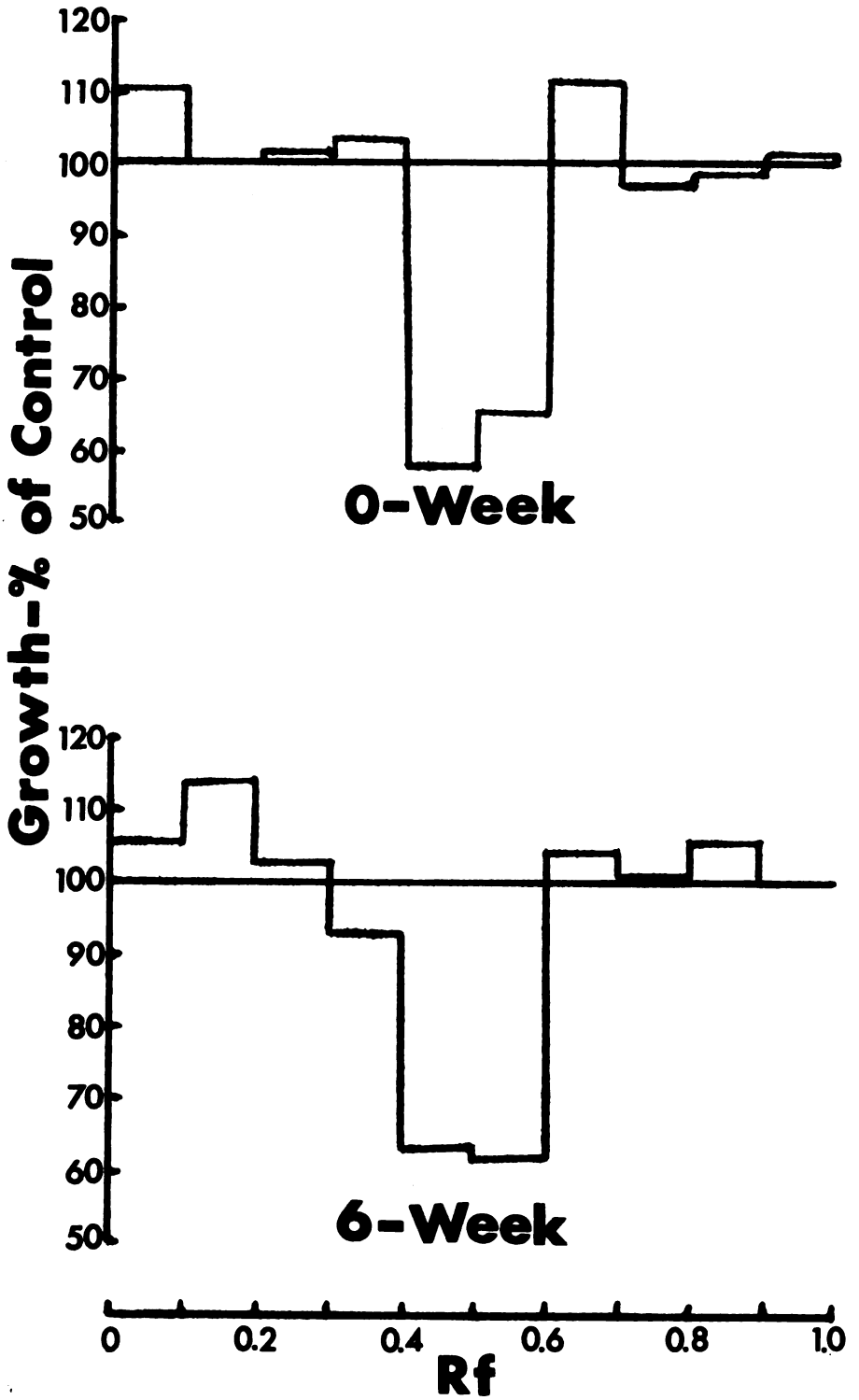

120
110
100
90
80
70
60
50
0-Week
Growth-% of Control
120
110
100
90
80
70
60
50
6-Week
0
0.2
0.4
0.6
0.8
1.0
Rf

Figure 21. UV spectra of eluates of thin-layer chromatograms of the acid fraction of whole 'Halford' seeds stratified at 3°C for 0 and 6 weeks. The chromatograms were developed in 10:1:1 isopropanol:ammonium hydroxide:water. Each curve represents one sample of 200 seeds.

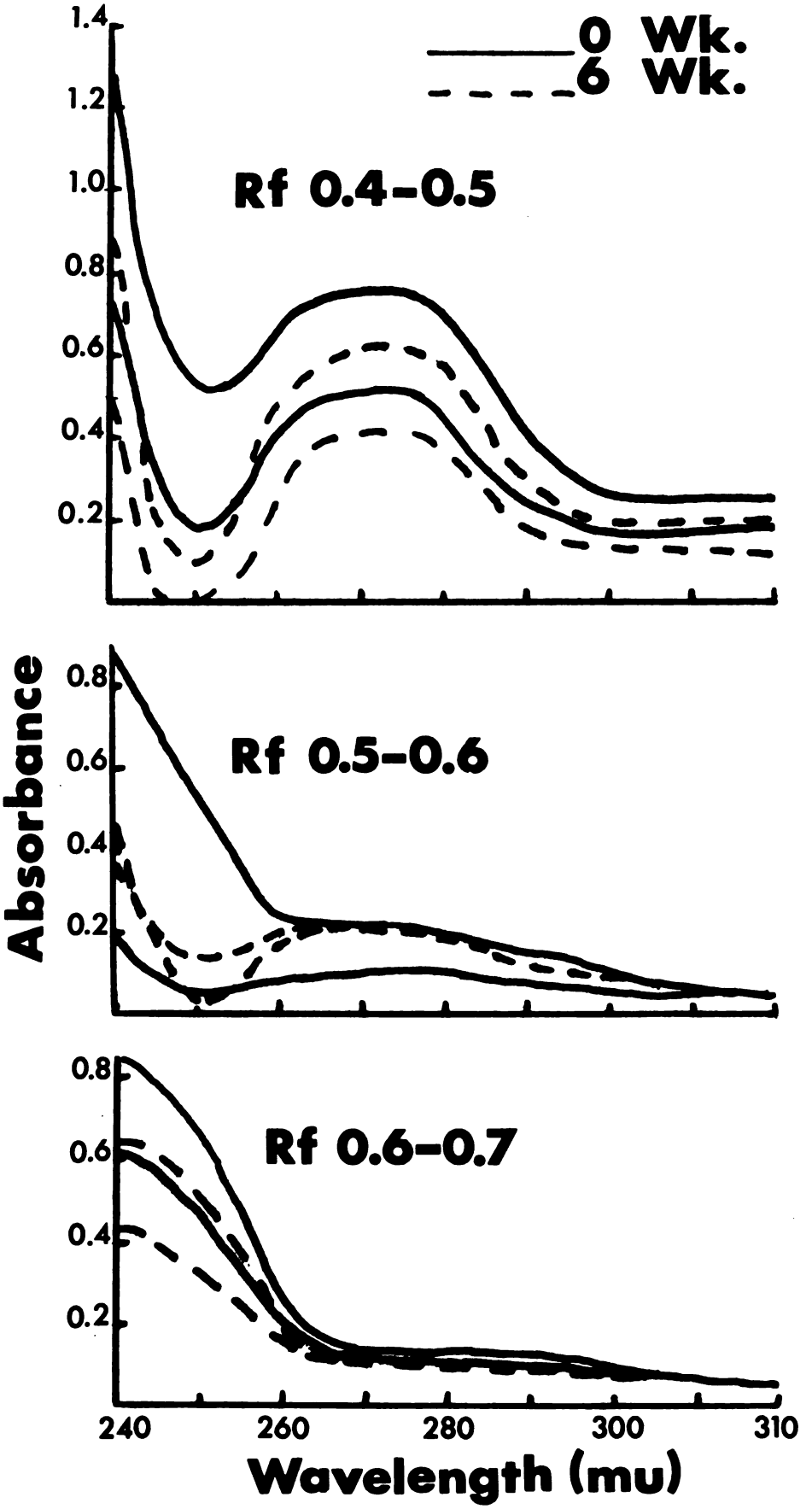
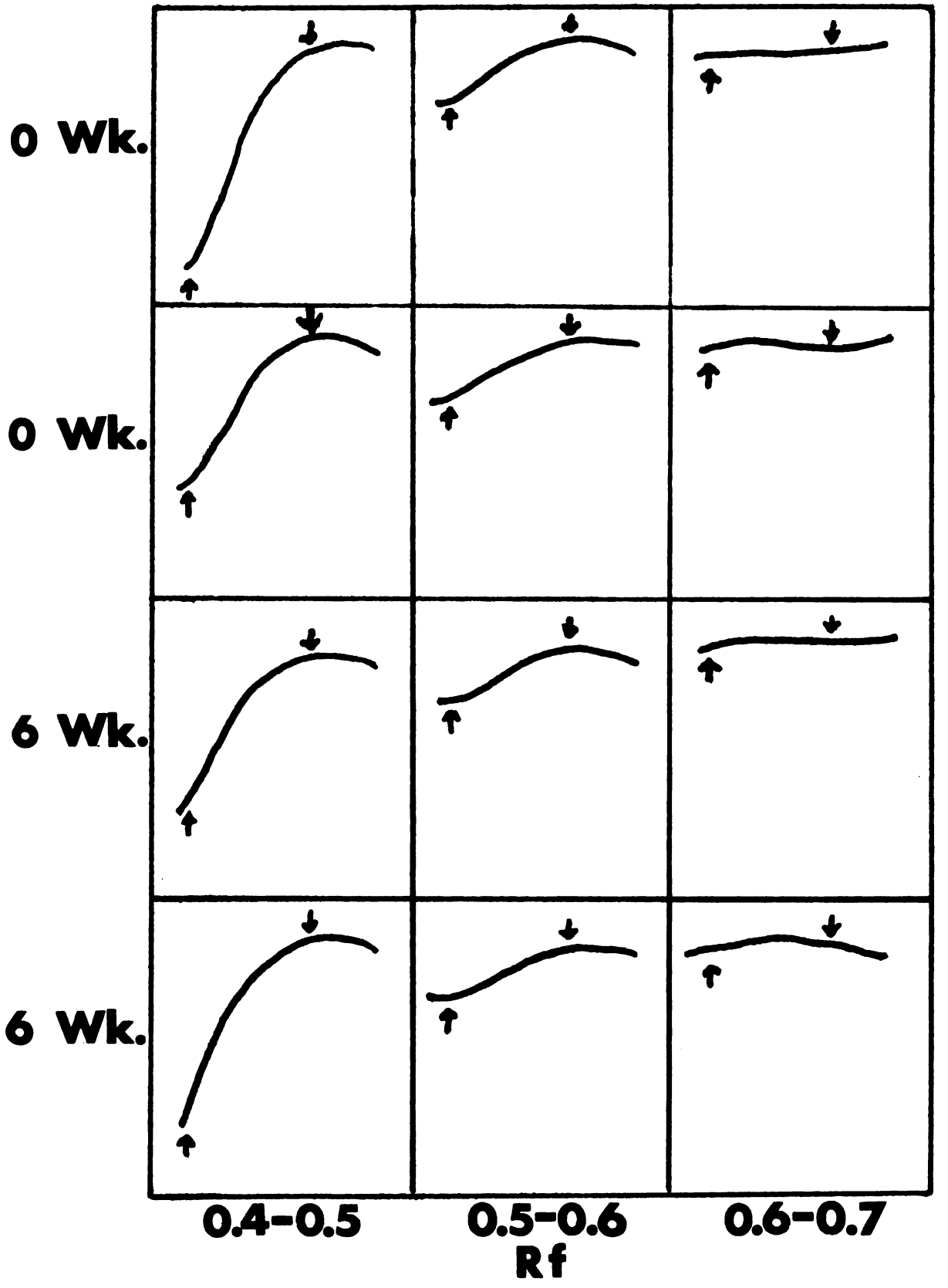

0 Wk.
6 Wk.
Rf 0.4–0.5
Rf 0.5–0.6
Rf 0.6–0.7
Absorbance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
240
260
280
300
310
Wavelength (mu)

Figure 22. ORD spectra of eluates of thin-layer chromatograms of the acid fraction of whole 'Halford' seeds stratified at 3°C for 0 and 6 weeks. The chromatograms were developed in 10:1:1 isopropanol:ammonium hydroxide:water. Each curve represents one sample of 200 seeds. The arrows indicate points of maximum (arrow on the right at 289 mμ) and minimum (arrow on the left at 246 mμ) dispersion for abscisic acid.


0 Wk.
0 Wk.
6 Wk.
6 Wk.
0.4-0.5
0.5-0.6
0.6-0.7
Rf

bioassay, did not decrease during cold temperature stratification (Figure 20).

The ultraviolet (Figure 21) and ORD (Figure 22) spectra of the eluates were then obtained. The spectra for the 0 and 6 weeks treatments are very similar in both cases. The ultraviolet spectra of the eluates from Rf 0.4-0.5 showed a maximum at approximately 270 mμ. The maximum for abscisic acid should be at 260 mμ. The absorption of the thin-layer blank, which was much higher at 260 mμ than at 270 mμ, could have affected the accuracy of the readings. Small differences between background of actual samples and the reference blank could have shifted the maximum from 260 to 270 mμ. Also, further purification of the sample would have been desirable. Nevertheless, a consistent difference between samples from unstratified and stratified seeds was not observed. The ORD data for the same samples (Figure 22) also showed little difference between unstratified and stratified seeds. The highest response was again obtained at Rf 0.4-0.5, followed by Rf 0.5-0.6. The ORD data therefore paralleled the ultraviolet absorption data.

Further Studies

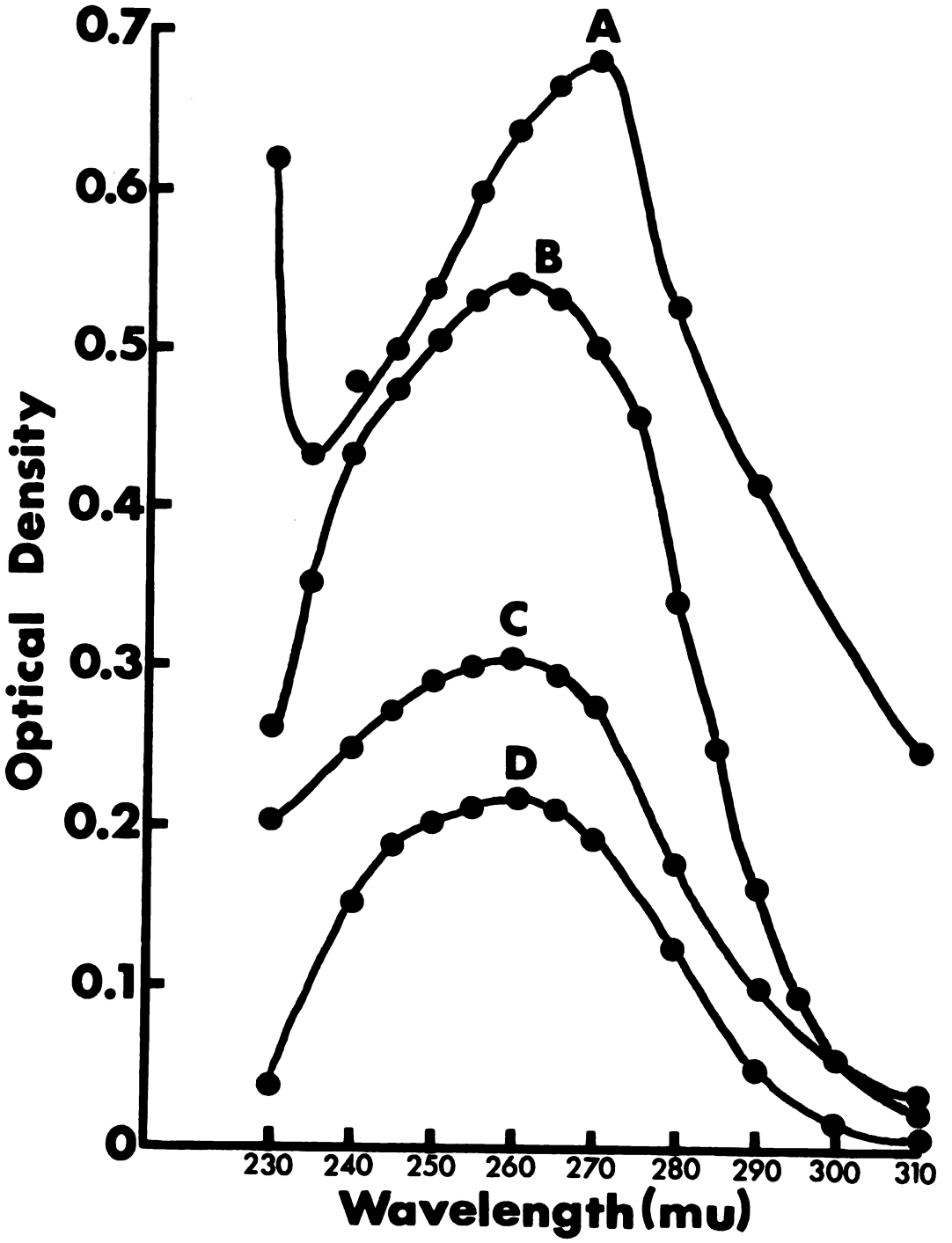
To clarify some of the initial results, solvent blanks and standard abscisic acid were treated as follows: (1) no treatment; (2) thin-layered on Eastman silica gel plates 100 μ thick with binder and fluorescent indicator; (3) thin-layered on plates 250 μ thick made with EM

Reagents silica gel H without binder or fluorescent indicator; and (4) processed to the acid fraction as shown in Figure 2. Ultraviolet spectra were then obtained, followed by methylation and analysis with gas-liquid chromatography.

The ultraviolet spectra indicated that substantial amounts of abscisic acid were lost in chromatography, but that the procedure did not increase the background appreciably or shift the wavelength of maximum absorption (Figure 23). However, during partitioning to the acid fraction there was not only a loss of abscisic acid but also a shifting of the maximum from 260 to 270 mμ (Figure 23). In the initial study samples suspected of containing abscisic acid often had an ultraviolet maximum of 270 mμ (Figure 21). The partitioning procedure may therefore be the source of trouble. It was not known if the problem was due to background or a conversion of abscisic acid to another form. Since later gas-liquid chromatography and gas-liquid chromatography-mass spectrometry analysis indicated that abscisic acid was present in the seed samples as unaltered abscisic it was unlikely that the shift was due to background from solvent residues.

These same samples were methylated and analyzed with a gas-liquid chromatograph with a flame ionization detector. Chromatography on either of the two types of silica gel plates or methylation alone did not cause a

Figure 23. Effects of solvent-solvent partitioning and thin-layer chromatography upon recovery and UV spectral characteristics of abscisic acid. UV spectra of abscisic acid: A--processed to the acid fraction as shown in Figure 2; B--neither partitioned nor chromatographed; C--chromatographed on Eastman silica gel thin-layer chromatograms (100 μ thick) with fluorescent indicator and eluted with ethanol; and D--chromatographed on EM Reagents silica gel H thin-layer chromatograms (250 μ thick) without binder and fluorescent indicator and eluted with ethanol. All samples were dissolved in 0.05 N formic acid in ethanol.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Optical Density
A
B
C
D
230
240
250
260
270
280
290
300
310
Wavelength (mu)

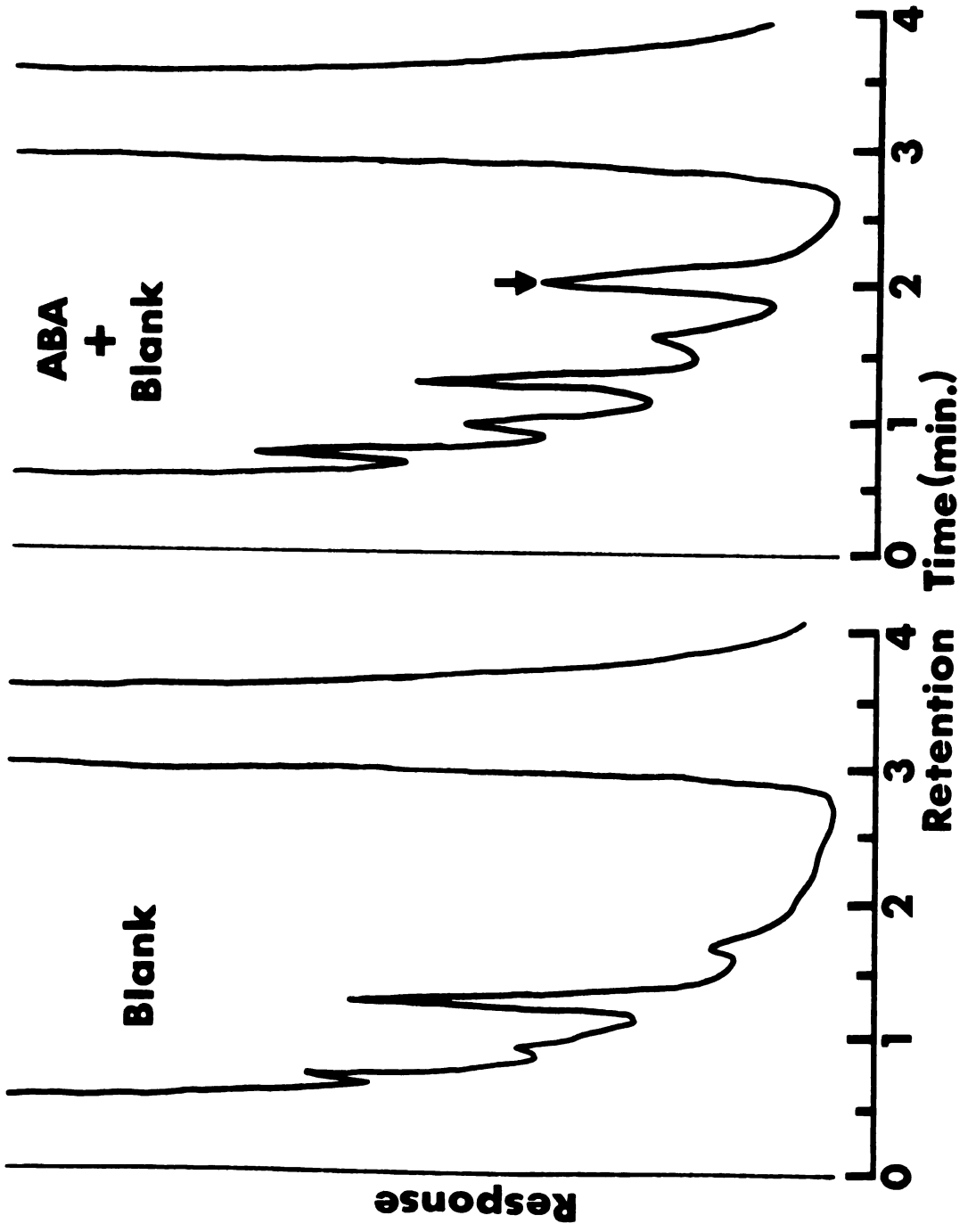
change of retention time of abscisic acid or give rise to interfering peaks. However, loss of abscisic acid was again noted during chromatography, and many high background peaks were present after the partitioning procedure to the acid fraction (Figure 24). Fortunately, none of these peaks coincided with cis-trans abscisic acid.

The acid fraction of dormant 'Halford' peach seeds was chromatographed on both Eastman silica gel thin-layer plates 100 μ thick containing binder and fluorescent indicator, and EM Reagents silica gel H thin-layer plates 250 μ thick containing no binder or fluorescent indicator. Aliquots of these samples were then bioassayed or methylated and analyzed with gas-liquid chromatography. Inhibition of wheat coleoptile sections occurred most at Rf 0.4-0.5, less at Rf 0.5-0.6, and little or none at Rf 0.2-0.4 on both types of plates. The dosage-response curve was log-linear, as is characteristic of abscisic acid (Table 4).

TABLE 4.--Linear elongation response of wheat coleoptile sections to logarithmic concentrations of the inhibitor from the acid fraction of 'Halford' peach seeds.

Peach seed equivalents	Average length of coleoptile sections (mm x 7.5)
0.5	68.4
5.0	55.0
50.0	43.0

Figure 24. Gas-liquid chromatograms of a methylated blank with and without abscisic acid after processing through the partitioning procedure to the acid fraction. Gas chromatograph conditions are given in Table 5 in the column listing 2% QF 1 on Gas Chrom Q. Arrow indicates retention time of abscisic acid.


Response
Blank
ABA
+
Blank
0
1
2
3
4
Retention Time (min.)

When eluates of Rf 0.3-0.5 of the Eastman plates were gas chromatographed, a large peak with a retention time slightly less than that of abscisic acid was observed (Figure 25-B). Estimates of abscisic acid equivalents based upon peak area were much larger than those based upon biological activity. A similar peak was observed in the initial study. A shoulder, with a retention time identical with that of abscisic acid, consistently occurred on this peak in the eluate from Rf 0.4-0.5, which exhibited the highest biological activity. A small peak occurred in the eluate from Rf 0.5-0.6, the quantity agreeing closely with the quantity of abscisic acid estimated by bioassay.

When eluates from Rf 0.3-0.4 of the EM Reagents thin-layer plates were gas chromatographed, a high peak occurred close to the retention time of abscisic acid (Figure 25-A). However, estimates of abscisic acid equivalents based upon peak area were much larger than those based upon biological activity, indicating lack of separation between an unknown compound and abscisic acid. In eluates from Rf 0.4-0.5, a peak occurred at the retention time of abscisic acid. Biological activity agreed with the quantity of abscisic acid estimated from peak area. This fraction was gas chromatographed on two additional columns and a distinct peak occurred on both at the precise retention time of cis-trans abscisic acid (Table 5). In addition gas-liquid chromatography-mass spectrometry was

Figure 25. Gas-liquid chromatograms of methylated eluates from two types of thin-layer chromatograms spotted with the acid fraction of dormant 'Halford' peach seeds. The two types of plates were: A--EM Reagents silica gel H (250 μ thick) with no indicator or binder, and B--Eastman silica gel thin-layer plates (100 μ thick) with fluorescent indicator and binder. Arrows indicate retention time of the methyl ester of cis-trans abscisic acid (1.96 minutes).

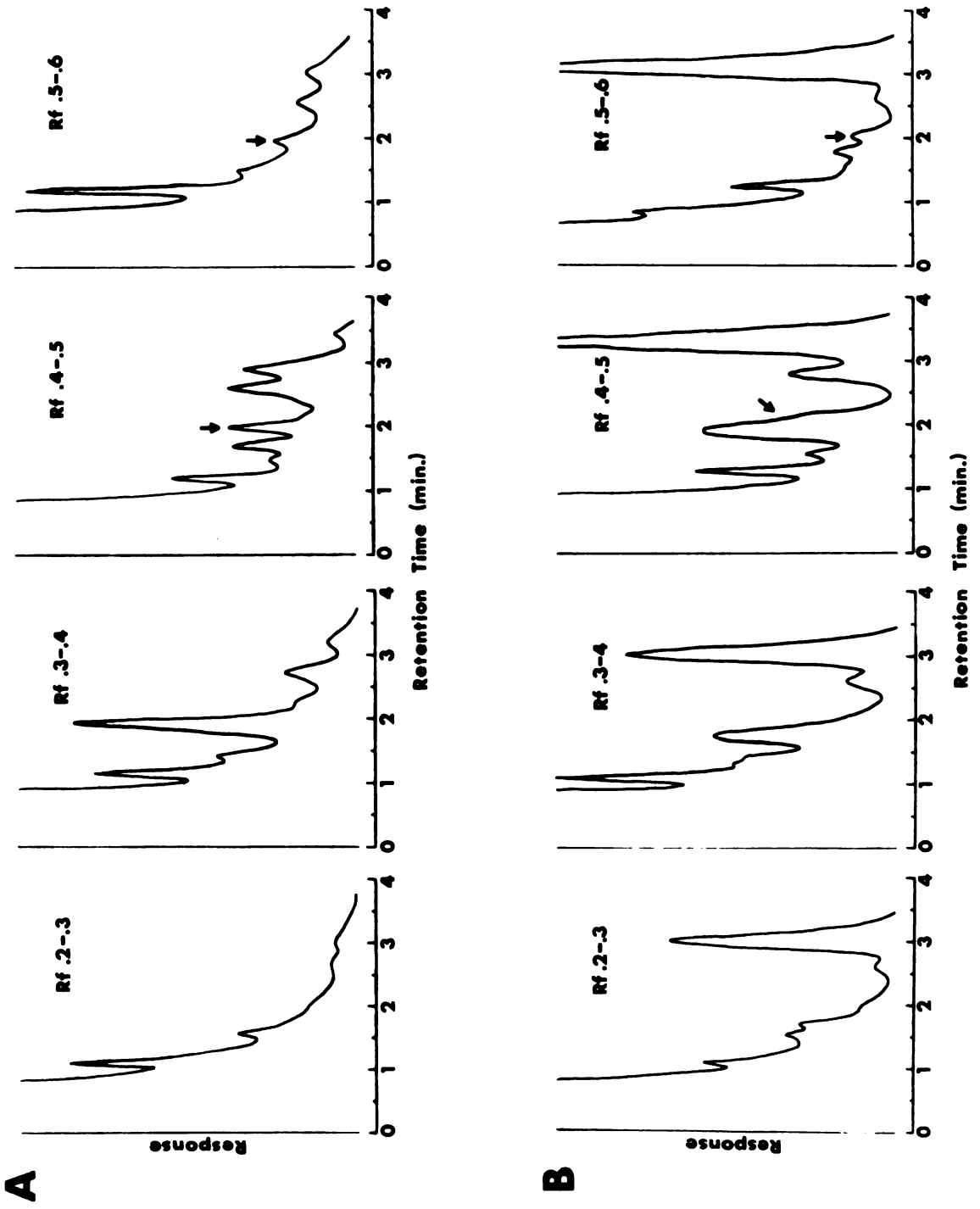

A
B
Response
Rf .2-.3
Rf .3-.4
Rf .4-.5
Rf .5-.6
0
1
2
3
4
Retention Time (min.)

TABLE 5.--Conditions of gas chromatograph and retention times of methylated samples of an extract of dormant 'Halford' peach seeds and of cis-trans abscisic acid. The seed sample represents the acid fraction after chromatography on EM Reagents silica gel H and elution from Rf 0.4-0.5.

GLC Make	Packard 7000 Series	Packard 7000 Series	LkB 9000 Gas Chromatograph-Mass Spectrometer
Column	6 ft U-shaped glass column 2 mm i.d.	6 ft U-shaped glass column 2 mm i.d.	6 ft spherical glass column 3 mm i.d.
Solid Support	Chromosorb W (80/100 mesh)	Gas Chrom Q (60/80 mesh)	Supeleo-port (80/100 mesh)
Liquid Phase	2% DC 200 (12500 cslk)	2% QF 1	3% SE-30
Detector	Electron Capture (15 mCi Ni^{63} at 5 V)	Flame Ionization	Total Ion Current
Gas Flow--H_2		30 ml/min	
N_2	30 ml/min	40 ml/min	
Air		375 ml/min	
He			30 ml/min
Temperature			
Inlet	250°C	240°C	220°C
Column	210°C	210°C	200°C
Detector	250°C	240°C	290°C
Attentuation	1×10^{-8}	3×10^{-10}	
Retention Time			
cis-trans ABA	1.46 min	1.96 min	4.34 min
Sample	1.46 min	1.96 min	4.34 min

used in an attempt to identify the compound with the same retention time as the methyl ester of cis-trans abscisic acid, as well as the largest peak. The latter had a retention time of 7.3 minutes using 2% QF 1 on Gas Chrom Q (Figure 26), and 6.05 minutes using 3% SE-30 on Supeleoport. Mass spectrometry confirmed the identification of the first compound as cis-trans abscisic acid (Table 6). Its molecular ion (parent peak) had a mass of 278, identical with that of the methyl ester of abscisic acid. In addition it had the same base peak and the same major peaks with similar relative abundances as abscisic acid.

The molecular ion of the compound responsible for the large peak was 294, 16 mass units (mass of oxygen is 16) higher than the methyl ester of abscisic acid (Table 6). The base peak was 43 and many of the major peaks of the methyl ester of abscisic acid were present. One fact should be considered at this point. The methyl ester of trans-trans abscisic acid had a retention time slightly less than that of the large peak. Therefore, some of the peaks in the spectrum could be due to the methyl ester of trans-trans abscisic acid. However, it is unlikely that the major component of the peak is the methyl ester of trans-trans abscisic acid for the following reasons: (1) the molecular ion was 294, 16 mass units higher than that of abscisic methyl ester; (2) the base peak occurred at 43 instead of 190; (3) a similar large peak was observed

Figure 26. Gas-liquid chromatogram of a methylated eluate of a thin-layer chromatogram spotted with the acid fraction of dormant 'Halford' peach seeds and developed in 10:1:1 isopropanol:ammonium hydroxide:water. Arrows indicate peaks for the methyl ester of abscisic acid (M^+278) and an unknown compound in high concentrations assumed to have a mass of 294.

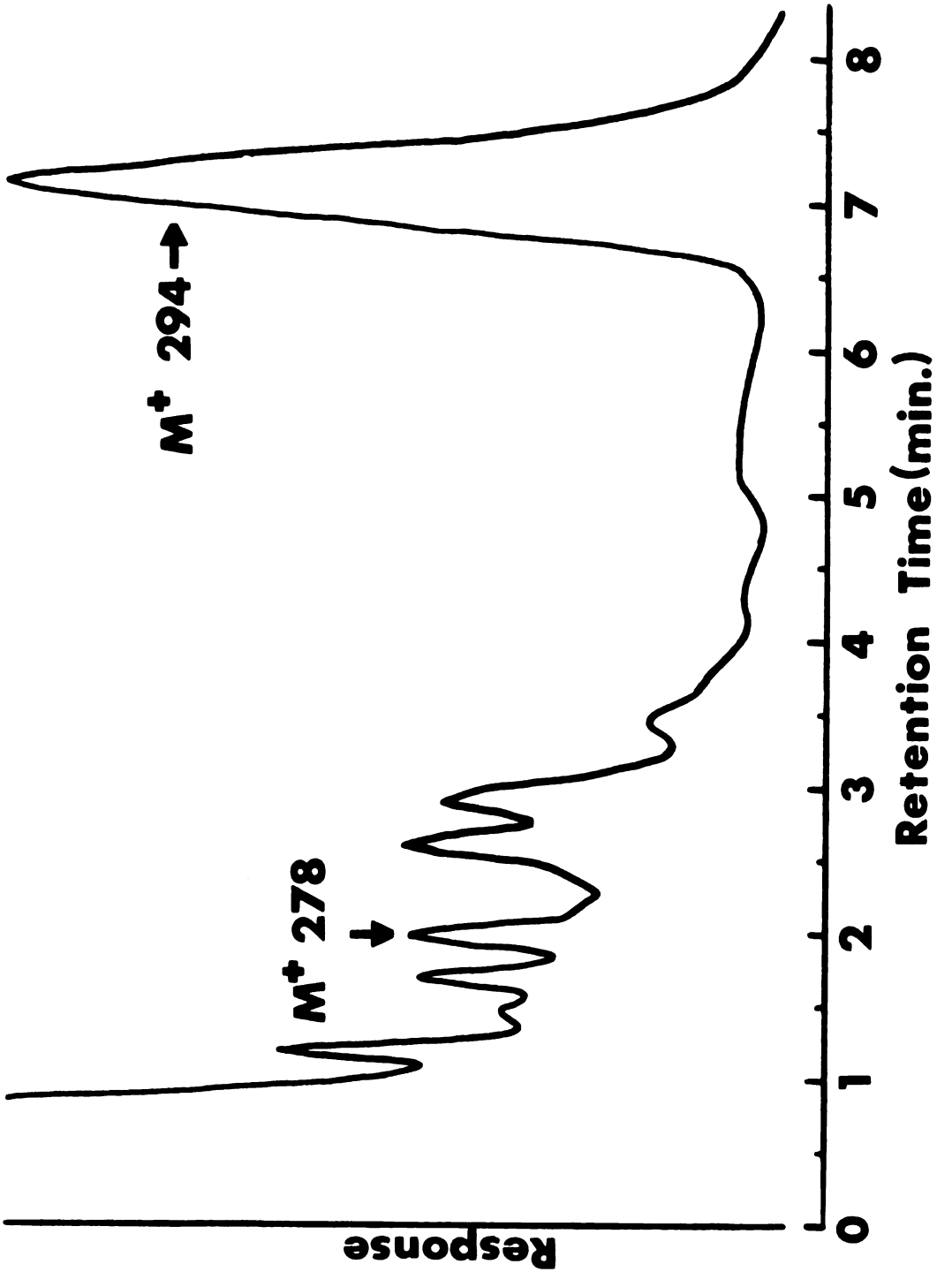

Response
0
1
2
3
4
5
6
7
8
Retention Time(min.)
M+ 278
M+ 294

TABLE 6.--Characteristic mass spectrometer peaks of the methyl ester of cis-trans abscisic acid expressed as a per cent of peak m/e 190, and a comparison with the methylated, biologically active zone of the acid fraction of dormant 'Halford' peach seeds (A-retention time of cis-trans abscisic acid, 4.34 min., B_1-half way up largest peak at retention time of 5.75 min., and B_2-at maximum of largest peak at retention time of 6.06 min.).

	Per cent of peak m/e 190				
	Abscisic acid		Sample		
m/e	Most, et al. (98)	Observed	A	B_1	B_2
190	100	100	100	100	100
125	28	33	42	20	91
162	25	29	34	28	40
134	24	30	31	31	36
91	14	15	28	23	41
191	12	14	18	15	18
260	4	3	5	3	4
278	0.6 (M^+)	1 (M^+)	2 (M^+)	2	4
294				2 (M^+)	5 (M^+)

when the same sample was chromatographed on another column, and this peak had a much longer retention time than did trans-trans abscisic acid; and (4) on columns which separated trans-trans abscisic acid from the large peak, peaks with retention times similar to that of trans-trans abscisic acid were relatively small. The possibility remains that the main component of the large peak could be an oxygenated derivative, possibly a precursor or metabolite of abscisic acid.

In summary, cis-trans abscisic acid is present in 'Halford' peach seeds in quantities which could account for the inhibition observed in the wheat coleoptile section bioassay.

VI. Seed Coat Studies

To determine if the seed coat weakens during cold temperature stratification, 'Halford' and 'Okinawa' seeds were sampled at various times during cold temperature stratification and the strength of the seed coats tested. There was no substantial decrease in strength of the seed coats during cold temperature stratification of either variety (Table 7). Thus, cold temperature stratification does not exert its effect upon germination by a weakening of the seed coat.

TABLE 7.--Seed coat strength of 'Halford' and 'Okinawa' seeds stratified at 3°C for various periods of time.

Variety	Weeks of stratification	Force (g) required to break seed coat
'Halford' (1st year)	0	244
	2	251
	4	237
	6	243
	8	238
'Halford' (2nd year)	0	243
	0.5	233
	4	238
	5	247
	6	263
'Okinawa'	0	229
	4	241

DISCUSSION

Chilling of buds and seeds has long been known to be necessary for the natural breaking of rest of many fruit tree species. Many theories have been proposed to explain this phenomenon, but definitive evidence is not available for any of the proposed theories. The widely accepted theories involve growth regulators, and hypothesize an increase of promoters, a decrease of inhibitors or a combination of both for the breaking of rest. In the last few years gibberellins and abscisic acid have been the growth regulators considered by most to fit the above models. In this thesis seed dormancy of peach seeds was studied in relation to levels of acidic inhibitors during cold temperature stratification.

One inhibitory zone was noted on thin-layer chromatograms of the acid fractions of seed coats, cotyledons, and embryonic axes of 'Halford' (a variety with a medium stratification requirement) and 'Okinawa' (a variety with a short stratification requirement) peach seeds. In 'Halford' seeds the relative inhibitor concentrations were approximately the same for seed coats and embryonic axes and about ten times less in the cotyledons. The existence of high amounts of inhibitors in the seed coats and low

amounts in the cotyledons is not surprising, since the role of the seed coat in dormancy was stressed as far back as the early 1930's in some of the early work of Flemion (42, 44, 45). She found that if the outer and inner coats of unstratified apple, peach, Crataegus, and Rhodotypos seeds were removed, the embryos germinated and developed hypocotyls and epicotyls within a week. However, the resultant plants were often dwarf. Lipe and Crane (88) found that the inhibitor studied in peach seeds was located primarily in the outer and inner integuments.

But investigators have failed to recognize the high levels of inhibitors in the embryonic axes of peach seeds. In most of the previous studies the embryonic axes were not separated from the cotyledons, and the high tissue weight of the cotyledons could, therefore, have masked the possible importance of the inhibitor levels in the embryonic axes. The embryonic axes were of interest again when it was found that the inhibitor did not decrease during cold temperature stratification in the seed coats or cotyledons, but decreased in the embryonic axes during stratification at both 3 and 20 °C. This decrease in inhibitor levels in the embryonic axes may be a prerequisite for germination even though other processes which only occur during cold temperature stratification, such as an increase in promoters, may also be required before radicle protrusion can occur.

Decreases in abscisic acid or abscisic acid-like compounds have been observed in seeds of peach (88), apple (112), walnut (91), and ash (124) during cold temperature stratification. However, Flemion and de Silva (50) studied extracts of peach seeds in various stages of dormancy and found no correlation between the breaking of rest and the levels of promoters and inhibitors. More recently, Strausz (125) was not able to detect significant changes of an inhibitor tentatively identified as abscisic acid in Pyrus fruit and seed coats during stratification. Bradbeer (15) observed high inhibitory activity in dormant hazel pericarp and testa together and lower amounts in the embryo, but abscisic acid, if present, accounted for less than two per cent of the inhibition. Ironically, high levels of abscisic acid have been found in actively growing tissues (104) and in seeds of Acer saccharinum L. (113), a non-dormant species.

In order to bring the work on the acid fraction into the broad perspective of the whole seed, five fractions of a peach seed extract were tested to determine if inhibitors occurred in any other than the acid fraction. Activity occurred in only one other fraction, the butanol-soluble fraction (see Figure 2). The compound(s) responsible for this activity could be glycosides since glycosides would generally partition into this phase. The glucoside of abscisic acid has been isolated, identified, and

found to be biologically active by Koshimizu, et al. (83). Glycosides of many phenolic compounds are also common in plant tissues.

Since naringenin has been implicated in bud dormancy of peach (65, 66, 100), thin-layer and paper chromatograms of the various fractions were tested for this compound and naringin, the rhamno-glucoside of naringenin. Results indicated little or no naringenin or naringin in any of the five fractions.

The inhibitor in the acid fraction had the same Rf as abscisic acid on silica gel thin-layer plates and Whatman no. 1 paper developed in 10:1:1 isopropanol:ammonium hydroxide:water. Logarithmic increases in this inhibitor caused a linear inhibitory response of wheat coleoptile sections. This type of response is typical of all plant growth regulators. Evidence from ultraviolet spectrophotometry and spectropolarimetry, while not conclusive, supported the tentative identification of the inhibitor as abscisic acid. Conclusive evidence for the presence of abscisic acid was obtained by gas-liquid chromatography on three different column materials and by mass spectrometry of the compound with the same retention time as the methyl ester of cis-trans abscisic acid. The levels of abscisic acid detected by gas-liquid chromatography accounted for most of the inhibition measured in the acid fraction via bioassay.

Mass spectrometry also revealed that the largest peak on gas-liquid chromatograms of the active zone had an apparent molecular ion of 294 and several peaks characteristic of abscisic acid. This compound could possibly be an active or inactive precursor or metabolite of abscisic acid. The methyl ester of phaseic acid has a molecular ion of 294 and is almost identical with the methyl ester of abscisic acid except for the addition of one oxygen molecule (90, 96). Milborrow (97) found that phaseic acid had less than 1/200 of the growth inhibitory activity of abscisic acid in the wheat-embryo germination bioassay. "Metabolite C" of Milborrow (96, 97) also is very similar to abscisic acid. "Metabolite C" differed from abscisic acid only in the addition of one hydroxyl group and rearranged to phaseic acid upon methylation. Radioactive abscisyl-β-D-glucopyranoside and "Metabolite C" were the two major compounds labeled when radioactive abscisic acid was metabolized by tomato shoots.

More work on the role of the seed coat would seem justifiable since high levels of inhibitors were found in the seed coats, and their removal from dormant seeds resulted in germination. The seed coat may be a physical barrier to germination, but, if so, it did not weaken during cold temperature stratification. Studies involving exchange of seed coats were not successful, but should be pursued since much pertinent information might be gained.

The genetical approach might also be useful. Crosses between peach cultivars having short and long stratification requirements should result in seeds with the same stratification requirement as the female parent if the seed coat (maternal tissue) is the primary site of dormancy.

SUMMARY AND CONCLUSIONS

1. One major inhibitory zone was noted in the acid fraction of extracts of seed coats, cotyledons, and embryonic axes of peach seeds.

2. The butanol-soluble fraction also contained considerable inhibitory activity.

3. Neither naringenin nor naringin was detected in any fraction of extracts of dormant seeds.

4. Levels of an acidic inhibitor, as measured by bioassay, did not decrease significantly in extracts of seed coats, cotyledons, or whole seeds during stratification at 3°C. Data for whole seeds were supported by optical rotatory dispersion measurements. However, the inhibitor decreased significantly in the embryonic axes during stratification at 3 or 20°C.

5. Abscisic acid was identified in the active zone of thin-layer chromatograms of the acid fractions using gas-liquid chromatography-mass spectrometry. The levels of abscisic acid detected by gas-liquid

chromatography accounted for most of the inhibition measured in the acid fraction via bioassay.

6. The above observations do not support the hypothesis that endogenous levels of abscisic acid are the primary cause of dormancy in peach seeds.

7. The seed coat did not weaken during a stratification period which was sufficient for high germination.

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