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RELEASE OF DORMANCY IN APPLE (Malus domestica Borkh.) SEEDS AND EMBRYOS: RESPONSE TO TEMPERATURE, CYTOLOGICAL CHANGES, AND METABOLISM OF GIBBERELLIN A12 ALDEHYDE

presented by

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has been accepted towards fulfillment
of the requirements for

Ph. D. degree in HORTICULTURE

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Date SEPTEMBER 26, 1991

RELEASE OF DORMANCY IN APPLE (*Malus domestica* Borkh.) SEEDS AND EMBRYOS: RESPONSES TO TEMPERATURE, CYTOLOGICAL CHANGES, AND METABOLISM OF GIBBERELLIN A12-ALDEHYDE.

BY

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture

1991

ABSTRACT

RELEASE OF DORMANCY IN APPLE (Malus domestica Borkh.) SEEDS AND EMBRYOS: RESPONSES TO TEMPERATURE, CYTOLOGICAL CHANGES, AND METABOLISM OF GIBBERELLIN A12-ALDEHYDE.

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The optimum temperature for breaking dormancy of 'Golden Delicious' and 'Paulared' apple embryos was 2.5 to 7C. Embryo germination was stimulated by alternating the stratification temperature between 5 and 10C (16/8 h) but temperatures higher than 10C either had no effect or were inhibitory. Chilling negation by high temperature was more dependent upon the temperature than upon cycle length. Seed germination was consistently inhibited by alternating between 5C and higher temperatures.

Seeds chilled in fruit germinated poorly in comparison with those chilled in Petri dishes; this effect was much less pronounced following embryo excision. Holding seeds under water (anaerobiosis) failed to break embryo dormancy at 20C, but was effective at 2.5C. Gibberellin A4+7, benzyladenine (BA), Promalin (GA4+7 and BA) and cyanamide all significantly stimulated the germination of non-chilled embryos, but were ineffective on intact seeds.

The effects of chilling vs. chemical treatments on cytological changes in the cells of the embryonic axes were compared. Dormancy release was always associated with degradation of protein, appearance of rough endoplasmic

reticulum and Golgi apparati, and increases in nucleolar volume and numbers of mitochondria.

14C-GA12-aldehyde was incubated with apple embryos, cotyledons, or embryonic axes, and methanolic extracts were partially purified by high performance liquid chromatography (HPLC). Six 14C-labelled metabolites were recovered. One metabolite had the same retention time as GA12, but none was eluted at the retention time of GA1 or GA4. There were no qualitative differences in metabolite profiles between chilled vs. dormant embryos, cotyledons, or embryonic axes, but one metabolite was more abundant in dormant, and another in chilled embryos. The rate of metabolism was higher in chilled than in dormant tissues.

DEDICATION

This thesis is dedicated to my mother, ENEGESI, who passed away when I was very young, but is still with me in Spirit.

ACKNOWLEDGMENTS

I express my deep appreciation and indebtedness to my major professor, Dr. Frank G. Dennis Jr. for his encouragement, direction, dedication and sense of humor during my program of research and preparation of this thesis.

I am also very grateful indeed to, in alphabetical order, Drs. D. Dickmann, J. A. Flore, K. Poff and K. Sink for their advice, encouragement and direction in the research, and critical review of the manuscript, as members of my guidance committee.

My special gratitude goes to Drs. Karen L. Klomparens and J. Heckman for their guidance and assistance in Transmission Electron Microscopy and also Dr. J. Everard for his assistance in computer programs for data processing.

My special thanks and appreciation go to the Malawi government and USAID for their financial and moral support which made this study possible.

I would like to thank my wife, Catherine, and children Agnes, Olive, Limbani and Chifundo for their support and love during my graduate study. Finally, my thanks go to my father, grandmother, father-in-law and mother-in-law for their patience and understanding during my long study leave away from them.

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To: The Guidance Committee,

This thesis is organized in Journal-style in accordance with departmental and university requirements. The format is that of the Journal of the American Society for Horticultural Science.

INTRODUCTION

The need for commercial production of deciduous fruits in many tropical regions of the world (Australia, Asia, Southern Africa, South America) has recently been emphasised for both economic and nutritional reasons. Dormancy is the major limiting factor to the successful production of deciduous fruits under tropical conditions. Buds and seeds enter an annual dormancy period in order to survive low winter temperatures. Dormancy is naturally broken by 1,000 to 3,000 hours of chilling temperatures, between 0 and 7C in winter, and this leads to resumption of normal growth, development, flowering and fruiting. The process of breaking dormancy of seeds by cold temperature is called stratification or after-ripening (Bennett 1950; Powell 1986; Saure 1985; Westwood 1978).

Under tropical conditions, buds and seeds do not receive sufficient chilling hours to break their dormancy fully; this results in delayed foliation or prolonged dormancy, erratic bud break and flowering. The trees may not come out of dormancy, hence they weaken and may eventually die. Alternative methods of breaking dormancy have been developed for commercial use under tropical

conditions; these include defoliation of trees soon after harvest, use of cultivars with low chilling requirements, use of dormancy-breaking chemical agents such as dinitro-ortho-cresol (DNOC), cyanamide, and plant growth regulators (Fuchigami and Nee 1987; Morimoto and Kumashiro 1978; Powell 1987). However, the application of these alternative methods for breaking dormancy has met with limited success.

The apple embryo which is like a miniature plant, which exhibits dormancy and hence makes a good model system for studies on induction and release of dormancy under controlled conditions (Come and Thevennot 1982).

Moderate temperatures of 10 to 15C can enhance the chilling effect of 5C , while high temperatures negate it (Erez and Lavee 1971; Erez et al. 1979a, 1979b). Under sub-tropical conditions, the daily temperatures during winter fluctuate between 2 and 30C. The beneficial effects of moderate temperatures have only been demonstrated in buds of peach (Erez and Couvillon 1987) and the mechanism involved in not known. Little attention has been paid to the ultrastructural changes which take place during the breaking of dormancy by either chilling or by treatment with chemical agents. An intricate balance between growth-promoting and growth-inhibiting substances appears to regulate bud and seed dormancy (Wareing and Saunders 1971) and gibberellins (GAs) are believed to play a major role in this process. GAs undergo significant shifts in free and

bound forms during stratification. GA4 is believed to be involved in the dormancy release process, for it increases during chilling as a result of either release from its bound forms or de novo synthesis (Sinska et al. 1973; Smolenska and Lewak 1975). The ability of the embryos to synthesize GA4 is dependent on the stage of the chilling period. (Sinska and Lewak 1977). Hence, dormancy may affect biosynthesis of GAs by influencing the biosynthetic pathway.

The goals of this research were to: a) determine the effects of alternating temperatures during the after-ripening process on the breaking of dormancy in apple seeds and embryos; b) observe ultrastructural changes associated with dormancy release by both chilling and chemical agents; and c) compare the metabolism of GA12-aldehyde in chilled vs. non chilled embryos.

LITERATURE REVIEW

Terminology Used

Dormancy.

The temporary suspension or arrest of active growth in plant organs containing meristems under conditions suitable for growth is called dormancy (Lang 1987). Three types of dormancy are identified, namely a) ecodormancy which is synonymous with quiescence or imposed dormancy and is regulated by environmental factors b) ectodormancy which is synonymous with correlative inhibition or summer dormancy and is regulated by physiological factors outside the affected structure, and lastly c) endodormancy which is synonymous with rest, winter dormancy or deep dormancy and is regulated by physiological factors inside the affected structure. Endodormant seeds or buds require chilling for the resumption of growth. Primary dormancy prevails during development and maturation of buds or seeds on the mother plant while secondary dormancy is re-imposed in partially chilled seeds or buds under conditions unfavorable for germination or growth (Karssen 1980/81).

Stratification

The holding of seeds under moist conditions at any temperature is referred to as stratification (Pellet 1973), although some would restrict the term to any temperatures that break dormancy.

After-ripening

After-ripening refers to changes that occur within the seed during storage as a result of which germination can take place or is improved (Nikolaeva 1969). After-ripening may occur at room temperature (grains), or only at low temperature (apple), depending upon species.

Seed and Bud Dormancy

The breaking of dormancy allows induction of active growth of an organ. Bud and seed dormancy have several common characteristics. The optimum temperature which breaks dormancy in buds and seeds is similar and so is the length of the chilling period required. Furthermore, secondary dormancy can be induced in both buds and seeds by high temperature during the early period of chilling.

Induction of Dormancy

Several external and internal factors are known to induce dormancy in fruit trees, including temperature, low oxygen levels, endogenous rhythms, growth inhibitors, and cell membrane integrity.

Temperature

Little is known about how temperature induces dormancy. Various investigations have demonstrated that chilling may intensify dormancy in the fall although it breaks dormancy

later (Ben-Ismail 1989; Hatch and Walker, 1969; Lavarenne 1975; Walser et al 1981). Dormancy of peach and apricot buds increased as chill units accumulated in the autumn (Hatch and Walker 1969). Ben-Ismail (1989) reported that growth of vegetative buds of apple was inhibited by artificial chilling of cuttings sampled in early October. However, the response varied depending on time of collection of shoots and length of the period of exposure to low temperature. Some physiologists contend that summer temperatures induce dormancy while cold temperatures in fall intensify it (Samish, 1954). The knowledge that both cold and warm temperatures induce dormancy and the reported dual role of cold temperature i.e., its ability to both induce and break dormancy, complicate the interpretation of the model of action of temperature in the induction and release of dormancy. Low temperature may not be essential for the induction of dormancy, for deciduous fruit trees become dormant in the tropics in areas where chilling temperatures do not occur.

Oxygen supply

The seed coat presumably restricts diffusion of oxygen to the embryo, thereby limiting respiration and inducing embryo dormancy. Seeds displayed no dormancy if the seed coat was removed (Crocker 1916). Visser (1956b) observed that restriction of oxygen uptake by apple seed coats

increased with temperature. If intact, partially stratified seeds were germinated at 25C, about 60% of the seeds entered into secondary dormancy. However, if the seed coat was removed and the endosperm ruptured, high germination levels were obtained (Visser 1956a). Come, et al. (1972) suggested that phenolic compounds in the testa react with oxygen, thereby reducing oxygen supply to the embryo resulting in dormancy. However, Tissaoui and Come (1973) were able to break apple embryo dormancy in the absence of chilling by anaerobic conditions under pure nitrogen alone. If the testa induces anaerobiosis, one would expect that embryo dormancy could be broken by holding embryos at room temperature, but this is not the case.

Growth Inhibitors

Abscisic acid is an endogenous growth hormone presumably involved in the control of dormancy in seeds and buds. Attempts to correlate the level of ABA with the induction of dormancy have resulted in conflicting evidence. Seeds of species which exhibit dormancy, i.e, hazel nut (Corylus avellana) and apple, contain relatively high levels of ABA (Williams et. al. 1973, Balboa-Zavala and Dennis 1977; Dudarichi 1969). The ABA declines rapidly during chilling, but several studies have indicated that the decline occurs under both high and low temperature regimes, although only chilling alleviates dormancy (Borkowska and

Powell 1982-1983; Balboa-Zavala and Dennis 1977). Strong evidence for the involvement of ABA in inducing dormancy comes from studies with ABA-deficient mutants of Arabidopsis thaliana L. Heynh. Mutant lines of Arabidopsis characterized by high transpiration rates (wilty mutant) and lack of seed dormancy contain low levels of ABA in leaves and mature seeds in comparison with the wild type (Karssen et al. 1983) which exhibits dormancy. Rudnicki (1969) found a good correlation between chilling and the disappearance of ABA in seeds during the first 3 weeks of stratification. Thus it appears that ABA can be relatively effective in inhibiting bud or seed growth where a) the potential for growth is low, thus allowing ABA to tip the balance towards a no-growth situation, and b) in situations where the initial events leading to growth are absent or have not progressed very far. Hence ABA may play a role in the early stages of dormancy but not in later stages; rather some kind of promoting force becomes dominant tending to override the possible effects of endogenous ABA.

Low Levels of Growth Promoters

Growth promoting hormones decrease to low levels in shoots late in the growing season and this alone could account for dormancy induction (Lewak 1985). This hypothesis is questionable because the application of growth-promoting substances might be expected to promote

growth in resting organs and this is not generally the case. Furthermore, rest gradually intensifies long after growth-promoting hormones have reached extremely low levels, suggesting the build-up of an inhibitory influence. This increasing intensity of dormancy could result from a gradual loss of an essential metabolic function rather than from the gradual increase of an inhibitory compound (Powell 1987).

Cell Membrane Integrity

The sites of metabolic activities in the living cell are separated from each other by membranes. The most important properties of the membranes are semipermeability, the activity of membrane-bound enzymes, and membrane potential (Bewley and Black 1982).

Membranes undergo transitions in their physical state at specific temperatures, and the activity of enzymes associated with them also changes. Therefore, the factors that induce dormancy may do so through changes in membranes and membrane-bound enzymes (Bewley and Black 1982). Membrane repair mechanisms and membrane development are under hormonal control and there is evidence that GA is involved in changing the permeability and also in the synthesis of some essential membrane constituents (Wood and Paleg 1974 ; Ben-Tal and Varner 1979). Similarly, an increase in membrane permeability may be the cause of

dormancy release (Doorenbos 1953).

Dormancy Release

Chilling temperatures

The optimum chilling temperature for breaking dormancy in buds and seeds varies with species. In most cases 5C is considered to be the optimum temperature (Erez and Lavee 1971; Perry 1971). Sarvas (1974) reported that 3.5C was the optimum for Betula pubescens. In peach buds (Erez and Lavee 1971) and pear seeds (Westwood and Bjornstad 1968) the optimum was found to be 6C, and between 7 to 10C, depending upon species, respectively. The effect of temperatures below 0C on overcoming rest is not clear. Generally temperatures just above freezing are more effective than lower temperatures. However, subfreezing temperatures are effective in overcoming rest in some species (Sparks 1976).

Sarvas (1974) found that temperatures above 10C were not effective in satisfying the chilling requirement of certain forest trees. However, such temperatures are effective in peach (Erez and Lavee 1971). Kobayashi et al. (1987) reported that temperatures up to 20C were effective in meeting the chilling requirement of red-osier dogwood (Cornus seriaca).

High Temperature

High temperatures can either prolong dormancy or induce

secondary dormancy in buds and seeds. Weinberger (1954) reported that longer chilling periods were required to overcome rest in peach during warm winters. In a few North American forest trees, a moderate temperature interruption can counteract all the previous chilling effects of low temperature (Nienstaedt 1966), but Erez and Lavee (1971) found that high temperature must occur within a few days after chilling in order to negate prior chilling; otherwise a fixation process will prevent reversal.

Short periods of high temperature during a daily cycle can negate chilling. Erez et al. (1979b) reported that exposure of dormant peach to 6 hr at 21C to 24C negated 18 hours at 6C. Thus the length of the high temperature treatment following chilling is critical. In apple buds greater chilling negation was found if the high temperature occurred every 2 days rather than every 4 days (Thompson et al. 1975).

The effect of high temperature in negating chilling depends on both the stage of rest and the temperature. The temperature range for induction of secondary dormancy decreases progressively during the post-dormancy period (Vegis 1964).

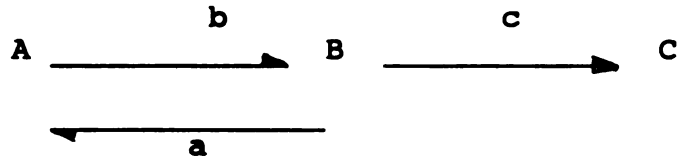
Alternating Temperatures

Fluctuating temperatures are more effective in breaking dormancy of peach buds than are constant temperatures. In

peach, daily fluctuation between 6 and 15C was more effective in breaking rest than constant 6C (Samish 1954; Erez et al. 1979). Erez et al. (1979) demonstrated that constant 15C alone was ineffective in breaking dormancy of peach buds. However, alternating temperatures (15C for 8 hr, 6 for 16 hr) were more effective than constant 4C for the same number of chilling hours. Temperatures higher than 15C, i.e. 18C had no effect, while 21 and 24C were inhibitory. Erez and Couvillon (1987) also demonstrated that the most efficient moderate temperature for alternation was 13C; alternation was effective only during the latter part of the chilling period and could even be inhibitory during the initial third. Later, Erez et al.(1979a) showed that temperatures higher than 15C were capable of enhancing the effects of chilling temperatures on breaking rest provided exposure time was reduced to 4 hours or less during a 24-hour cycle.

The promotive effects of alternating temperatures were confirmed in nectarine buds (Gilreath and Buchanan 1981) and peach seeds (Aduib and Seeley 1986; Mahhou 1991). However, in peach seeds, 10C was promotive while 15C was inhibitory. In contrast Sarvas (1974) found no beneficial effects of alternating temperatures in breaking rest of several forest species, and Del Real Laborde (1987) observed that alternating temperatures never released apple seed dormancy.

Erez et al. (1979, 1986) proposed a two-step model to explain temperature effects on rest completion in peach buds as follows:



where A = the resting state

B = the product of low temperature exposure, which can revert to "A" at high temperature;

C = the product of B, which is fixed and thus irreversible;

b = the chilling reaction (favored by low temperature); and

a = the reverse reaction (favored by high temperature)

c = the reaction converting B to C at moderate temperature, which fixes the chilling effect.

Of the two steps, the first one is reversible while the second is irreversible. The reactions have different temperature response curves. The chilling reaction (b) occurs at temperatures between 0C and 13C. Chilling efficiency decreases as the temperature rises above 8C, reaching zero at 14C. Reaction 'a' occurs at temperatures above 16C, reaching high levels of activity at 24C. The influence of temperatures greater than 24C and less than 0C is unknown. Reaction 'c' occurs at a wide range of temperatures but is most rapid at moderate temperatures i.e., 13 to 15C, and thus overlaps reactions 'a' and 'b'. Reaction 'c' can occur at 0C since budbreak will occur on

plants chilled continuously at this temperature.

Reactions 'a' and 'c' compete for the same substrate 'B', but reaction 'c' has a much lower Q10 than reaction 'a' which partly or fully masks it at a temperature of 13C. Reaction 'a' seems to be affected by the level and duration of the high temperature. Short diurnal periods of exposure to 20C may divide the substrate 'B' between 'a' and 'c' resulting in no negative effect.

Erez and Couvillon (1986) demonstrated that the chilling efficiency of low temperature was indeed increased by cycling low temperature (5C) with moderate temperature (15C). They later suggested that the level of the day temperature in a diurnal cycle is of critical importance under marginal growing conditions with warm winters, i.e., the temperature can either negate or enhance budbreak depending on its level and duration. Fishman et al. (1987), developed a mathematical model to explain the mechanism of chilling promotion by moderate and high temperatures. Mahhou (1991), working with peach seeds, observed that cycling was unnecessary. Germination of seeds held at constant temperatures for three 3-week periods was promoted or inhibited by raising the temperature from 5C to 10C or 15C, depending upon the time the higher temperature was applied. This casts doubt on the cyclical scheme proposed by Fishman et al. (1987) for peach buds.

Chemical treatments

Several chemicals have been reported to break dormancy of fruit trees, including mineral oils and dinitro-ortho-cresol (DNOC) (Jeffrey 1951); nitrogen containing compounds like thiourea (Blommaert 1965); cyanamide (Morimoto and Kumashiro 1978; Shulman et al. 1983). How these chemicals break rest in deciduous fruit plants is still unknown. It is highly unlikely that low temperature, heat, injury, oils, toxic substances and hormones all act at the same site in plants. One hypothesis is that agents which produce sublethal stress cause plants to produce 'necrohormones' (Doorenbos 1953; Erez and Lavee 1974). The 'necrohormone' may be ethylene (Fuchigami and Nee 1987). In crabapple and red-osier dogwood, a positive correlation between ethylene production and the breaking of rest has been established with several rest-breaking agents (Nee 1986). Therefore sublethal stresses may overcome rest by stimulating ethylene production and/or increasing membrane permeability. Increased production of ethylene due to sublethal stresses may be due to the release or activation of the ethylene forming enzyme (EFE) that is reported to be associated with membranes and is required for the conversion of ACC to ethylene (Mayak et al. 1981).

Hormones

Gibberellins. Among the promoters of seed germination special emphasis has been given to gibberellin (GA) because of its pronounced germination-stimulatory effect and its common occurrence in seeds during dormancy removal. Endogenous gibberellins are presumed to play a role in the chill-related dormancy mechanism. In general, dormant seeds contain minute amounts of gibberellins and these increase during the chilling process. In contrast, Ross and Bradbeer (1971) reported that chilled hazel nut seeds had to be transferred to warm temperatures before substantial amounts of gibberellin were detectable, suggesting that chilling removes blocks to gibberellin biosynthesis.

Several inhibitors of GA biosynthesis were ineffective during chilling but inhibited germination following chilling (Ross and Bradbeer 1971). The first evidence supporting the involvement of GA in dormancy release came from exogenous application of GA3, which breaks the dormancy of peach buds and hazel nuts (Walker and Donoho 1959, Jarvis et. al. 1968). In general, however, treatment with GAs cannot entirely replace the chilling requirement (Walker and Donoho 1959; Powell (1987).

Work with physiological dwarf plants has also implicated the involvement of gibberellins in chill-related dormancy. Seedlings developing from unchilled embryos are dwarfs, but normal growth can be stimulated by chilling the

seedlings or treating them with GA's (Barton 1956; Bloomaert and Hurter 1959). However, a single application of GA is not sufficient; a continuous supply is necessary for sustained growth, suggesting that the chilling process activates the biosynthesis of gibberellins (Powell 1987). Furthermore, the treated seedlings often exhibit abnormalities associated with insufficient chilling. Current evidence suggests that GA may may break dormancy through the stimulation of an enzyme, acid lipase, which breaks down reserve lipids. However, the gibberellin-mediated hydrolysis of reserve lipids cannot be the only mechanism involved in the removal of apple embryo dormancy (Zarska-Macikerska et al. 1980). The presence of acid lipase with an optimum at 5C was also observed in hazel nut (Ross 1983).

Cytokinins. Both bound and free cytokinin-like compounds occur in mature apple seeds and increase during stratification (Borkowska and Rudnicki 1974). Cytokinin-like activity reached a maximum in the 5th week, but the increased level of cytokinins was not directly correlated with the ability of the seeds to germinate (Borkowska and Rudnicki 1974; Kopecky et al. 1975). Among the 6-substituted purines tested, 6-benzylaminopurine (BA) was especially active in stimulating germination of seeds in a number of species (Van Overbeek 1966). Other investigators

have broken apple embryo dormancy with 10 to 25 mg/l BA (Badizadegan 1967; Zhang and Lespinasse 1991; Lewak and Bryzek 1974). Although application of cytokinins to dormant or partially chilled seeds has promoted growth in some cases, results have been inconsistent (Lewak and Bryzek 1974; Tzou et al. 1973).

Auxins and ethylene. There is no convincing evidence that these two hormones play an important role in regulating dormancy in buds or seeds in which chilling is required to break dormancy. In studies where a positive correlation between ethylene and dormancy release was obtained it was generally attributed to ethylene action on events after partial or full release from dormancy by chilling (Powell 1987; Ozga 1988).

Enzymes

Dormancy release has been closely associated with an increase in activity of enzymes. The activity of peroxidase, succinate dehydrogenase, lipases, and proteases in apple embryos increased seven-fold during chilling, and interruption of the cold stratification period caused a sharp decline in enzyme activity (Nikolaeva and Yankelevich 1974). The biosynthesis or release of hydrolytic enzymes is presumably under hormonal control (Burg and Burg 1962) and treatment of dormant seeds with GA4 or benzyladenine

increases the activity of lipase, phosphatase and peroxidases (Rychter and Lewak 1971; Rychter et al. 1971). Although a gradual rise in hydrolytic enzymes could be responsible for breaking of dormancy, such changes may be a result , rather than the cause of dormancy removal.

Anaerobiosis

Anaerobic conditions have been reported to break dormancy (Tissaoui and Come 1973). Apple embryo dormancy was eliminated at room temperature when imbibed embryos were held under nitrogen for 7 days (Barthe and Bulard 1983). More evidence was provided by Esashi et al. (1976) who broke the dormancy of cocklebur (Xanthium) seed by anaerobic treatment. Anaerobiosis may induce changes in membrane permeability leading to the leaching of cell constituents including hormones implicated in the regulation of embryo dormancy.

Ultrastructural changes.

Dormant embryos have a minimal rate of metabolism and are highly resistant to adverse environmental conditions. Many reports are available concerning metabolic aspects of dormancy release but little is known about ultrastructural changes associated with them. Biosynthesis of and interconversions between the major reserves and the formation and proliferation of cellular organelles and

membranes that take place during stratification have been reported in many species (Villiers 1971; Lewak et al. 1975; Dawidowcez-Grzegorzewska and Maciejewska 1979; Bouvier-Durand et al. 1981; Kupila-Ahvenniemi et al. 1978). The followimg changes have been observed in the embryo meristem cells as a result of stratification: a) lipid and protein bodies decrease; b) development of organized endoplasmic reticulum lined with ribosomes develop d) Golgi bodies become numerous and active; e) plastids differentiate and starch grains appear and f) volume of the vacuole and nucleus increases. These changes take place during the breaking of dormancy by cold temperature, but little is known about the effects of other treatments which break dormancy, e.g., anaerobiosis or treatment with gibberellins, cytokinins, cyanamide or DNOC.

Summary

Mature apple seeds exhibit dormancy which may be released by either chilling at 5C for 2 to 3 months or treatment of the embryos with dormancy breaking chemicals. Many studies have emphasized seed dormancy and paid little attention to embryo dormancy which is the site of dormancy in seeds.

Recent studies with peach have demonstrated that the chilling effects of low temperature (5C) can be enhanced by moderate temperatures (10-15C) if alternated during

stratification. However the promotive effect of moderate temperatures has not been demonstrated in apple buds or embryos. High temperatures (20-30C) are known to negate the previous chilling effect, resulting in induction of secondary dormancy.

Current evidence suggests that dormancy is regulated by a balance between growth inhibitors and promoters. Dormancy appears to be the result of low levels of gibberellins and cytokinins and high levels of ABA. Chilling apparently reduces the levels of ABA while promoting the synthesis of promoters and hydrolytic enzymes. GAs and cytokinins stimulate germination of non-chilled apple embryos and their effects are greater when the embryos are partially chilled. On the other hand ABA inhibits the germination of non-dormant embryos and counteracts the effects of promoters. Light and photoperiod appear to have little or no effect on dormancy release in apples.

This thesis was designed to explore several aspects of dormancy in apple seeds, including response to alternating temperatures, comparison of the cytological effects of chilling with those of chemicals which break rest, and the effect of chilling on gibberellin metabolism.

CHAPTER ONE, SECTION ONE

THE EFFECTIVENESS OF CONSTANT VS ALTERNATING TEMPERATURES IN BREAKING THE DORMANCY OF APPLE SEEDS AND EMBRYOS

THE EFFECTIVENESS OF CONSTANT VS. ALTERNATING TEMPERATURES IN BREAKING THE DORMANCY OF APPLE SEEDS AND EMBRYOS

Abstract. The effects of constant vs. alternating temperatures in breaking dormancy of apple embryos and seeds were investigated. The germination response of the embryos at constant temperatures was bell-shaped, but skewed to the cold temperature side. Temperatures between 2.5 and 7C were the most effective in breaking dormancy, while -2.5, 0, and 15C had marginal effects. Temperatures higher than 15C either had no effect or were inhibitory. The chilling requirement of embryos was fulfilled earlier (6 weeks) than that of seeds (8 to 12 weeks). The rate of germination increased with increasing time of stratification. Mean time to germination was two days in fully chilled embryos and 7 days in non-chilled embryos. The chilling effect of 5C was enhanced by alternating with 10C on a daily cycle (16 h at 5C, 8 h at 10C), the effects of the two temperatures being additive. Higher temperatures either had no effect or were inhibitory. Parallel data were obtained with 3-week and 6-week cycles. The degree of negation by high temperature depended more on the temperature of alternation than on cycle length. Inhibition increased as temperature increased from 15 to 25C. Germination of seeds was nil following exposure to all alternating temperatures except 5/10C; in this case 10C negated the chilling effect of 5C.

Introduction

Dormancy limits the production of deciduous fruits under tropical climates. The dormancy of both buds and seeds was broken by temperatures between 0 and 10C, with the optimum near 5C. (Abbott 1955; Gilreath and Buchanan 1981; Seeley and Damavandy 1985, Westwood and Bjornstad 1968; Erez et al. 1979a).

Constant chilling temperatures broke dormancy while high temperatures interspersed with chilling temperatures negated the chilling effect (Couvillon and Erez 1985). However, moderate temperature (i.e, 15C), when alternated with a chilling temperature (5C), in a daily cycle, enhanced the chilling effect in peach buds. Constant 15C was ineffective while 21 or 24C was inhibitory. Moderate temperatures promoted rest completion only when applied at later stages of the chilling period. Temperatures of 21 and above negated the chilling effect if the exposure time was more than 4 hours per day, but became less effective as cycle time increased (Convillon and Erez 1985; Erez and Couvillon 1987; Erez et al. 1979a, 1979b).

Aduib and Seeley (1985) and Mahhou (1991), using peach seeds, observed chilling enhancement by alternating 5C with 10C in a daily cycle, whereas chilling negation occurred when 5C was alternated with 15, 20 or 25C. Mahhou (1991) also observed that cycling was not necessary because a 3-week period at 10C given at the end of the stratification

period was as effective as the same total time of exposure to 10C on daily cycles. The effect of moderate temperatures on hastening dormancy release has been confirmed in nectarine buds (Prunus persica nectarina) (Gilreath and Buchanan 1981) and sour cherry (Prunus cerasus L.) (Felker and Robitaille 1985).

The objectives of this study were : a) to determine the response of apple seeds and embryos to alternating temperatures in both daily and longer cycles; and b) attempt to explain the mechanisms involved during chilling enhancement or negation by alternating temperatures on the basis of the Erez et al. 1979, 1986 and Fishman et al. (1987a, 1987b) two-step model for induction and release of dormancy.

Materials and Methods

Plant material

Mature apple (Malus domestica, cv. Golden Delicious and Paulared) fruits were harvested from mature trees at the Horticultural Research center, Michigan State University, East Lansing, in 1989 and 1990. The seeds were extracted from the fruits, air-dried and stored at room temperature (21C).

Stratification and germination

Seeds were soaked for 48 hours in distilled water, then placed in Petri dishes lined with Whatmann no.1 filter paper (Whatman No.1) wetted with 1% Captan [N-(trichloromethyl thio)-4-cyclohexene-1,2-dicarboximide] solution. The Petri dishes were placed in growth chambers at various temperatures in the darkness.

The germination capacity of the seeds or embryos was used as a measure of the degree of dormancy release. No germination occurred during stratification, regardless of treatment. At the end of each stratification period the seeds or excised embryos were germinated in Petri dishes lined with filter paper wetted with distilled water at 20C in darkness.

Each Petri dish contained 20 seeds or embryos; 3 replicate dishes were used per treatment. The seeds or embryos were considered germinated when the radicle had grown at least 3 mm. Germination counts were made daily over a 10-day period and used to calculate germination percentages, and, in some cases, the rate of germination (mean days to germination or MDG) according to the formula of Tincker (1925) as follows:-

$$MDG = \frac{\sum_{n=1}^{n=c} (Xn)(n)}{X}$$

where Xn = number of seeds or embryos germinating on day n

n = day on which germination occured in days

c = duration of germination test in days

X = total number of seeds or embryos which germinated over the germination period.

Example

If all seeds germinate on day 1, MDG = 1 x 20/20 = 1 day

If all seeds germinate on day 10, MDG = 10 x 20/20 = 10 days

If 5, 10 and 5 seeds germinated on day 3, 4 and 5, respectively, MDG = 15/20 + 40/20 + 25/20 + 80/20 = 4 days

Experimental design

Treatments were arranged factorially in a completely random design. The data were subjected to analysis of variance (ANOVA) and were analyzed both as percentages and as arcsin transformations. The data were also subjected to regression analysis, however no regression equation satisfactorily described the germination response curves. Consequently the data are presented as means of 3 replicates plus or minus standard error.

Experiments

Experiment 1. Effect of stratifying seed at constant temperatures on subsquent germination of the embryos at 20C.

'Golden Delicious' and 'Paulared' apple seeds were stratified at -2.5, 0, 2.5, 5, 7, 10, 15 and 20C for 2, 4, 6, and 8 weeks. Embryos were excised from the seeds at the end of each stratification period and germinated at 20C for 10 days. A control treatment of non- stratified seeds was included. The germination percentage and rate of germination were calculated from the germination counts.

Experiment 2. Effect of stratifying seed at constant 5C vs alternating temperatures in a diurnal cycle on subsquent germination of the embryos and seeds at 20C.

'Golden Delicious' and 'Paulared' seeds were stratified for 2, 4, 6, 8, 10, 12, 15 and 18 weeks at constant temperatures (5, 10, 15 and 20C) and at alternating temperatures of 5/10, 5/15, 5/20 and 5/25 C in a diurnal cycle (16 hrs at 5C and 8 hr at the higher temperature). An additional treatment of non-stratified embryos was included. Total stratification time for alternating temperatures was adjusted so that all seeds were exposed to 5C for the same period of time. At the end of this time the seeds were germinated at 20C over a 10-day period.

Experiment 3. Effects of stratifying seeds at constant 5C or alternating temperatures in a 24-hour or 3-week cycle on subsquent germination of the embryos at 20C. Seeds of 'Golden Delicious' and 'Paulared' apple were held for 4 weeks at constant 5C, for 6 weeks at alternating temperatures in a 24-hour cycle (5/10) or for 6 weeks on a 3-week cycle with 5-5-10C or 10-5-5C for a total of 2 cycles. Total time at 5C was 4 weeks in all cases. At the end of this time, the embryos were excised from the seeds and germinated at 20C.

Experiment 4. Effect of stratifying seeds at constant 5C or alternating temperatures in a 6-week cycle on subsquent germination of the embryos during 6 days at 20C. 'Golden Delicious' and 'Paulared' seeds were stratified for 4 weeks at constant 5C or for 6 weeks at alternating temperatures, to give an equivalent time of 4 weeks at 5C in a long cycle (six weeks total, two consecutive weeks at each temperature). The temperature was held constant at 5C or alternated between 5C and 10, 15, 20 or 25C, as follows : 5-5-10, 5-10-5, 10-5-5; 5-5-15, 5-15-5, 15-5-5; 5-5-20, 5-20-5, 20-5-5; 5-5-25, 5-25-5, and 25-5-5. The embryos were excised at the end of the stratification period and germinated at 20C for 10 days.

Results

Experiment 1. Effect of stratifying seed at constant temperatures on subsquent germination of the embryos at 20C. The germination capacity of non-chilled embryos of 'Golden Delicious' and 'Paulared' from 1990 harvest ranged from 28 to 38%. The germination response curves of the two cultivars at constant temperatures ranging from -2.5 to 20C were similar (Fig. 1). Temperatures most effective in releasing dormancy were between 0 and 10C with the optimum between 2.5 and 7C. Temperatures of -2.5C and 15C had a slight chilling effect while 20C was ineffective. The response of embryos chilled for 8 weeks was similar to that of embryos chilled for 6 weeks (data not shown)

At the optimum temperature 5C, germination capacity increased with increasing time of stratification, reaching 100% after 6 weeks. The germination rate increased significantly with increasing period of stratification (2 days for fully chilled embryos vs. 7 for non-chilled embryos). Non-chilled 'Golden Delicious' embryos required seven days for germination while all embryos chilled for 6 or 8 weeks germinated within three days (Table 1).

Experiment 2. Effect of stratifying seeds at constant 5C vs alternating temperatures in a diurnal cycle on subsequent germination of the embryos and seeds at 20C. Germination of 'Paulared' embryos after 2 weeks at 5C was

significantly enhanced when 5C was alternated with 10C, but the difference was non-significant in 'Golden Delicious' (Fig. 2). The effect in 'Paulared' can be explained by the additive effects of exposure time at 10C. With more than 2 weeks at 5C, 5/10C had no significant promotive effect. All other temperatures negated the chilling effect, and the degree of negation increased with increasing temperatures.

In the case of seeds, the moderate to high temperatures either had no effect or negated the chilling effect of 5C during stratification in both 'Golden Delicious' and 'Paulared' (Fig, 3) No seeds germinated following exposure to 5/15, 5/20 or 5/25C.

Experiment 3. Effect of stratifying seeds at constant 5C or alternating temperatures in a 24 or 3-week cycle on subsequent germination of the embryos at 20C. Germination of embryos was enhanced by alternating temperatures, regardless of cycle time, with one exception (10-5-5) for 'Paulared' (Fig. 4). However, there were no significant differences between 24-hr vs. 3-week cycles.

Experiment 4. Effect of stratifying seeds at constant 5C or alternating temperatures in a 6-week cycle on subsequent germination of the embryos at 20C. The germination response differed between cultivars (Fig. 5), therefore each will be discussed separately. No treatment

enhanced germination of 'Golden Delicious' embryos significantly; however, 20 or 25C in the last step of the cycle significantly inhibited germination. Exposure to 15C in the third step significanlty reduced germination in comparison with that of seeds exposed to 15C earlier in the cycle, but not in comparison with the control. Thus, a one week exposure to temperatures of 15 to 25C was inhibitory only when applied at the end of the 3-week cycle.

Germination of 'Paulared' embryos was promoted by only one treatment , 10C during the last week of the cycle and many other treatments significantly inhibited germination. Exposure to 25C inhibited germination regardless of timing, 20C was inhibitory only in the first or second weeks of the cycle, 15C inhibited only in the 2nd week, and 10C was not inhibitory at any time.

Comparison of effects of constant vs. alternating temperatures and of cycle length on the breaking of apple embryo dormancy

Golden Delicious: Alternating temperatures of 5-5-10 in a 3-wk cycle significantly promoted germination (Fig 6). The effects of constant 5 and 10C were similar and the germination responses declined as temperatures increased from 10 to 25C. Daily alternating temperatures inhibited germination whenever the high temperature was 20 or 25C. The inhibitory effects of 15, 20 and 25C in the daily cycle

were similar to those in the 6-wk cycle. Exposure to 25C completely negated the effects of chilling regardless of cycle time.

'Paulared': The data for' Paulared' generally paralleled those for 'Golden Delicious' (Fig. 6). However, differences between treatments were more pronounced on daily cycles and less pronounced on longer cycles. Alternating temperatures of 5-5-10 in a 3 or 6-wk cycle promoted germination significantly relative to the control.

Discussion

Seed dormancy is a complex phenomenon controlled by a large number of factors. Embryos were used in this study because they are more sensitive than seeds to factors that induce or release dormancy. About 35% of the embryos were not dormant at harvest time. This may be attributed to the effect of environmental factors such as temperature during seed development, or to genetic variability as a result of cross pollination, although both 'Golden Delicious' and 'Paulared' are self-fruitful.

The chilling requirement of apple embryos was fulfilled earlier (6 to 8 weeks) than that of seeds (10 to 12 weeks). Thus, the seed coat has an inhibitory effect. A longer chilling period may be required for softening the seed coat, or for leaching of inhibitors from it, or may merely

increase the vigor of the radicle sufficiently to enable it to break through the seed coat.

The effective constant chilling temperature range of 0-10C was similar to that reported for apple seeds (Abbott 1955; Aduib and Seeley, 1985; Del Real Laborde 1987; Ozga 1989; Purwoko 1990; Seeley and Damavandy 1985). However, the optimum chilling temperature range (0-10C) for embryos was broader than that of seeds. It is known that the effectiveness of chilling temperatures is dependent on the chilling requirement of the cultivar (Gilreath and Buchanan 1981). The effective chilling temperature range is narrower for high than low chilling requirement cultivars. It is therefore suggested that low chilling requirement cultivars are adapted to mild climatic conditons due to the widening of the temperature range over which chilling is effective. It is also reported that species originating from warmer climates have a lower chilling requirement, broader temperature response curve and a higher optimum temperature range than those from colder climates (Gilreath and Buchanan 1981b; Seeley and Damavandy 1985; Westwood and Bjornstad 1968). These observations imply that the greater the intensity of dormancy the narrower the effective chilling temperature range. Embryo dormancy is less than that of whole seed dormancy which is attributed to both the embryo and seed coat. A temperature of 15C had a marginal chilling effect while 20C and 25C had none. The rate of germination

increased with increasing periods of stratification.

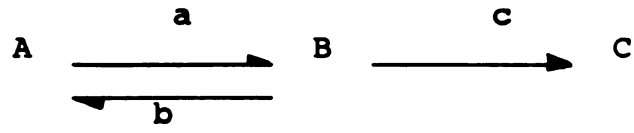
Alternating 5C with 10C in a daily cycle promoted the germination of embryos but temperatures higher than 10C either had no effect or were inhibitory. Parallel data were obtained with 3- and 6- week cycles. The stimulatory effect of 5/10C was largely the result of additive effects of the two temperatures. The inhibition increased as temperatures increased from 15C to 25C, although 25C was often no more inhibitory than 20C. These data are in agreement with those obtained by Aduib and Seeley (1985) with apple seeds and Mahhou (1991) with peach seeds. Alternating 5 with 10C reduced the germination capacity of seeds whereas no seed germinated following exposure to 15, 20, 5/15, 5/20 and 5/25. These data corroborate those of Porwoko (1991) and Del Real Laborde (1987). Therefore the degree of negation by high temperatures depended more on the temperature of alternation than on cycle length, i.e., the higher the temperature the greater the chilling negation. Weinberger (1954) reported that the opening of peach leaf buds was reduced by 33% when the temperature was raised from 10C to 18C for 15 days while raising the temperature to 22.2C for the same period reduced the bud break by 80%. Furthermore chilling negation by high temperature is dependent on the chilling requirement of the cultivar. Chilling negation by high temperature is less pronounced in low than high chilling peach cultivars (Gilreath and Buchanan 1981a). As

a result, low chilling requirement species have a higher optimum chilling temperature than high chilling requirement species and such species could be more tolerant to high temperatures. Why embryo dormancy is broken by 5/10C while seed dormancy is not remains an enigma. Conceivably the seed coat could restrict respiration at the higher temperature, leading to the accumulation of toxic products. The intensity of whole seed dormancy is higher than that of embryo, therefore the latter has a broader effective chilling temperature range than the former.

The beneficial effects of moderate temperatures during the last stages of chilling for both 'Golden Delicious' and 'Paulared' cultivars may be attributed to the promotion of germination processes, chilling effects due to a change in optimum temperature, and widening of the effective chilling temperature range as stratification proceeded. The inhibitory effect of moderate temperatures given in the middle of the chilling period is attributed to the sensitivity of partially chilled seeds or embryos to high temperatures. However, it is difficult to explain why the moderate temperatures would inhibit germination when applied at the beginning of the chilling period since such temperatures would be expected to have no effect on subsequent chilling.

The following model was proposed by Erez and Couvillon (1986) to explain the promotive effect of moderate

temperatures on breaking dormancy of peach buds.



Chilling converts a precursor (A) to an intermediate (B) which , upon further chilling, is fixed by conversion to a dormancy breaking factor (C). High temperatures enhance dormancy release by promoting the conversion of B to C. Alternating temperatures enhance the effect of chilling in peach buds provided the higher temperature does not exceed 15C (Erez and Couvillon 1987). This scheme does not explain the inhibitory effect of moderate temperatures during the early stages of seed stratification as shown by these results. Similar effects were apparent but ignored in the studies with peach buds (Erez and Couvillon 1987). Fishman et al. (1987) provided a mathematical analysis which attempted to rationalize the effects of moderate temperatures during cycling. The scheme, as shown below, assumes the existence of a thermally unstable precursor (PDBF) formed from the dormant state.

Once PDBF reaches a critical level it undergoes an irreversible conversion to the dormancy breaking factor (DBF) by chilling temperatures. The basis for the promotive effects of alternating temperatures between 4 and 15C is

that the initial exposure to 15C causes PDBF to accumulate faster innitially. When the temperature is shifted to 4C, less time is needed to reach the critical level. Hence more DBF accumulates over a given time period. Cycle time is crucial in this scheme if dormancy is to be broken more rapidly. The critical level of PDBP is reached faster when low temperature is alternated with moderate temperature than 5C, hence the chilling efficiency is enhanced. The total time required to accumulate the amount of chilling units is shorter at cycling temperatures than at constant low temperature. However, alternating temperatures do not enhance the effect of chilling in apple seeds and embryos supporting previous work with apple (Porwoko 1980; Del Real Laborde 1987; Aduib and Seeley 1985) and peach seeds (Mahhou 1991; Aduib and Seeley 1985). Therefore if these models are valid they must apply only to buds of peach. A somewhat different set of temperature conditions may be optimal, with seeds responding better to constant temperature. This hypothesis can only be tested by comparing seed and bud responses to alternating temperatures in the same experiment. These data show that cycling was either ineffective or inhibitory; the promotive effect of 10C on embryo germination was largely the result of additive effects of the two temperatures (5/10C).

Saure (1985) suggested the existence of two distinct but overlapping temperature reactions; one producing a

dormancy breaking factor by chilling temperature while the other produces a factor which maintains and/or enforces dormancy by high temperature. The first reaction has a low temperature range at first, but as chilling accumulates the effective temperature range widens. The inhibitory potential is higher during the early stages of chilling hence could be negated by both moderate and high temperatures. As chilling proceeds the buds accumulate sufficient chilling units that the second reaction can proceed and the range of temperatures capable of negating subsequent chilling becomes narrower (> 23C). These data partially support the hypothesis based on widening of the temperature range for the breaking of dormancy as the chilling process proceeds at low temperature.

Figure 1. Effect of stratifying seed at constant temperatures on subsquent germination of the embryos at 20C. (1990 seed source, Expt. 1)

Figure 1.

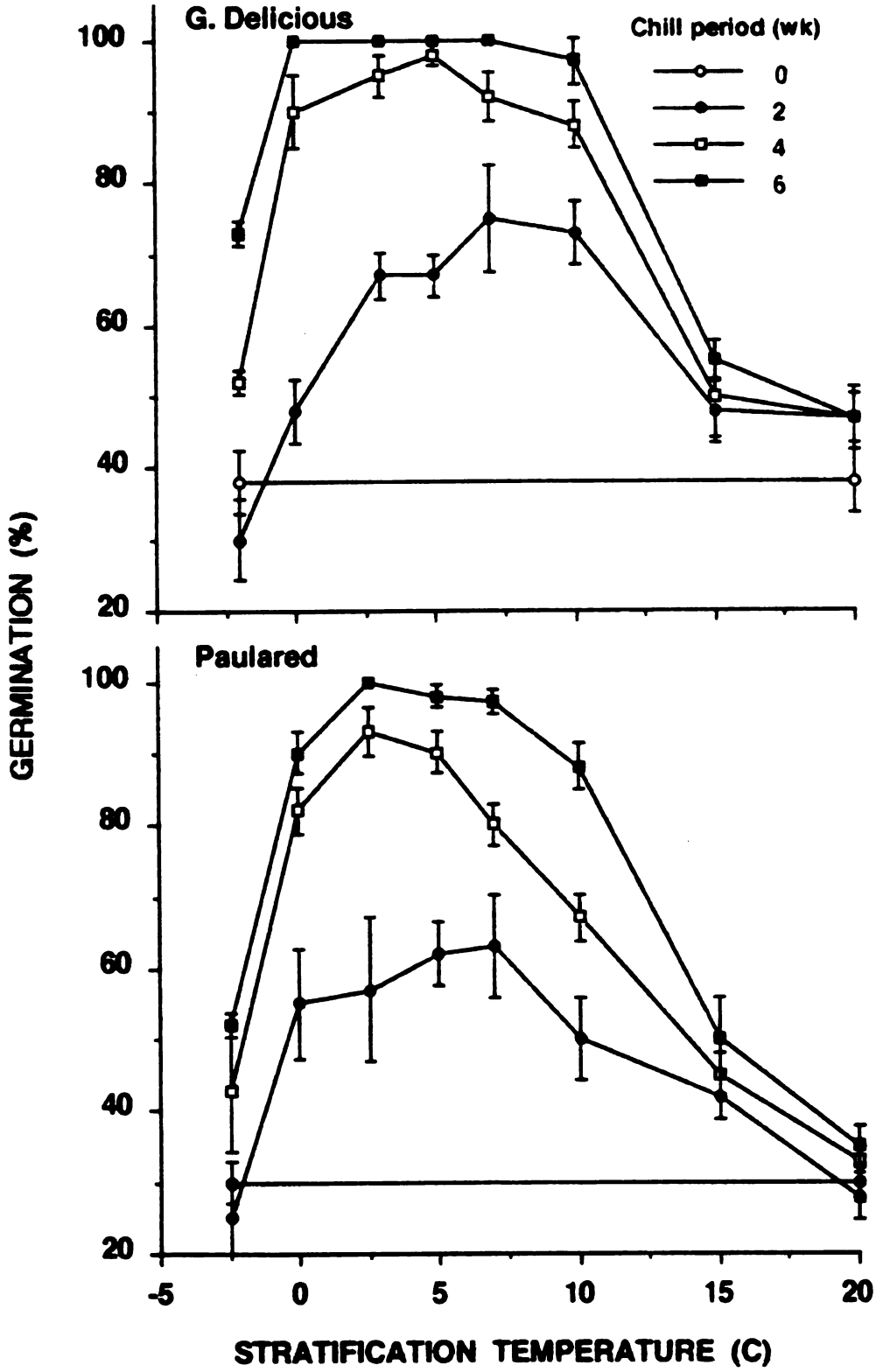

G. Delicious
Chill period (wk)
0
2
4
6
Paulared
GERMINATION (%)
100
80
60
40
20
-5
0
5
10
15
20
STRATIFICATION TEMPERATURE (C)

Table 1. Effects of time of stratification of 'Golden Delicious' and 'Paulared' seeds at 5C on subsequent germination (%) of the seeds and embryos and on germination rate (MDG) of 'Golden Delicious' embryos

Cultivar	Stratification time (wk)				
	0	3	6	9	12
			Embryo germination (%)		
G. Delicious	50b[Z]	97a	100a	100a	-
Paulared	45c	87b	100a	100a	-
			Seed germination (%)		
G. Delicious	-	-	7c	88b	100a
Paulared	-	-	35b	90a	97a
			Embryo germination rate (MDG)[Y]		
G. Delicious	7a	4b	2.9c	2.1c	-

Z : Treatment mean separation within rows by DMRT, $P<0.05$.
Y : Mean days to germination (MDG)

Figure 2. Effects of stratifying seeds at constant 5C vs. alternating temperatures in a diurnal cycle on subsequent germination of the embryos at 20C.

Figure 2.

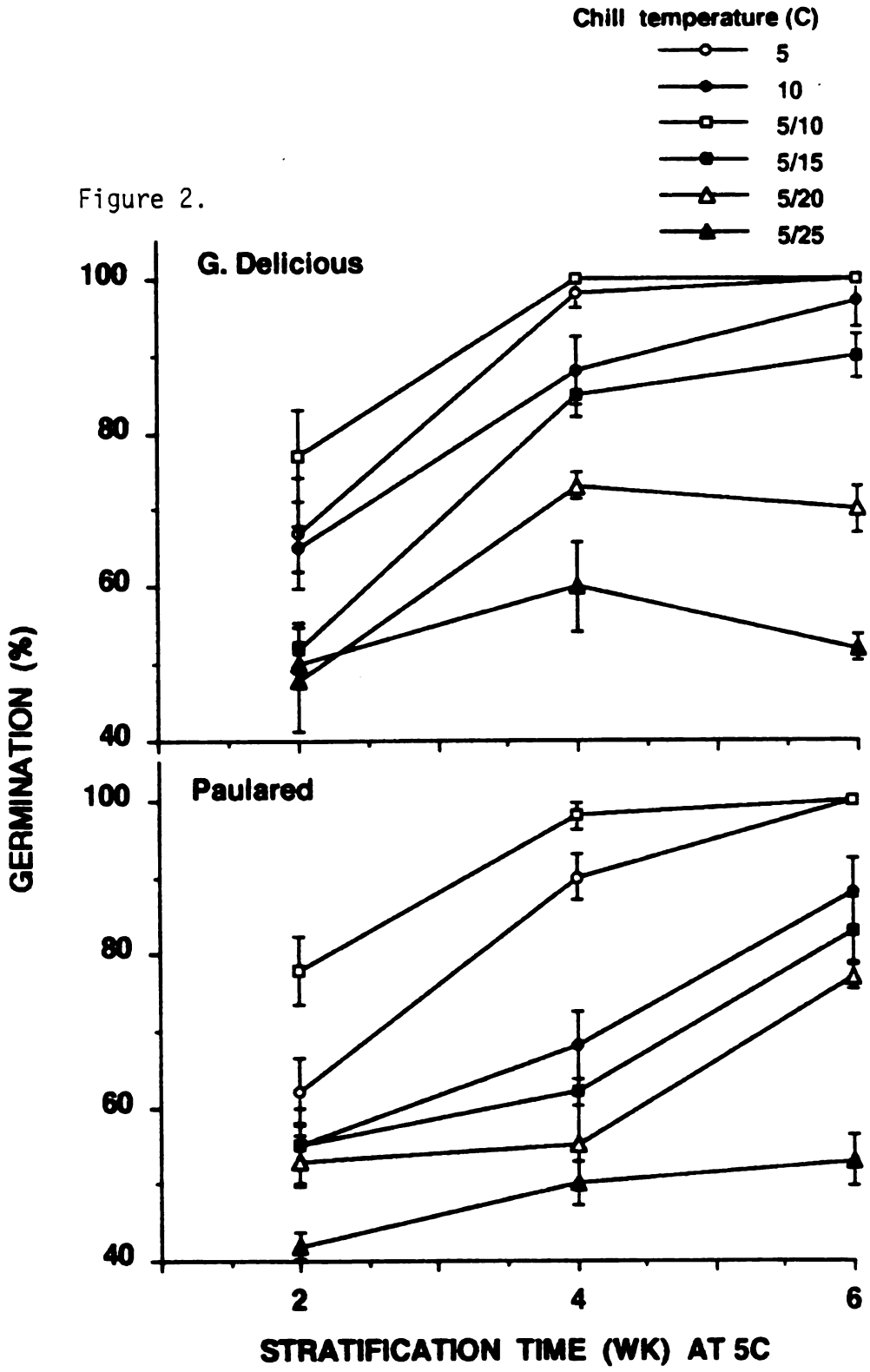

Chill temperature (C)
5
10
5/10
5/15
5/20
5/25
G. Delicious
Paulared
100
80
60
40
GERMINATION (%)
2
4
6
STRATIFICATION TIME (WK) AT 5C

Figure 3. Effects of stratifying seeds at constant 5C vs. alternating temperatures in a diurnal cycle on subsequent germination of the seeds at 20C.

Figure 3.

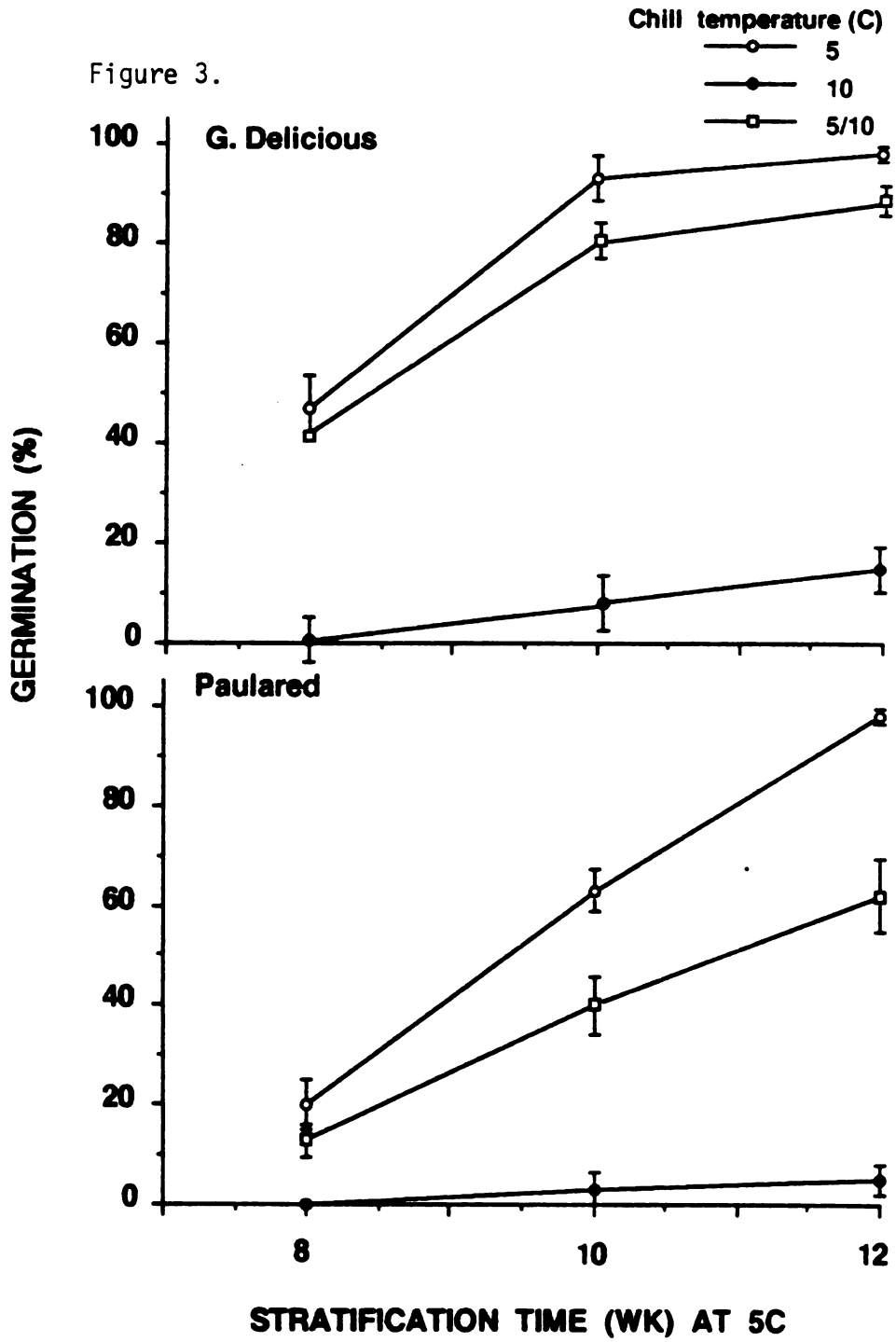

Chill temperature (C)
5
10
5/10
G. Delicious
Paulared
GERMINATION (%)
100
80
60
40
20
0
8
10
12
STRATIFICATION TIME (WK) AT 5C

Figure 4. Effects of stratifying seeds at constant 5C or alternating temperatures in a 24-hour or 3-week cycle on subsequent germination of the embryos at 20C. [5C = constant 5C′ 5/10 = daily cycle (16 hr at 5C, 8 hr at 10C); 5-5-10 = 3-week cycle (2 wk at 5C, 1 wk at 10 C); 10-5-5 = 1 wk cycle (1 wk at 10C, 2 wk at 5C). All seeds were exposed to 5C for a total of 4 wk.]

Figure 4.

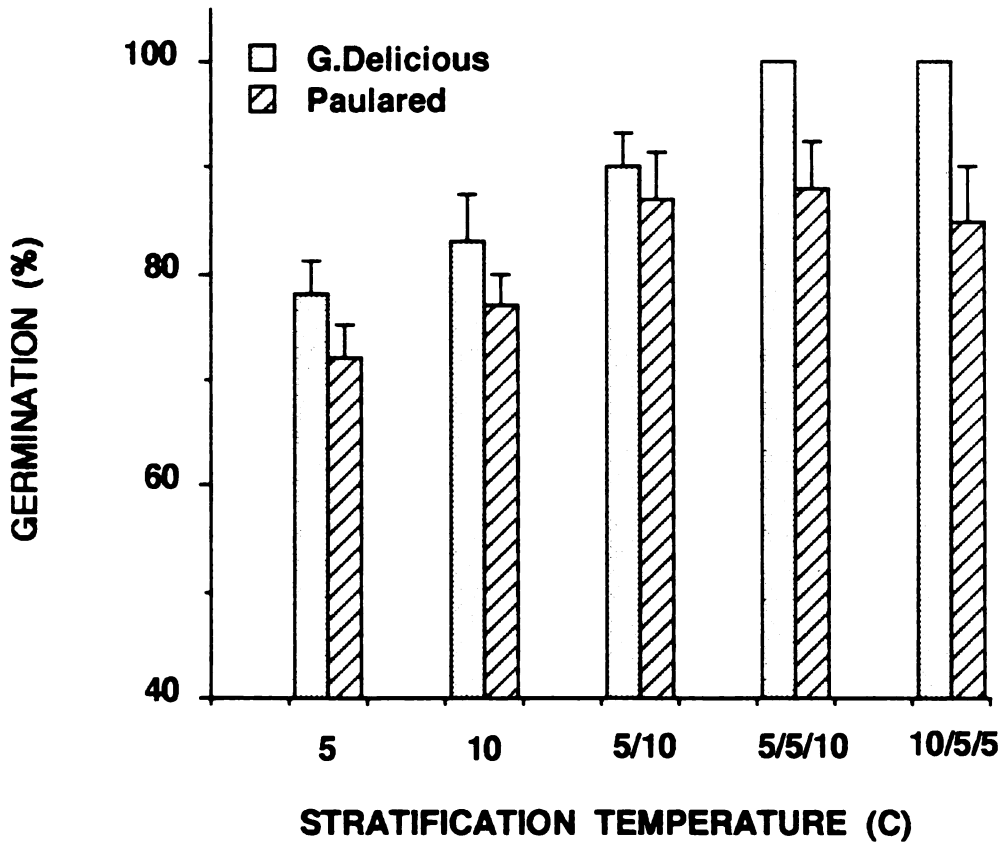

100
80
60
40
G.Delicious
Paulared
GERMINATION (%)
5
10
5/10
5/5/10
10/5/5
STRATIFICATION TEMPERATURE (C)

Figure 5. Effects of stratifying seeds at constant 5C vs. alternating temperatures in a 6-wk cycle on subsequent germination of the embryos during 6 days at 20C. (All seeds were exposed to a total of 4 wk at 5C).

Figure 5.

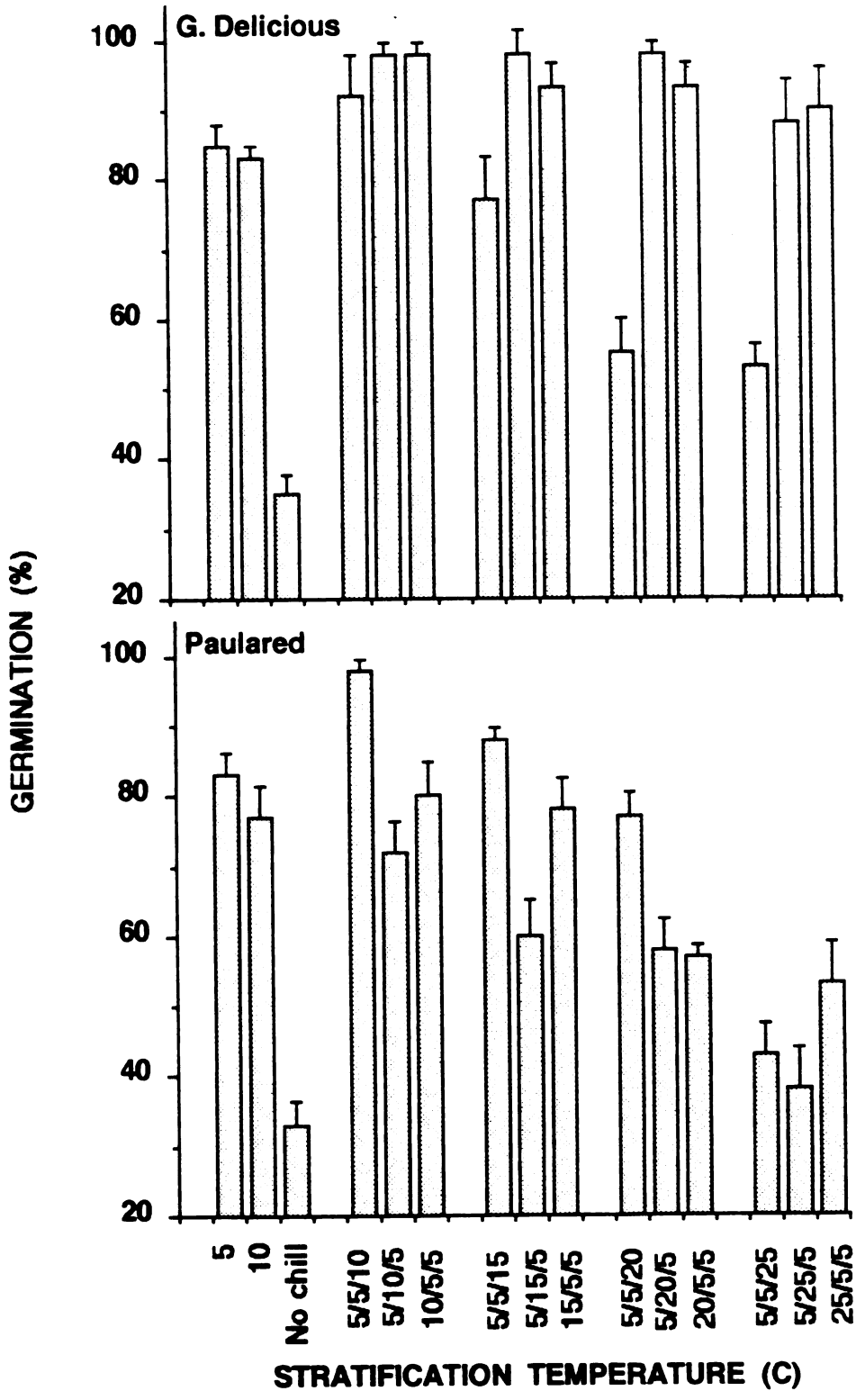
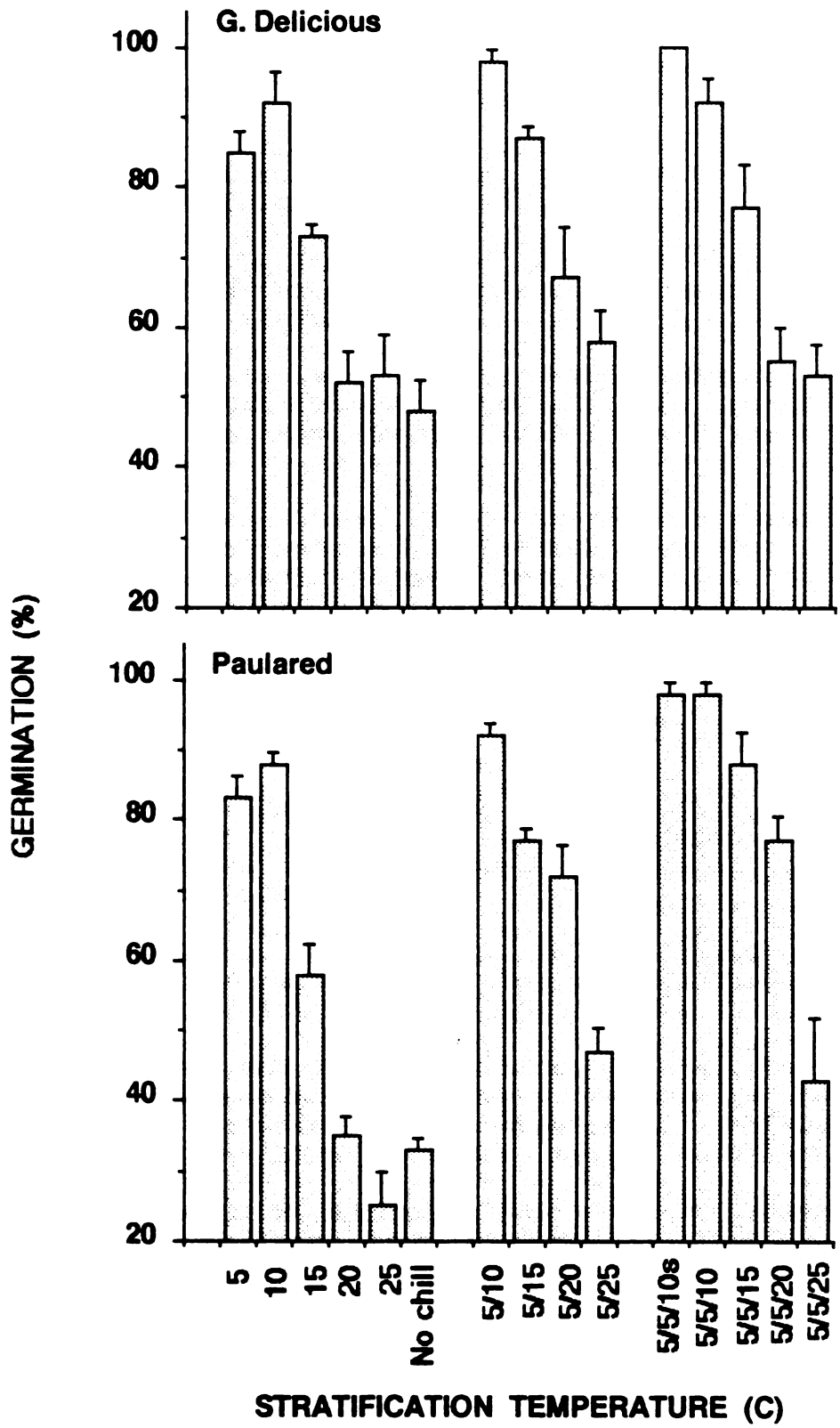

G. Delicious
Paulared
100
80
60
40
20
GERMINATION (%)
5
10
No chill
5/5/10
5/10/5
10/5/5
5/5/15
5/15/5
15/5/5
5/5/20
5/20/5
20/5/5
5/5/25
5/25/5
25/5/5
STRATIFICATION TEMPERATURE (C)

Figure 6. Comparison of effects of constant vs. alternating temperatures and of cycle length on the breaking of apple embryo dormancy. (Constant temperatures 5, 10, 15, 20 and 25; temperatures alternating in daily cycles: 5, 5/10, 5/15, 5/20 and 5/25 with 16 h at 5C and 8 h at higher temeprature;3-week cycle, 5-5-10,i.e. 2 wk at 5C, 1 wk at 10C; 6-week cycle, 5-5-10, 5-5-15, 5-5-20 and 5-5-25, 4 wk at 5C and 2 wk at the higher temperatures).

Figure 6.


G. Delicious
Paulared
100
80
60
40
20
GERMINATION (%)
5
10
15
20
25
No chill
5/10
5/15
5/20
5/25
5/5/10s
5/5/10
5/5/15
5/5/20
5/5/25
STRATIFICATION TEMPERATURE (C)

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CHAPTER ONE, SECTION TWO

NEGATION OF THE CHILLING EFFECT BY HIGH TEMPERATURE FOLLOWING SEED STRATIFICATION

NEGATION OF THE CHILLING EFFECT BY HIGH TEMPERATURE FOLLOWING SEED STRATIFICATION

Abstract. The chilling period of apple (*Malus domestica* Borkh.) embryos and seeds was interrupted by high temperature (15 to 30C) to determine the degree of negation on chilling and the stage of the chilling period at which the tissues were most sensitive. About 20 to 35% of the embryos excised from non-chilled seeds were capable of germinating and their germination capacity could not be reduced by high temperature. High temperatures, 20 to 30C, reduced the germination capacity of both seeds and embryos during the early stages of the chilling period. The degree of inhibition depended on the temperature of exposure during the interruption of the chilling period. As stratification at 5C proceeded , the embryos and seeds became less sensitive to high temperature. Secondary dormancy was induced in seeds fully chilled within fruit, but not in those chilled in Petri dishes by exposure to 30C for 10 days. Reduction in seed germination was less when the seeds were left in the fruit during exposure to 30C than when the seeds were removed.

Introduction

Apple embryos are dormant at harvest time. Two to three months of seed stratification are required to break dormancy and permit normal seed germination and seedling growth. However, chilled embryos can be induced into secondary dormancy by high temperature, anaerobiosis (Come and Thevenot 1982) or treatment with abscisic acid (Bouvier-Durand et al. 1977). Moderate temperatures (10-15C) enhanced the breaking of peach bud dormancy when alternated with 5C in a daily cycle, while temperatures of 20C and above negate the chilling effect (Erez and Couvillon 1987). The mechanisms involved in both primary and secondary dormancy are not known despite the numerous publications on this problem (Come and Thevenot 1982; Karssen 1980/81). Many studies have emphasized primary and secondary dormancy of seeds, yet the actual site of dormancy is in the embryonic axis (Come and Thevenot 1982). The objectives of this investigation were: a) to determine the temperatures which are most effective in negating the chilling effect, and b) determine the time during the chilling period when the embryos are most sensitive to high temperature.

Materials and Methods

Plant material.

Mature 'Golden Delicious' apple (Malus domestica Borkh.) fruits were harvested at Michigan State University

Horticultural Research center in 1989 and 1990. Some seeds were extracted, air dried, and stored at room temperature; others were left in the fruits.

Stratification and germination.

Except where otherwise noted, dry seeds were imbibed in water for 48 hr prior to stratification at 5C on filter paper. After various periods at 5C or 5C followed by 30C, seeds or embryos excised from the seeds were placed in Petri dishes lined with moistened filter paper and held at 20C in darkness. Germination counts were made daily for 10 days and germination percentages calculated. Germination capacity of the embryos or seeds was used as a measure of the degree of secondary dormancy induced.

Experimental design.

Each treatment included three replicates of 20 seeds or embryos arranged in a completely random design. Percentages were transformed into arcsin and both percentages and arcsin transformed data were subjected to an analysis of variance. No regression equation satisfactorily described the germination respone curves, hence the data are presented as means of three replicates plus or minus the standard error.

Experiments

Experiment 1. Effects of method of stratification and length of exposure to 30C after 10 wk at 2.5C on the germination capacity of embryos at 20C. Seeds were chilled for 10 wk either in the fruit or in Petri dishes prior to exposure to 30C for 0 to 4 weeks. Fruits were harvested and seeds were removed from some fruits immediately after harvest. On removal from the fruit after chilling , the seeds were soaked in water for 48 hours. The embryos were then excised and germinated at 20C on one layer of moist Whatmann No.1 filter paper in Petri dishes. Germination was recorded over a 10-day period.

Experiment 2. Effects of stratification period and length of exposure to 30C on the germination capacity of seeds at 20C. Seeds were stratified at 5C in Petri dishes for 6, 8 and 10 wk, then exposed to 30C for 0, 1, 2, and 3 weeks. At the end of the high temperature exposure the seeds were germinated at 20C and the germination percentages calculated.

Experiment 3. Effect of temperature of exposure after chilling on induction of secondary dormancy in apple embryos. Seeds were stratified in Petri dishes at 5C for 0 to 5 weeks, and then exposed to 15, 20, 25 or 30C for 10 days. Thereafter the embryos were excised and germinated at

20C. Germination counts were made and germination percentages calculated. Seeds that germinated during the high temperature exposure were also recorded.

Experiment 4. Effects of stratifying seeds at 5C before or after a 10-day exposure to 30C on induction of secondary dormancy in the seeds. Seeds were stratified in Petri dishes at 5C for 0, 3, 6, 9 or 12 wk, then subjected to 30C for 10 days, and then re-stratified at 5C for 0, 3, 6 or 9 weeks. At the end of each treatment the seeds were germinated at 20C.

Results

Experiment 1. Effects of method of stratification and length of exposure to 30C after 10 weeks at 5C on the germination capacity of embryos at 20C. High temperature neither reduced the germination capacity of embryos excised from seeds fully chilled in Petri dishes nor increased the intensity of dormancy of non-chilled embryos regardless of the length of time at 30C (Fig.1).

Germination capacity of embryos was significantly reduced by high temperature when seeds were chilled within the fruit. The effect of 30C was greater when seeds chilled in the fruit were removed prior to exposure to 30C. Most of the responses to high temperature were saturated within the first two weeks. However, the germination

capacity of the embryos was never reduced to a level below the initial germination capacity.

Experiment 2. Effects of stratification period and length of exposure to 30C on the germination capacity of seeds at 20C. The germination capacity of seeds chilled for 6 or 8 weeks at 5C was reduced significantly by high temperature (30C) while that for seeds chilled for 10 weeks was not (Fig. 2). Germination of seeds chilled for 6 weeks was totally inhibited after exposure to high temperature for 2 weeks. Germination of seeds chilled for 8 weeks was reduced to about 40% by one week of exposure to high temperature while longer exposures 2 or 3 weeks had no additional effect.

Experiment 3. Effects of temperature of exposure after chilling on induction of secondary dormancy in apple embryos. The germination capacity of control embryos increased to 100% as time of stratification increased from 0 to 4 weeks (Fig. 3). Exposure to 15C promoted germination, whereas temperatures above 15C reduced it. The degree of inhibition increased with temperature and decreased with time at 5C when the temperature did not exceed 25C. Response to 30C was less consistent, but germination was lower than in all other treatments once chilling exceeded one week.

Experiment 4. Effects of stratifying seed at 5C before or after a 10-day exposure to 30C on induction of secondary dormancy in apple seeds. Seed germination capacity increased with increasing time of stratification reaching 100% in 9 weeks for the control treatment (Fig. 4). High temperature treatment before stratification decreased the germination capacity of seeds stratified for 6 weeks but not those stratified for 9 weeks.

Interruption of the stratification period after 3 weeks of chilling reduced the germination capacity of seed re-stratified for 6 weeks only. Interruption after 6 weeks of chilling decreased the germination percentage of the seeds re-stratified for 6, but not 9 wk. High temperature treatment had no significant effect on the germination capacity of seeds stratified for 9 weeks. The data show that secondary dormancy can be induced only in partially chilled seeds.

Discussion

High temperature reduced the germination capacity of partially chilled embryos but was ineffective on embryos from seeds that were fully chilled in petri dishes. The first week of exposure to high temperature had the greatest effect in reducing germination of the seeds. As stratification proceeded at 5C, the embryos became less sensitive to the induction of secondary dormancy by high

temperature. The degree of negation depended on temperature of exposure (the higher the temperature above 15C the greater the chilling negation effect) and the amount of chilling previously accumulated. This suggests that the effect of chilling is fixed. Attempts to intensify the dormancy of the embryos further than the natural level failed.

In contrast, secondary dormancy was induced in seeds and embryos chilled within fruit for 10-12 weeks. The reduction in germination capacity was less when the seeds were left in the fruits during exposure to high temperature than when the seeds were removed. The degree of the reversal of chilling by high temperature could be attributed to the seed coat as a physical barrier to germination of the embryos or a chemical barrier by releasing germination inhibitors to the embryo or restricting oxygen supply to the embryo, conditions all of which could reduce the chilling efficiency making the seeds or embryos sensitive to high temperature. This agrees with the notion that secondary dormancy or chilling negation effect can be induced by exposure to high temperature or ABA if the germination or growth potential is low (Samish 1954; Saure 1985). Furthermore the effectiveness of the dormancy induction or release processes is maximized if the seeds are fully hydrated (Stokes 1965). Seeds chilled within fruit are not fully hydrated,i.e., they contain 47% moisture as compared

to 57% for seeds chilled in petri dishes (Wan 1980). Consequently neither the effect of chilling nor the induction of dormancy was complete under such conditions.

Figure 1. Effects of method of stratification and length of exposure to 30C after 10 weeks at 5C on the germination capacity of the embryos at 20C. [(Abbreviations: Embryos excised from non-chilled seeds (C); seed chilled in Petri dish (CPD); seeds chilled within the fruit then removed before exposure to 30C (CFT)].

Figure 1.

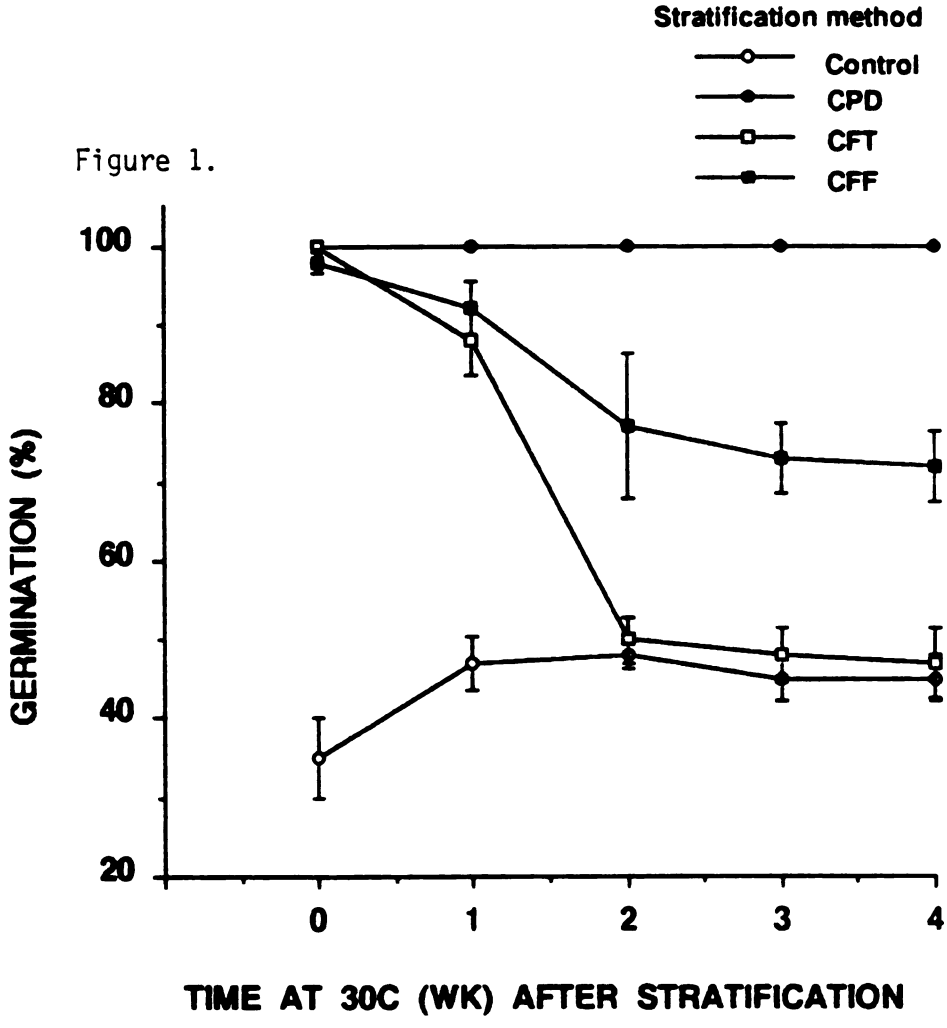

Stratification method
Control
CPD
CFT
CFF
100
80
60
40
20
GERMINATION (%)
0
1
2
3
4
TIME AT 30C (WK) AFTER STRATIFICATION

Figure 2. Effects of stratification period and length of exposure to 30C on the germination capacity of seeds at 20C.

Figure 2.

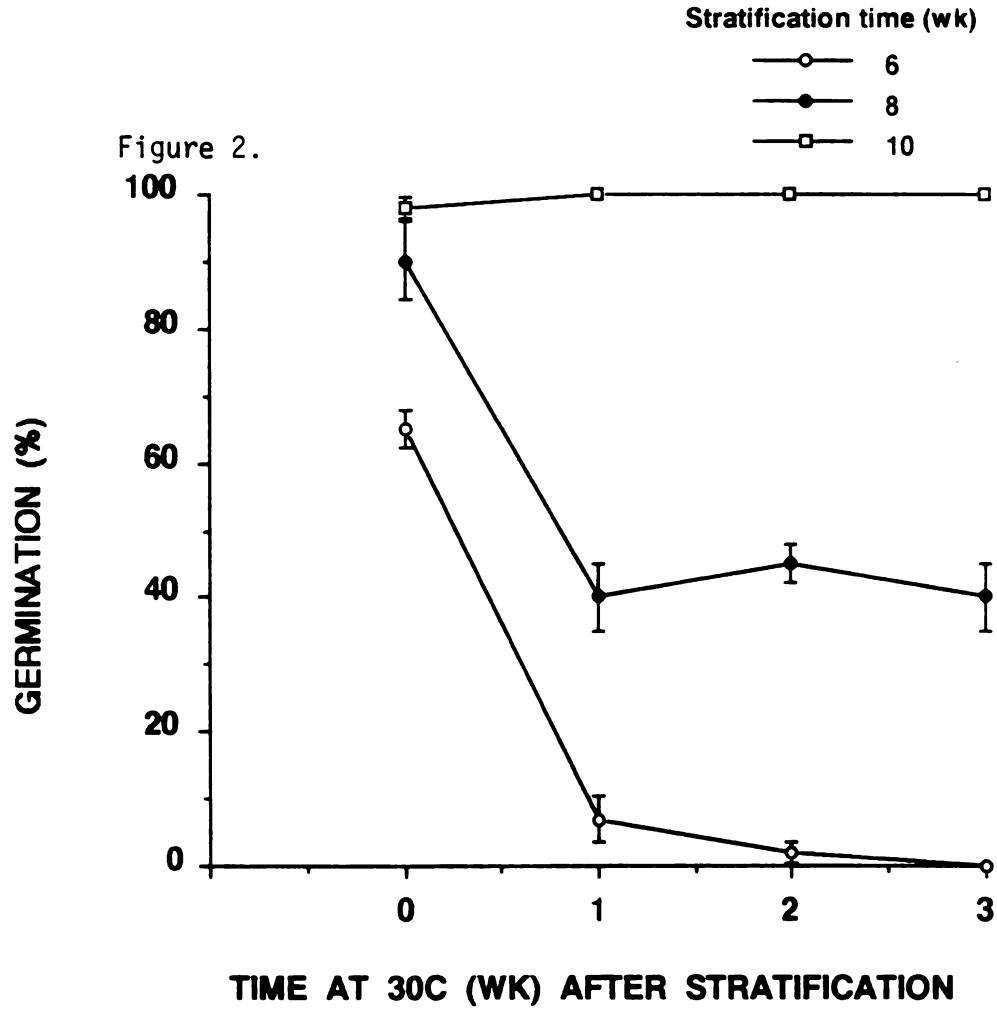

Stratification time (wk)
6
8
10
GERMINATION (%)
100
80
60
40
20
0
0
1
2
3
TIME AT 30C (WK) AFTER STRATIFICATION

Figure 3. Effect of temperature of exposure after chilling on induction of secondary dormancy in apple embryos.

Figure 3.

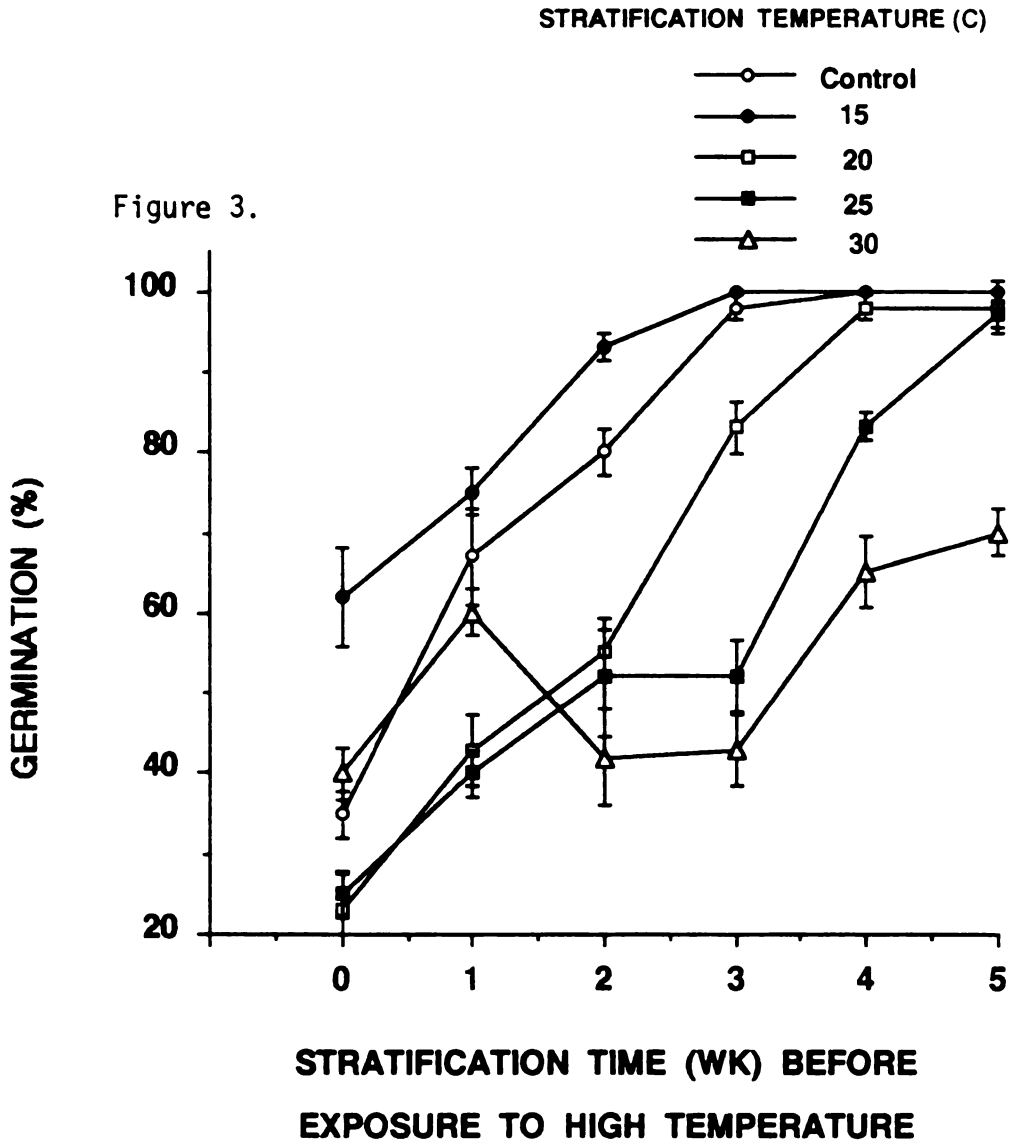
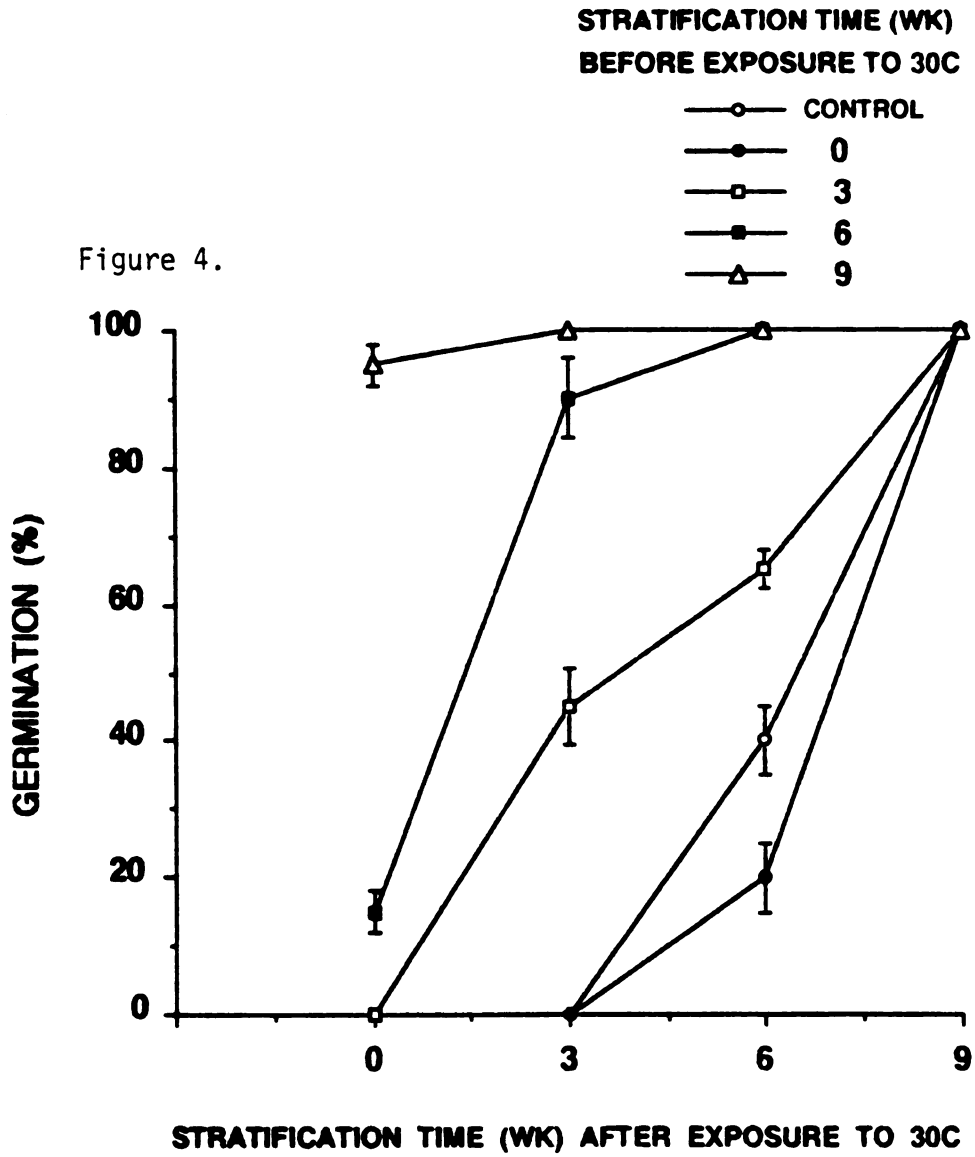

STRATIFICATION TEMPERATURE (C)
Control
15
20
25
30
GERMINATION (%)
100
80
60
40
20
0
1
2
3
4
5
STRATIFICATION TIME (WK) BEFORE
EXPOSURE TO HIGH TEMPERATURE

Figure 4. Effect of stratifying seed at 5C before or after exposure to 30C on induction of secondary dormancy in apple seeds.

Figure 4.


STRATIFICATION TIME (WK)
BEFORE EXPOSURE TO 30C
CONTROL
0
3
6
9
GERMINATION (%)
100
80
60
40
20
0
0
3
6
9
STRATIFICATION TIME (WK) AFTER EXPOSURE TO 30C

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CHAPTER TWO, SECTION ONE

BREAKING OF APPLE EMBRYO DORMANCY WITH LOW TEMPERATURE, HORMONES, CHEMICALS, AND ANOXIA. I. EVALUATION OF METHODS

BREAKING OF APPLE EMBRYO DORMANCY WITH LOW TEMPERATURE, HORMONES, CHEMICALS, AND ANOXIA. 1. EVALUATION OF METHODS

Abstract. The effectiveness of selected phytohormones and other chemicals was compared to that of low temperature (5C) in breaking dormancy of apple embryos. The germination capacities at 20C of embryos excised from seeds chilled in Petri dishes vs. within fruit were similar and reached 100% in 6 weeks. However, embryos chilled within fruit had a slower rate of germination than those chilled in Petri dishes. Intact seeds chilled in Petri dishes required 10 weeks to reach 100% germination. Those chilled within fruit began to germinate after 10 weeks of chilling; only 40% germinated after 15 weeks of stratification. Anaerobiosis, achieved by holding seeds under water at 20C, failed to break embryo dormancy, however, seeds held under water at 2.5C germinated as well as those chilled in Petri dishes. Gibberellin A4+7, benzyladenine (BA) , Promalin (a 1:1 mixture of GA 4+7 and BA) and Dormex (cyanamide) significantly promoted the germination of non-chilled apple embryos at concentrations of 20, 10, 10, and 20 to 80 mg/L respectively. However, maximum germination ranged only between 70 and 90%. Germination of dormant seeds was not stimulated by any of the chemicals.

Introduction

Dormancy is the major limiting factor to successful production of deciduous fruits under tropical conditions. Buds and seeds of apple require about 1000 to 3000 hours of chilling at 0 to 7C during winter to break dormancy and resume normal growth (Powell 1986; Saure 1985; Westwood 1978). Under tropical conditions the buds and seeds do not receive sufficient chilling to break dormancy fully, resulting in delayed foliation or prolonged dormancy.

Alternative methods to stratification involve the use of phytohormones and other dormancy breaking chemical agents (Powell 1986; Westwood 1978). Gibberellic acid (GA3) stimulates the germination of dormant apple embryos, although it cannot completely replace the chilling requirement in most cases (Durand 1974). Benzyladenine (BA) stimulates the germination of dormant apple and peach embryos (Rouskas et. al. 1980, Zhang and Lespinasse 1991; Badizadegan 1967). In some cases, BA permits normal germination and development of the plant whereas in others subsequent growth of the seedlings is abnormal. Come and Thevenot (1982) noted that dormant embryos of apple may germinate well if placed for 2 weeks at 15C following treatment with high concentrations of potassium cyanide. Come and Tissaoui (1973) reported that embryo dormancy can also be broken by placing the embryos under anaerobic conditions.

Thus, various treatments break embryo dormancy, some being more effective than others, but these methods have not been compared simultaneously. The purpose of this investigation was to compare these methods and determine:

1) the most effective treatments for stimulating embryo germination;

2) optimum chilling-time and/or concentration of the dormancy breaking chemical agents;

3) those treatments that break dormancy without allowing the processes of germination to occur at the same time.

The information obtained from these experiments was subsequently used to initiate studies on the ultrastructural changes occurring during the release of dormancy using transmission electron microscopy (see section two).

Materials and Methods

Apple (Malus domestica Bork, cv. 'Golden Delicious' and 'Paulared') fruits were harvested in 1989 and 1990 at the Horticultural Research Center, Michigan State University. Most of the seeds were removed at harvest time and were either used immediately or air dried and stored in an open bottle at room temperature; some fruits were stored at 2.5C for subsequent seed removal. Both seeds and embryos were germinated at 20C.

Experimental design

The treatments were arranged in a completely random, factorial design. Three replicates of 20 seeds or embryos each were used for each treatment. An analysis of variance was performed on the data and standard errors were used for comparison of treatment means.

Experiment 1. Effect of after-ripening apple seed at 2.5C within fruit vs. in Petri dishes on germination of the seeds and embryos at 20C. 'Golden Delicious' and 'Paulared' seeds were either held in the fruit or stratified in Petri dishes lined with moist filter paper at 2.5C for 0, 2, 4, 6, 8, 10, and 12 weeks.Additional seeds were left in fruits held at 21C for 4 or 8 weeks. Seeds held in the fruit were soaked in water for 48 hours prior to embryo excision or germination. Both seeds and excised embryos were germinated at 20C.

Experiment 2. Effect of after-ripening apple seed at 2.5C under water with or without sparging with N2 on subsequent germination of the embryos at 20C. About 500 'Golden Delicious' seeds were placed in each bottle (17 cm high by 9 cm in diameter), which was then filled with water and placed at 2.5C,or 20C for 2, 4, 6 or 8 weeks. Half the bottles were sparged with nitrogen for one hour prior to chilling to displace all dissolved oxygen and create an anoxic

condition. Additional seeds were chilled in Petri dishes (control treatment) lined with moistened Whatmann No. 1 filter paper for 2, 4, 6 or 8 weeks.

Experiment 3 Effect of hormone treatment of non-chilled apple embryos on subsequent germination at 20C.
Dormant seeds of 'Golden Delicious' and 'Paulared' were soaked in water for 48 hours. The embryos were then excised, rinsed in water and soaked for 24 hours in several concentrations of: a) gibberellin A4+7 b) benzyladenine (BA); c) Promalin (commercial mixture of GA4+7 and BA in 1:1 ratio); or d) Dormex (commercial preparation of cyanamide, H2CN). The following concentrations were used: 0, 10, 20, 40, 80 and 160 mg/l. After 24-hour of soaking, the embryos were rinsed three times with sterile distilled water and germinated at 20C in darkness over a 10-day period.

Results

Experiment 1 Effect of stratifying seed within fruit vs. in Petri dishes on germination of the seeds and embryos at 20C. About 25 to 38% of the embryos were not dormant at harvest time (Fig. 1). Similar germination percentages were obtained with embryos excised from seeds in fruits held at 20C for 4 and 8 weeks, i.e., 35 and 37%, respectively. Germination capacity of the embryos increased with time of stratification at 2.5C, reaching 100% at 6 to 8 weeks.

Methods of chilling did not significantly affect this response. Germination at 20C of seed chilled in Petri dishes started after 8 weeks and reached full capacity after 12 weeks of stratification. Seeds started germinating at the temperature of stratification (3C) in the 11th week. Seeds chilled within fruit commenced germination at 20C after 10 weeks but reached only 40% after 12 weeks, with no further increase even after 15 weeks (data not shown) of after-ripening. Therefore, the first 6 to 8 weeks of after-ripening broke embryo dormancy while the last 6 to 12 weeks broke whole seed dormancy.

The germination rate of embryos increased significantly with increasing stratification period up to 6 weeks (Table 1). Non-stratified embryos required 6 to 7 days to germinate, those excised from seeds stratified for 8 weeks in Petri dishes about 2 days, while those excised from seeds chilled within fruit for 8 weeks required about 3.5 days. The germination rate at 6 weeks did not differ from that at 8 weeks except for 'Paulared' seed chilled in Petri dishes. The germination rate of embryos excised from seed chilled within the fruit was similar to that of embryos excised from seeds chilled in Petri dishes for the first 4 weeks. However, differences were apparent in 'Golden Delicious' at 6 and 8 weeks of stratification; embryos from Petri dishes germinating more rapidly than those from fruits.

Experiment 2. Effect of after-ripening 'Golden Delicious' apple seed at 2.5C under water on subsequent germination of the embryos at 20C. Embryos excised from seed stratified in Petri dishes at 2.5C reached full germination capacity at 20C within 4 weeks (Table 2). Those from seeds stratified under water at 2.5C reached full germination capacity in 6 weeks regardless of sparging the water with nitrogen or not. Creating total anoxia by sparging the water with nitrogen before stratification did not affect germination as compared to the non-sparged control. Neither intact seeds held at 2.5C nor embryos from seeds held under water at 20C germinated.

Experiment 3. Effect of chemical treatment of non-chilled 'Golden Delicious' and 'Paulared' apple embryos on subsequent germination at 20C. The optimum concentration of gibberellin A4+7 for stimulating embryo germination was 20 mg/L, which induced about 85% germination in 'Golden Delicious' and about 78% in 'Paulared" (Fig. 2) . Promalin and BA were more effective than GA4+7 in stimulating germination. At 10 mg/L, BA induced 83 and 67% germination in 'Golden Delicious' and 'Paulared,' respectively. Promalin was the most effective treatment, giving about 85 and 88% germination for 'Golden Delicious' and 'Paulared', respectively. However, higher concentrations of both BA and Promalin were less effective, becoming inhibitory at 160

mg/L.

Dormex (cyanamide) was effective over a broader concentration range than any of the other chemicals. Concentrations ranging from 10 to 80 mg/L and 20 to 160 mg/L were effective on 'Golden Delicious' and 'Paulared', respectively, and the highest germination percentages achieved were 80 and 68%, respectively.

GA4+7 and cyanamide were not toxic and most of the seedlings were normal, whereas those treated with BA and promalin had abnormally thick hypocotyls.

Discussion

Embryo dormancy was broken earlier than whole seed dormancy. This implies that the seed coat either contains germination inhibitors or physically restricts protrusion of the radicle. Seeds started germinating at the temperature of stratification (2.5C) as soon as they attained full germination potential. Thus chilling widens the temperature range for germination as suggested by Samish (1954) and Saure (1985).

During the first 4 weeks of chilling, the rate of embryo germination was not affected by method of stratification. Thereafter embryos from seed chilled within fruit had a slower rate of germination than those from seeds chilled in Petri dishes. Thus the fruit inhibited after-ripening. The chemicals in the fruit (Evenari 1949) and/or

seed coat (Luckwill 1952) may reduce the effectiveness of the stratification process. Alternatively low moisture content of the seeds stratified within fruit may not permit full effectiveness of the stratification process (Wan 1980). Chilling seeds within fruit is considered one of the best methods of breaking dormancy without allowing the processes of germination to occur simultaneously (Come and Thevenot 1982).

Stratifying seeds under water permits dormancy release without allowing germination. The chilling requirement of the embryos was fulfilled within 6 to 8 weeks of stratifying the seeds under water at 2.5C, but not at 20C. These data indicate that low temperature broke the dormancy whereas anaerobiosis failed to do so. Sparging the water with nitrogen gas to displace any dissolved oxygen and create total anoxia did not affect the response. Traces of oxygen present in the water could maintain dormancy, for Tissaoui and Come (1973) reported that the medium must be completely free of oxygen because as little as 0.2% oxygen may block the chilling effect. The embryos excised from seeds chilled in Petri dishes germinated faster than those chilled under water. The absence of oxygen may have delayed the process of germination prior to transfer to 20C.

GA4+7 partially broke embryo dormancy but failed to break whole seed dormancy, whereas chilling broke both. In seeds, GA promotes the biosynthesis of several hydrolytic

enzymes (e.g., amylase, proteases, lipases and ribonucleases) that are required for the germination process and also for the development of endoplasmic reticulum, which acts as an intracellular transport system (Walton 1980). Amen (1968) considered GA to be a germination agent whose continued presence is required, unlike a triggering agent whose continued presence is not essential for germination. This hypothesis was supported by Bradbeer (1968) who suggested that GA synthesis was not needed for dormancy release but rather for germination.

Cynanamide is used commercially to break dormancy of grapevine in Israel (Shulman et. al. 1982), raspberries (Snir 1983) and many deciduous fruit species in Japan (Morimoto and Kumashiro 1978), but its mode of action is not known. No published data are known to the author on the effect of cyanamide on seed and/or embryo dormancy. These results have demonstrated that cyanamide is capable of breaking apple embryo, but not seed dormancy, and has a broad concentration range of effectiveness without phytotoxicity.

Benzyladenine also broke embryo dormancy but not seed dormancy. Concentrations higher than 10 mg/l led to the formation of deformed radicles which were short and thick, while the highest concentrations actually killed the embryos. BA is known to complement or permit the activity of GA in dormancy release (Ryc and Lewak 1982).

Promalin was the most effective treatment used, indicating the complementary effects of BA and GA. Arias and Crabbe (1975) observed that isolated buds of sweet cherry (Prunus avium) responded better to cytokinins during the early phase of true dormancy, whereas the response to GA was better in the later phase of true dormancy. This biphasic character in the complementary actions of GA and BA was also observed in dormant apple embryos (Ryc and Lewak 1982).

The failure of the chemicals (GA4+7, BA, promalin and cyanamide) and anoxia to break whole seed dormancy supports the concept of dormancy being a quantitative state. Brief chilling results in embryo germination, but the germination is weak. Vigor of germination increases with chilling until the radicle can penetrate the seed coat. GA4+7, BA, cyanamide and chilling under water break dormancy but the radicles do not have enough vigor to penetrate the seed coat.

Figure 1. Effect of stratifying 'Golden Delicious' and 'Paulared'apple seed at 2.5C within fruit vs. in Petri dishes on germination of the seeds and embryos at 20C. [Abbreviations; embryos excised from seeds chilled in Petri dish (FTE); embryos excised from seeds chilled within fruit (FTE); seeds chilled in Petri dishes (PDS); seeds chilled within fruit (FTS)].

Figure 1.

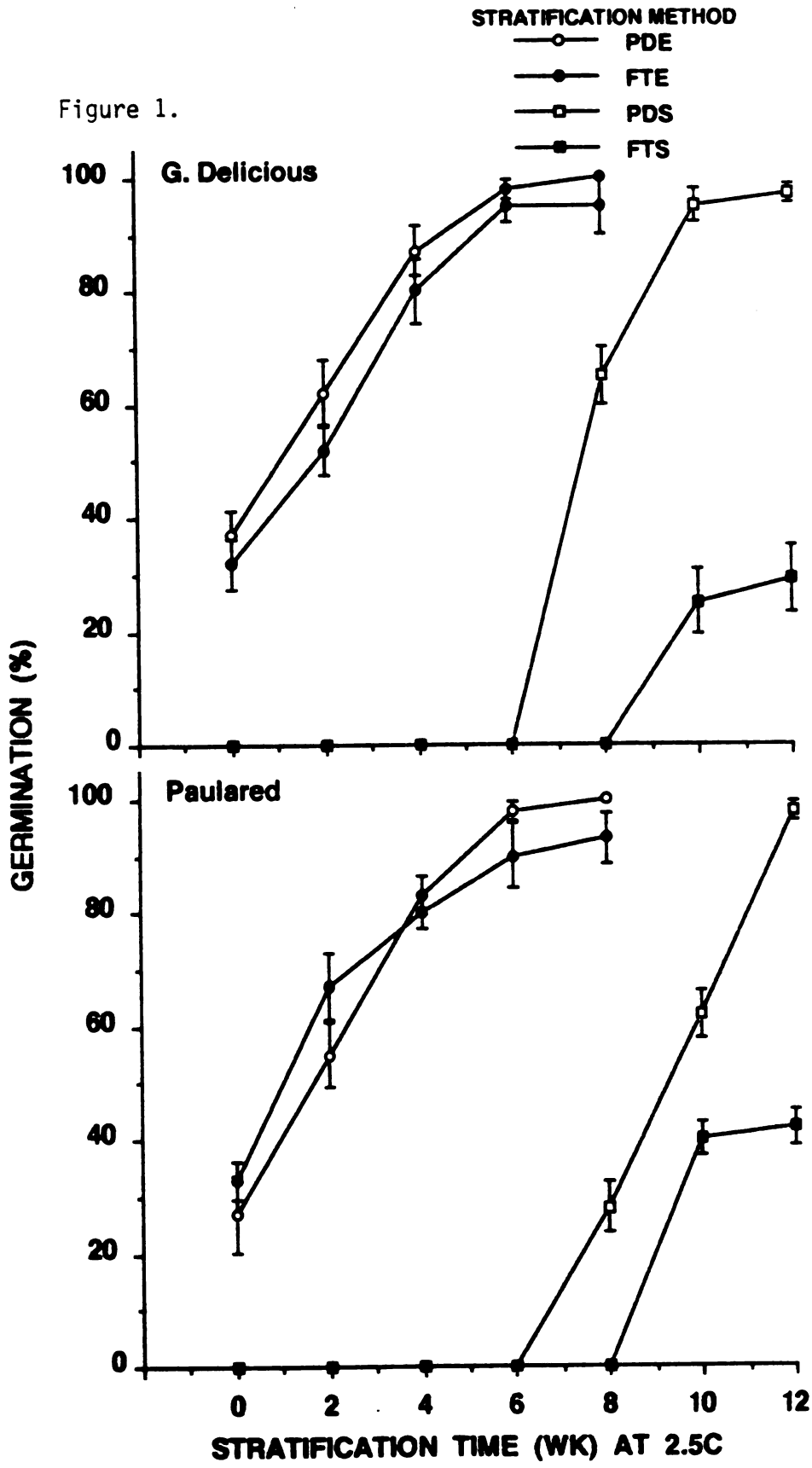

STRATIFICATION METHOD
PDE
FTE
PDS
FTS
G. Delicious
Paulared
GERMINATION (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
STRATIFICATION TIME (WK) AT 2.5C

Table 1. The germination rate of 'Golden Delicious' and 'Paulared' apple embryos excised from seeds stratified at 2.5C in Petri dishes or within fruits.

Cultivar	Chill medium	Stratification time (wk) at 2.5C				
		0	2	4	6	8
G. Delicious	P. dish	6.9a[Z]	5.3b	3.9c	2.5d	2.2d
	Fruit	6.7a	5.3b	4.3c	4.1c	3.7c
Paulared	P. dish	6.4a	5.1b	3.8c	3.8c	2.2d
	Fruit	6.3a	5.5b	4.1c	3.7cd	3.4d

Z = Treatment mean separation within rows by DMRT, $P<0.05$

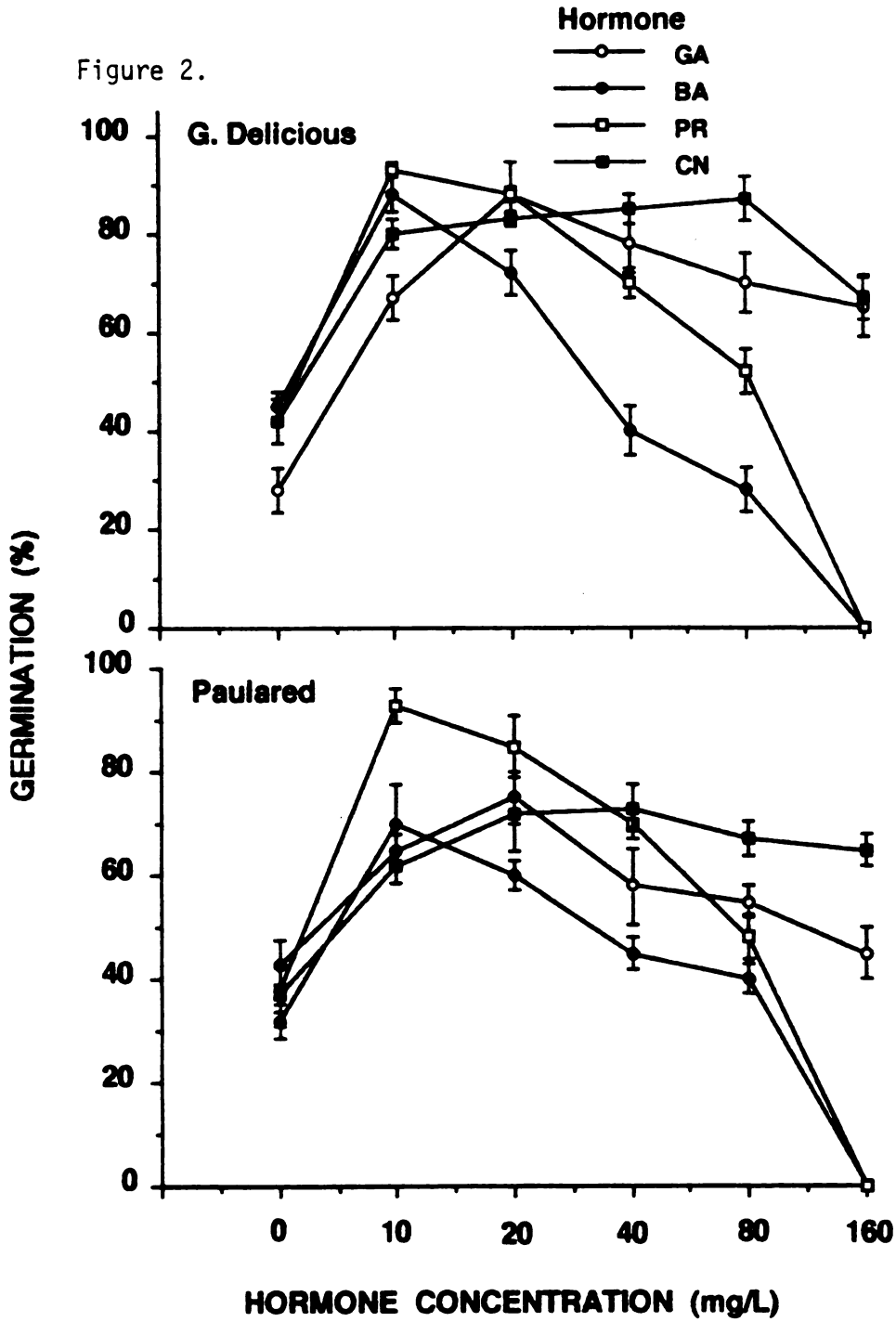
Table 2. Effect of after-ripening 'Golden Delicious' apple seed at 2.5C under water with or without sparging with nitrogen on subsequent germination of the embryos at 20C.

Chill period (wk)	Petri dish	Submerged in Water	
		Not sparged	Sparged
0	35c[Z]	38c	38c
2	87b	75b	70b
4	100a	93a	93a
6	100a	100a	97a
8	100a	100a	98a

Z = separation within column by DMRT, P<0.05

Figure 2. Effect of hormone treatment of 'G. Delicious' and 'Paulared' apple embryos on subsequent germination at 20C. [Abbreviations: gibberellin A4 + 7 (GA); benzyladenine (BA); Promalin (PR); cyanamide (CN)].

Figure 2.


Hormone
GA
BA
PR
CN
G. Delicious
Paulared
GERMINATION (%)
100
80
60
40
20
0
0
10
20
40
80
160
HORMONE CONCENTRATION (mg/L)

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CHAPTER TWO, SECTION TWO

BREAKING OF APPLE EMBRYO DORMANCY WITH LOW TEMPERATURE, HORMONES, CHEMICALS, AND ANOXIA. II. CYTOLOGICAL CHANGES

BREAKING OF EMBRYO DORMANCY WITH TEMPERATURE, HORMONES, CHEMICALS, AND ANOXIA II. CYTOLOGICAL CHANGES

Abstract. The effects of chilling (in fruit, in Petri dishes, and under water)and of chemical treatments [GA4+7, BA, and Dormex (cyanamide)] on cytological changes in the meristematic cells of embryonic axes were compared. Chilling seeds within fruit or under water breaks dormancy without permitting germination, whereas the other methods do not block germination. Cells in dormant embryos were observed to contain a distinct nucleus and nucleolus, nuclear membrane and plasma membrane, as well as large number of reserve lipid and protein bodies that were closely associated with one another. Regardless of the method used, the major changes observed as a result of dormancy release were degradation of protein bodies, and concomitant appearance of organized long, rough endoplasmic reticulum, Golgi apparati, and plastids and an increase in nucleolar volume. Cytological changes were least evident in embryos in which dormancy had been broken by chilling within the fruit, and most extensive in those chilled in Petri dishes or treated with chemicals. However, the development and organization of the rough ER and degradation of the protein bodies in embryos excised from seeds chilled under water were as advanced as those chilled in Petri dishes. The relationship between cytological changes and dormancy release is discussed.

Introduction

Many governments in the tropics (Australia, Asia, Africa and South America) have recently advocated diversification and sustainability of agricultural production, including the production of deciduous fruits for their economic and nutritional importance. However, the need for chilling to break bud dormancy is the major limiting factor to the successful production of deciduous fruits under tropical conditions.

Extensive research has been conducted on deciduous fruit crops to identify the physiological and hormonal mechanisms involved in the induction and release of dormancy. In nature, dormancy is released by chilling temperatures (3 to 7C) for about 8 to 12 weeks (Samish 1954; Wareing 1969). Alternatively, dormancy may be broken by chemical agents like gibberellins (Come and Durand 1971; Kollman 1970; Jarvis et al. 1968; Walker and Donoho 1959), cytokinins (Badizadegan 1967; Borkowska 1980; Broome and Zimmerman 1976) and hydrogen cyanamide (Amberger 1984; Nir et al. 1984; Shulman et al. 1983).

Many investigators have inquired if there is any structural basis for maintenance of dormancy or release of dormancy. Hydrolysis of storage proteins occurs in the embryonic axis of apple seeds during after-ripening at 5C but not at 20C, and this was correlated with the removal of embryo dormancy (Lewak et al. 1975). In another study

Dawidowicz - Grzegorzewska and Zarska-Maciejewska (1979) observed degradation of proteins, lipids, and starch in the embryonic axis during stratification, and in the cotyledons during germination. These changes were accompanied by an increase in nucleolar volume and RNA synthesis.

Highly organized stacks of endoplasmic reticulum (ER) appeared in apple embryos progressively as a function of time at 0C, while at 20C the ER resembled that of dormant embryos (Bouvier-Durand et al. 1981). The change in ER was associated with dormancy release. The appearance of ER in stacks was also correlated with the release of dormancy in microsporangiate strobili of Scotch pine (Kupila-Ahvenniemi et al. 1978).

Since the organelles which contain stored reserves do not synthesize proteins, catabolism of reserves is dependent on the intracellular transport, through the ER, of the newly synthesized catabolic enzymes from their sites of synthesis in the cytoplasm to the reserve-containing organelles (Walton 1980; Chrispeels and Jones 1980/81.)

Protein degradation and appearance of rough ER may either be directly associated with the dormancy release process or are merely non-specific responses to environmental stimuli. Therefore the objective of this study was to compare the ultrastructural changes associated with the release of embryo dormancy by several methods, namely, chilling temperatures, and chemical agents,

including gibberellin A4+7, benzyladenine (BA) and cyanamide (Dormex). Emphasis was placed on treatments which break dormancy without allowing the processes of germination to occur simultaneously.

Materials and Methods

Plant material

Apple fruits (Malus domestica Borkh. cv., Golden Delicious) were collected at harvest time in 1989 and 1990 at the Horticultural Teaching and Research Center, Michigan State University. Some fruits were stored at room temperature (21C), others at 2.5C. Seeds were extracted from the remaining fruit, dried and stored at room temperature (21C) until used.

Treatments

Selection of the following treatments was based on their relative effectiveness in breaking embryo dormancy and their differences in mode of action. All chilling treatments (2.5C) were applied for 8 weeks.

1) Dormant embryos removed from fruits at harvest;
2) Seed held in Petri dishes lined with moist filter paper. at 20C for 8 weeks
3) Seed chilled in Petri dishes lined with moist filter paper
4) Seed chilled within fruit.

5) Seed chilled under water in tightly covered bottles (17 cm tall by 9 cm in diameter).

6-8) Dormant embryos soaked in 40 mg/L gibberellin A4+7, 20 mg/L BA or 80 mg/L cyanamide for 24 hours, rinsed 3 times with distilled water and left to stand in Petri dishes lined with moist filter paper at 20C for 24 hours.

Treatments 1 and 2 served as controls. Treatment 3 releases dormancy without blocking germination, while treatments 4 and 5 break dormancy without permitting germination. Embryos used for treatments 6-8 were incubated at 20C for 24 hr after soaking to allow time for the chemicals to exert their effects.

Transmission electron microscopy (TEM)

Sample fixation. Embryos were excised from seeds, and the embryonic axes were separated from the cotyledons and fixed in a mixture of 1% paraformaldehyde and 5% glutaraldehyde in PIPES buffer (pH 6.8) at 4C for 12 hours. After fixing, the specimens were rinsed 3 times (3 x 1 h) with the same buffer, then post fixed in 1% osmium tetroxide for 4 hours at room temperature with gentle agitation before rinsing 3 times with deionized water. The specimens were later dehydrated in a series of acetone-water mixtures (25,50,75,and 100% acetone for 1 hr each then 100% acetone overnight. Following dehydration, the specimens were infiltrated with Spurs medium-hard resin using a graded

series of acetone-Spurs resin (25, 50, 100 and 100% resin with 24 h in between solutions). Following the last resin change, the embryonic axes were transferred into fresh resin in mold blocks, which were then polymerized at 65C for 24 h

Sectioning. Thin sections (90 um) were made with a diamond knife, on a Solvo ultramicrotome. Thereafter the sections were mounted on flamed 300-mesh copper grids, and stained with uranyl acetate for 30 minutes followed by Reynolds lead citrate for 4 minutes. The sections were viewed in a Phillips TM 201 transmission electron microscope operating at 60 or 80 Kv. Photographs of areas in the meristematic zone of the embryonic axes were taken at several magnifications. At least four specimens were sectioned per treatment and only the most representative structures were photographed.

Results

Dormant embryos. Cell walls varied in thickness and the plasmalemma were not distinct (Fig. 1A). The well-defined nucleus was bordered by a double membrane unit (Fig. 1D). The chromatin was homogeneous and separated into light and dark zones, the latter being larger in area than the former (Fig.1B,C,D). The nucleolus was distinct, spherical and dense and was uniformly dark (Fig.1C, D). Some dark-staining particles were observed scattered around the nucleolus (Fig. 1C, D); their identity is not known.

The cells contained large amounts of reserve material in discrete lipid and protein bodies (Fig.1A). The protein bodies were spherical or irregular in outline and varied in diameter. Some protein bodies were homogeneous while others contained electron-transparent spherical regions. The lipid bodies were spherical, homogeneous and moderately electron dense and were smaller than the protein bodies. They were distributed throughout the cell but were frequently attached to the protein bodies and around the periphery of the cytoplasm along the cell wall.

Mitochondria varied in size and shape and exhibited a well-defined outer double membrane (Fig.1C,D) . Endoplasmic reticula were occasionally seen, being essentially smooth with very unorganized short cisternae (Fig.1D). The large number of lipid and protein bodies made observation of other organelles difficult. However, a careful search of areas free from lipid and protein bodies revealed no additional organelles.

Sections from embryos excised from seeds held moist at 20C for 8 weeks were similar to those from dormant embryos processed soon after harvest (data not shown).

Embryos from seeds chilled in Petri dish. Among all treatments tested chilling in Petri dishes induced the most pronounced ultrastructural changes (Fig. 2). The most dramatic change was the disappearance of almost all protein

bodies, leaving vacuoles, and most lipid bodies (Fig. 2A,C). Increase in nucleolar volume was also greatest in these cells (Fig. 2A,C). Organized long endoplasmic reticula (ER) appeared in single strands (saccule) or in small stacks (Fig.2B,C,D). The ER were loaded with ribosomes evenly arranged on the outer face of each saccule (Fig. 2D). Mitochondria, Golgi bodies and plastids containing starch grains were common (Fig. 2A,B). Ribosomes were also scattered in the ground cytoplasm (Fig. 2D).

Embryos from seeds chilled within fruit. Embryos from seeds chilled in fruit differed only slightly from dormant embryos. The protein bodies were still intact but appeared smaller than in dormant embryos, while lipid bodies were more dispersed (Fig. 3A,B). ER with short cisternae were occasionally observed (Fig. 3C,D). Some mitochondria were observed (Fig. 3C,D), but starch grains were absent.

Embryos from seeds chilled under water. Degradation of the protein bodies and scattering of the lipid bodies in the cytoplasm were evident (Fig. 4A,B,C). The protein bodies were irregular in shape, and varied in size, and the proteinaceous matrix varied in density. Some protein bodies were dense and dark while others were electron-transparent with a membrane around them resembling a vacuole (Fig.4B,C). The ER were organized in long parallel saccules with ribosomes orderly arranged on the outer face of each

saccule (Fig.4D). The mitochondria and plastids appeared less dense and could not be distinguished from one another (Fig.4C,D). No starch granules were observed. Ribosomes were scattered throughout the cytoplasm (Fig.4D).

Dormant embryos treated with GA. Protein bodies appeared degraded and contained electron-transparent areas (Fig.5A). These areas resembled vacuoles, for they were bound by an outer membrane. The lipid bodies were scattered in the cytoplasm (Fig.5C). The ER consisted of many short cisternae with attached ribosomes (Fig. 5C,D). Mitochondria, plastids and Golgi bodies occurred frequently (Fig. 5B,C).

Dormant embryos treated with BA. BA induced ultrastructural changes similar to those induced by GA (Fig. 6A,B,D,E). However, mitochondria appeared more frequently in BA-treated than in GA treated embryos (Fig. 6A,B). Invaginations in the plasma membrane along the cell wall were common (Fig.6C,F)

Dormant embryos treated with cyanamide; Ultrastructural changes were similar to those induced by GA (Fig.7A,B,C,D). However, the ER was longer and more organized (Fig. 7B,C,D) and starch grains were more abundant (Fig. 7A).

General ultrastructural organization. Most of the organelles and membranes, including mitochondria, ER, nucleus, double membrane nuclear envelope, nucleolus, vacuole-like degraded protein bodies (Fig.8 C,D), and Golgi apparati (Fig. 8E), remained intact. In embryos from seeds chilled under water the mitochondria appeared more electron-transparent (Fig. 8A,B) than those of embryos chilled in air (Fig.8C,D). The plasmalemma was distinct and separate from the cell wall in all treatments except the dormant embryos. Furthermore, more invaginations of the plasmalemma occurred in embryos treated with dormancy-breaking chemicals than in embryos whose dormancy was broken by chilling. These could have resulted from endocytosis or exocytosis. Some microbodies, either prolamellar bodies or glyoxisomes, were observed in one cell (Fig.8E,F). Prolamellar bodies are common in plastids of dark-grown seedlings while glyoxisomes contain enzymes which degrade fatty acids and amino acids (Darnell et al. 1986). In the absence of light, major polypeptides, such as chlorophyll-binding proteins, are not synthesized. Plastids from such etiolated cells contain membranes that consist of primary lamellar and interconnected vesicles containing some chloroplast pigments. Light triggers the synthesis of additional chloroplast membranes and membrane proteins to form the flattened and stacked thylakoid vesicles (Darnell et al. 1986)

Discussion

These data confirm that ultrastructural changes do indeed occur during the after-ripening of apple embryos. The dormant embryo is characterized by the presence of large amounts of reserve lipid and protein materials and a general lack of some of the organelles normally present in metabolically active cells. These observations confirm those of many other investigators (Paulson and Srivastava 1968; Srivastava and Paulson 1968; Mia and Durzan 1974; Bouvier-Durand et al. 1981).

The major ultrastructural changes observed during the breaking of dormancy were the degradation of protein bodies,and the appearance of endoplasmic reticulum (ER) and Golgi apparati, regardless of whether dormancy was released by chilling temperature or chemical agents. Bouvier-Durand et al. (1981) associated the appearance of ER in the embryonic axis with the dormancy release process, while Dawidowicz-Grzegorzewska and Zarska-Maciejewska (1979) associated protein degradation in the embryonic axis with dormancy release. In vegetative buds of apple, stacks of ER are generally more abundant at the end than at the beginning of the winter (Dereuddre 1971, 1972). These data indicate that both protein degradation and the appearance of organized ER, accompanied by development of Golgi apparati, were concomitant with dormancy release. Such changes did not occur in embryos excised from seeds at harvest time or

those from seeds held at 20C in a moist medium for 8 weeks.

Ultrastructural changes were greatest in embryos from seeds chilled in Petri dishes. However, this treatment permitted germination to occur as dormancy was broken. Therefore one cannot distinguish between the effects of chilling on the two processes. In contrast chilling the seeds within the fruit or under water breaks dormancy without permitting germination. Embryos after-ripened within fruit exhibited the least ultrastructural changes, but were intermediate between dormant embryos and those from seeds after-ripened in Petri dishes or treated with dormancy-breaking chemicals. Ultrastructural changes in embryos from seeds after-ripened under water were much more extensive than those in seeds chilled within the fruit ,but less than those chilled in Petri dishes. The ER was well developed and organized, while some of the protein bodies had been completely degraded, leaving only vacuoles.

Changes in embryos chilled within fruit may have been inhibited by chemicals in the seed coats (Wareing 1969, Samish 1954) or by limited contact with water (Wan 1980). In contrast, any inhibitors in embryos from seeds chilled under water should have been leached out.

Bouvier-Durand et al. (1981) observed extensive organized stacks of rough ER in embryos chilled within fruit for 6 months. These changes were similar to those that occurred in embryos chilled for 8 weeks in Petri dishes in

this study. However, Bouvier-Durand et al. (1981) never observed significant ultrastructural changes in embryos chilled within fruit for 6 weeks. Likewise, Dawidowicz-Grzegorzewska et al. (1982) found that cold treatment of seeds within fruit never induced changes in the free amino acid level or in the structure of storage protein bodies. These results suggest that the development of ER is not a major factor in dormancy release. However, the small size of the protein bodies indicates that protein degradation occurred; this process may be essential for dormancy

The endoplasmic reticulum (ER) takes two forms namely smooth ER (without ribosomes attached) and rough ER (with ribosomes attached). The smooth ER is the site for synthesis and metabolism of fatty acids and phospholipids whereas the rough ER is involved in the synthesis of membrane and organelle proteins and also synthesis of secretory proteins (Chrispeels and Jones 1980/81). The completion of seed maturation is paralleled by a decline in polysome content, cessation of reserve protein synthesis, reduction of the cisternae to a few residual crescents, and the formation of many vesicles and tubules, indicating that the ER is being transformed to be conserved during desiccation. Following imbibition and chilling the conserved ER are re-activated and modified or new ER is formed. There is a dramatic decline in tubular ER and increase in cisternal ER. The changes in the activity of

the ER may occur independently whether lipid and protein reserves are broken down or not. The newly formed or modified ER are involved in the sequestration and subsequent transport of de novo synthesized catabolic enzymes from their site of synthesis in the cytoplasm to the organelles which contain the reserve macromolecules. The ER also plays a role in the biogenesis or modification of cytoplasmic organelles necessary for reserve catabolism and also in the synthesis of phospholipids and proteins and assembly of the membrane system essential for dormancy release.

Tissaoui and Come (1973) broke embryo dormancy by anaerobiosis without chilling. This was confirmed with buds of poplar cuttings, in which the dormancy-breaking effect of oxygen deprivation was more rapid at higher than at lower temperature (Soudain and Regnard 1982). Furthermore anaerobiosis and low temperature had additive effects. Erez et al. (1980) obtained similar effects with peach leaf buds. The rate of dormancy release was negatively related to the oxygen level and increased with duration of exposure. Products of anaerobic respiration such as acetaldehyde reportedly break dormancy (Samish 1954). Dengler (1967) observed that stacked ER were induced in Clarkia embryos by anoxia. My observations indicate that apple embryos in seeds chilled under water were normal even after 8 weeks of treatment and that the chilling process proceeded successfully. However, a reduced oxygen supply

sometimes prevents dormancy release in seeds (Saure 1985). This agrees with these data in that chilling under water broke the dormancy of apple embryos but not of whole seeds.

The effects of oxygen level on mitochondrial ultrastructure varies with species. Some investigators have observed normal intact functional mitochondria (Kennedy et al. 1991), or elongate mitochondria with complex cristae arrangement (Vartapetian et al. 1977). Others have observed large changes in number and size of mitochondria (Oliveira 1977, Rasao et al. 1987). I observed more electron-transparent mitochondria under anoxic conditions than under aerobic ones.

Changes in dormant embryos treated with GA, BA, or cyanamide paralleled those in seeds chilled in Petri dishes. Protein and lipid body degradation patterns were evident and accompanied by appearance of rough ER, Golgi bodies and light areas in the nucleolus.

These data indicate that the chemicals activated the cells, directly or indirectly driving the processes of dormancy release and/or germination. Of the chemicals tested cyanamide induced the most dramatic changes. However, mitochondria were most abundant in BA-treated embryos.

The nucleolus is responsible for synthesis of ribosomal RNA and of ribosomal subunits. The appearance of light areas in the nucleolus indicates activity in RNA

synthesis and was evident in all chilled embryos and those treated with chemicals.

The chromatin contains the DNA of the nucleus and associated with it are histone and non-histone proteins. The more open regions of the chromatin reflect active transcription while the dense regions are inactive. The chromatin of cells from chilled embryos and those treated with dormancy-breaking chemicals was less dense, indicating active transcription. The appearance of numerous mitochondria in the chemically-treated embryos reflects high respiratory/metabolic activity.

Different parts of the embryo are activated at different times. Storage protein degradation localized in the embryonic axis may be attributed to the breaking of dormancy. Degradation of storage proteins in cotyledons starts after full stratification (second month of stratification) at 5C and may be required for the initiation of germination (Thevenot and Come 1974, Dawidowicz-Grzegorzewska and Lewak 1978, Dawidowicz-Grzegorzewska 1981, Dawidowicz-Grzegorzewska and Zarska-Maciejewska 1979). Protein bodies serve as sources of a variety of hydrolytic enzymes that function during dormancy release and germination (Van Wilden et al. 1980). Protein body degradation may be stimulated by an amino-peptidase, which was found by Zarska-Maciejewska and Lewak (1983) to have its highest activity at about 5C. This enzyme may also be

involved in the release of conjugated hormones, which may regulate the activation and/or synthesis of more hydrolytic enzymes (Amen 1968). GA promotes the biosynthesis and/or activation of hydrolytic enzymes like lipase, amylase, protease and ribonuclease, as well as the development of ER as an intracellular transport system for the mobilization of food reserves and other macromolecules needed for bud growth or embryo germination. Other dormancy-breaking chemicals may exert similar effects.

Lipid bodies are closely associated with protein bodies and the ER (Fernandez and Staehelin 1987) and these data confirm this. This relationship may enable lipase to be transferred from protein bodies (storage form) to lipid bodies (active form as acid lipase) by lateral diffusion, for triacylglycerols found in the lipid bodies are the apparent substrate for acid lipase (Rost 1972; Fernandez and Staehelin 1987). The lipases are considered to be important in the removal of the primary cause of dormancy (Smolenska and Lewak 1974; Zarska- Maciejewska and Lewak 1976; and Zarska-Maciejewska et al. 1980).

Thus the ultrastructural changes observed in this study corroborate the biochemical changes reviewed and proposed for the release of dormancy.

Figure 1. Transmission electron micrographs of whole or portions of cells in the meristematic regions of dormant apple embryonic axes. [Key to lettering: N = nucleus; NL = nucleolus; CW = Cell wall; M = mitochondria; G = Golgi body; L = lipid body; Pb = protein body; Length of bars (μm): A = 2; B = 1; C = 1; D = 1].

Figure 1.

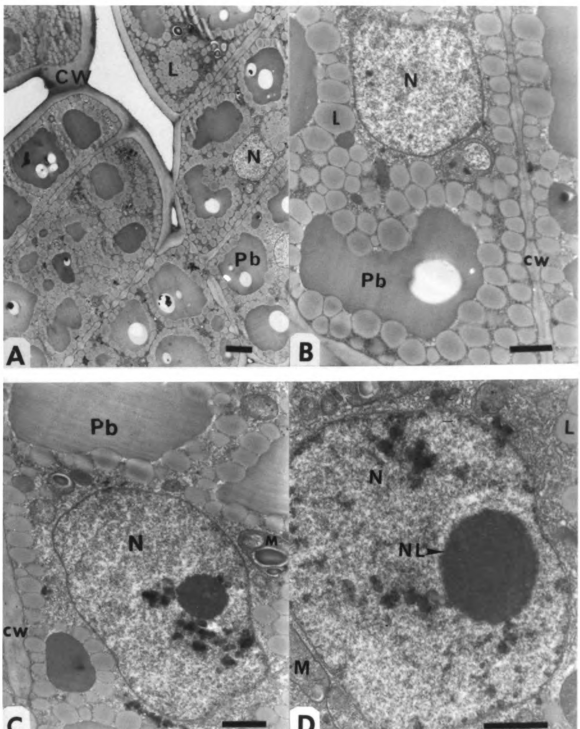

Figure 2. Transmission electron micrographs of whole or portions of cells in the meristematic regions of apple embryos chilled for 8 weeks in Petri dishes at 2.5C. [Key to lettering: as indicated in Fig.1 plus ER - endoplasmic reticulum; G - Golgi body; s - starch grain; Rb - ribosome: Length of bars (μm): A = 1; B = 2; C = 1; D = 0.3)].

Figure 2.

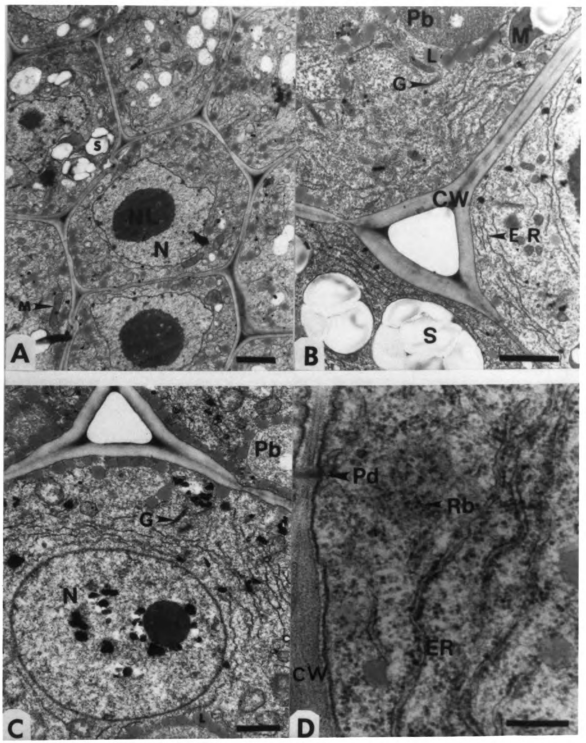

Figure 3. Transmission electron micrographs of whole or portions of cells in the meristematic regions of apple embryos chilled within fruit for 8 weeks at 2.5C [Key to lettering: see Figs. 1 & 2: Length of bars (μM): A = 2; B = 2; C = 2; D = 0.3].

Figure 3.

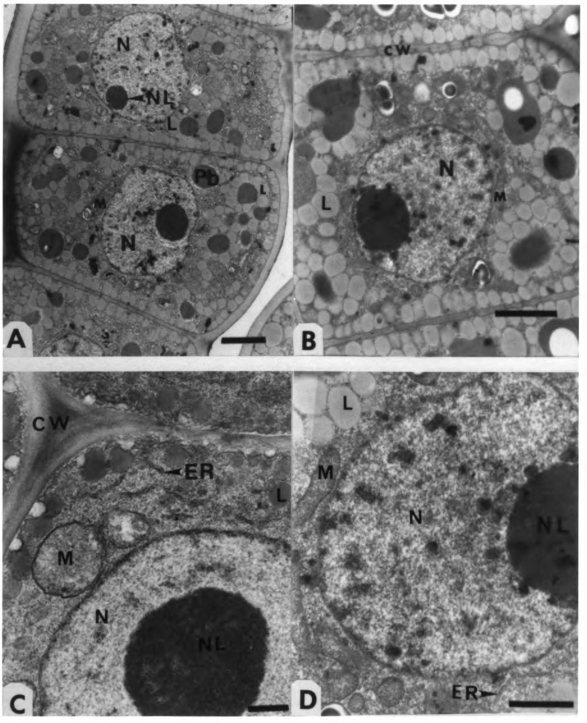

Figure 4. Transmission electron micrographs of whole or portions of cells in the meristematic regions of apple embryos chilled under water at 2.5C for 8 weeks. [Key to lettering: see Figs. 1 & 2 plus NM = nuclear membrane: Lenghth of bars (μm): A = 2; B = 1; C = 1; D = 3].

Figure 4.

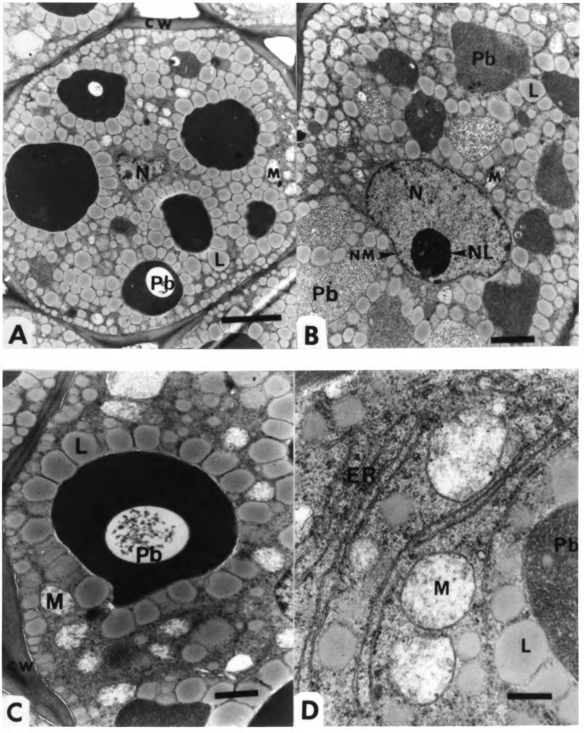

Figure 5. Transmission electron micrographs of whole or portions of cells in the meristematic regions of non-chilled apple embryos after treatment with gibberellin A4 + 7 at 40 mg/l for 24 hours. [Key to lettering: see Figs. 1 & 2 plus P = plastids; Pi = pinocyte: Length of bars (μm): A = 2; B = 1; C - 1; D - 0.3; E =0.3].

Figure 5

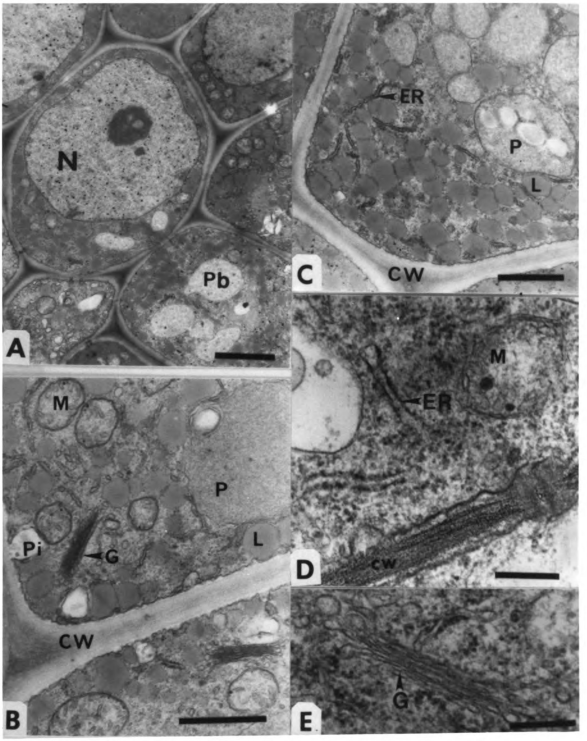

Figure 6. Transmission electron micrographs of whole or portions of cells in the meristematic regions of non-chilled apple embryos after treatment with benzyladenine at 20 mg/l for 24 hours. [Key to lettering: see Figs. 1 & 2: Length of bars (μm): A = 2; B = 2; C = 1; D = 1; E = 0.3; F = 0.3].

Figure 6.

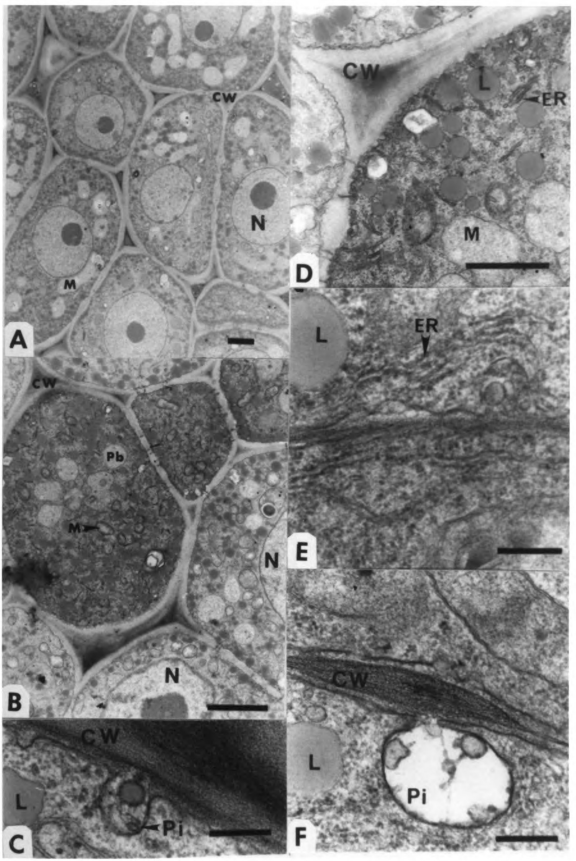

Figure 7. Transmission electron micrographs of whole or portions of cells in the meristematic regions of non-chilled apple embryos after treatment with Dormex (cyanamide) at 80 mg/L for 24 h.[Key to lettering: see Figs. 1 & 2 plus V = vacuole: Length of bars (μm): A = 1; B = 1; C = 1; D = 0.25].

Figure 7.

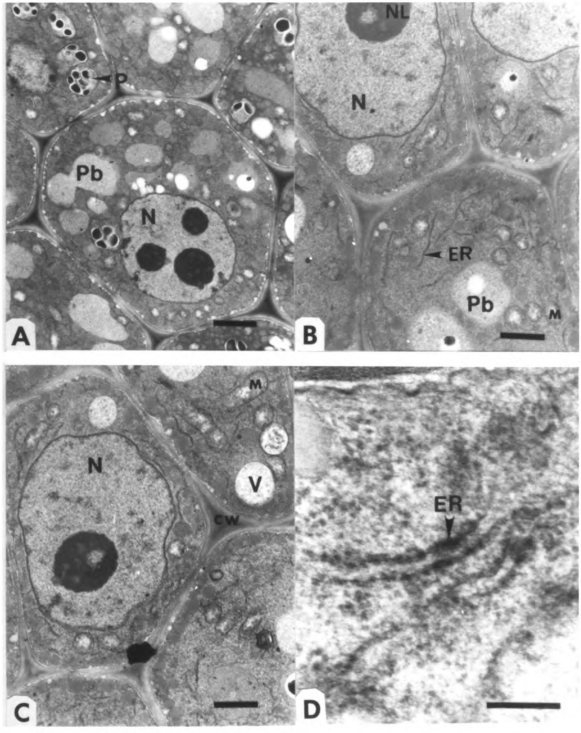

Figure 8. Transmission electron micrographs illustrating organelles in selected cells in the meristematic regions of apple embryos chilled in Petri dishes or under water at 2.5C for 8 wk.[Key to lettering: see Figs. 1 & 2 plus glyoxisome: Length of bars (μm): A = 1; B = 1; C = 0.3; D = 1; E = 0.3; F = 0.1].

Figure 8.

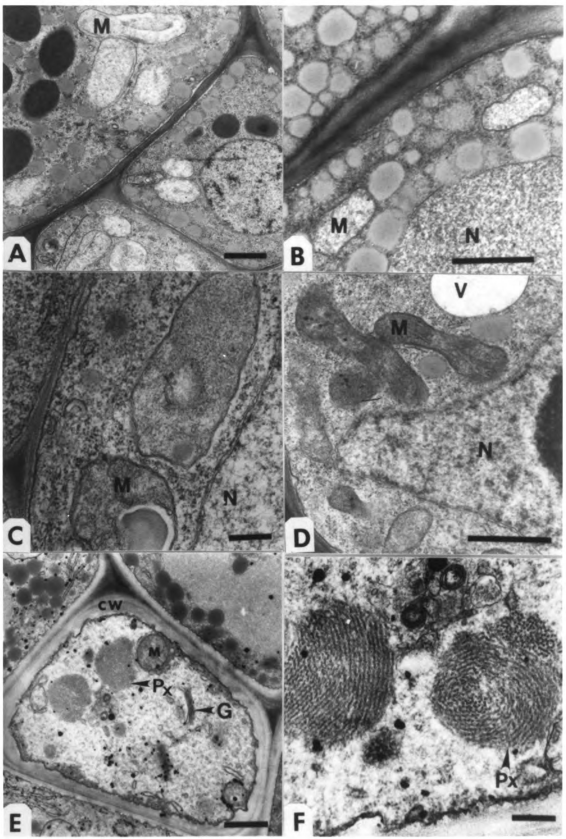

Table1. Summary of cytological changes induced by dormancy-breaking treatments.

Response	Treatment						
	No Chill	Chilled (PD)	Under Water	GA	BA	H2CN	Chilled
Protein Degradation	__	*****	***	**	**	**	*
ER Development	__	*****	***	**	**	**	*
Nucleus Volume	__	*****	***	**	**	**	*

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CHAPTER THREE

METABOLISM OF 14C-GA12-ALDEHYDE BY APPLE EMBRYOS IN RELATION TO DORMANCY RELEASE

METABOLISM OF 14C-GA12-ALDEHYDE BY APPLE EMBRYOS IN RELATION TO DORMANCY RELEASE

Abstract. The metabolism of 14C-GA12-aldehyde was investigated in apple embryos in relation to the release of dormancy by chilling. Half-embryos, cotyledons, or embryonic axes were incubated with 14C-GA12-aldehyde for periods up to 96 hr, then frozen and extracted. The methanolic extracts were subjected to high performance liquid chromatography (HPLC) on a reverse phase C18 column, using a discontinuous gradient of acetonitrile in water. During 1 hr incubation, 14C-GA12-aldehyde was oxidized to putative GA12, with a retention time of 30 minutes (F30). After 3 hr incubation some 14C-label was detected in fraction 26 (F26). As the incubation period was extended to 96 hr, a total of six metabolites appeared at retention times of 30, 26, 21, 18, 12, and 9 minutes and these were referred to as metabolite F30, F26, F21, F18, F12, and F9 respectively. The retention times for GA12 aldehyde, GA12, GA4 and GA1 standards were 33, 30, 23 and 11 minutes, respectively.

Metabolites F26 and F12 accumulated in the largest amounts. There were no qualitative differences in metabolite profiles between chilled vs. dormant embryos, cotyledons or embryonic axes except for F18, which was absent in chilled tissues. However, the rate of metabolism was higher in chilled than in dormant tissues.

Introduction

In temperate regions deciduous fruit crops become dormant each year as a means of survival during cold winters. In the tropics, where cold winters do not occur, prolonged dormancy is an important obstacle to the economic production of such crops. The dyanamics of dormancy induction and release are not fully understood. Many hypotheses and theories have been suggested, only to be rejected later (Saure 1985; Smith and Kefford 1964; Powell 1987).

Phytohormones, especially abscisic acid (ABA), gibberellins (GA), and cytokinins are regarded as important factors in the regulation of dormancy (Lewak et al. 1975; Halinska and Lewak 1987; Halinska et al. 1987; Saure 1985; Powell 1987).

The involvement of GA's in dormancy release has been suggested by many authors (Sinska and Lewak 1974, 1977; Ross and Bradbeer 1968, 1971). An increase in GA's has been observed during chilling of buds and seeds of many species, e.g. apple, peach, hazel nut, apricot (Eagles and Wareing 1964; Smith and Kefford 1964; Bachelard and Wightman 1974; Wareing and Saunders 1971; Zarska-Maciejewska et al. 1980; Gianfagna and Rachmiel 1986; Bradbeer 1968). In apple seeds GA4 undergoes marked shifts in free and bound forms during stratification (Halinska and Lewak 1978; Isaia and Bulard 1978; Sinska and Lewak 1970). The increase in GA4 during

stratification may be a result of either release from conjugated inactive forms or from 'de novo' synthesis (Sinska and Lewak 1973, 1977).

Physiological dwarf seedlings produced by partially chilled seeds also appear to be deficient in GA's. Such dwarfs will grow into normal plants if chilled for a period of time or if treated repeatedly with GA's (Barton 1956; Blommaert and Hurter 1959; Flemion and Beardow 1965). Exogenous applications of GAs may also replace at least part of the chilling requirement in buds and seeds of peach, apricot, pear, black currant, apple and hazel nut (Bradbeer 1968; Brown et al. 1960; Hatch and Walker 1969; Modlibowska 1960; Walser et. al. 1981).

The role of GAs in breaking dormancy has been questioned by many scientists who believe that GA may not be the primary cause of dormancy release but is required in later steps of bud development and seed germination (Bradbeer 1968; Brown et al. 1960; Gianfagna and Rachmiel 1986; Wareing and Saunders 1971). However, GA may promote the biosynthesis of several hydrolytic enzymes, e.g., amylase, protease, ribonuclease, and lipase, that are required for germination (Lewak 1985). GA also promotes the development of the endoplasmic reticulum which acts as an intracellular transport system (Chrispeels and Jones 1980/1981; Walton 1980; Zarska-Maciejewska et al. 1980).

Chilling temperatures apparently promote the

biosynthesis of GA4, which activates acid lipase, which in turn hydrolyzes storage lipids (Sinska and Lewak 1977). The metabolism of such lipids may be essential for germination (Smolenska and Lewak 1974; Zarska-Maciejewska and Lewak 1976).

The objective of this study was to follow the metabolism of 14C-gibberellin A12-aldehyde, a precursor of all GA's, during the after-ripening of apple embryos, to determine: a) if chilling affects the ability to metabolize GAs and/or the products formed; b) if the 14C-GA12-aldehyde is metabolized to GA4 which is presumed to be responsible for dormancy release; and lastly c) the relative ability of the different organs of the embryo, i.e. axis and cotyledon, to metabolize 14C-GA12-aldehyde.

Materials and Methods

Plant material

'Golden Delicious' apple (Malus domestica Borkh.) fruits were harvested from the orchard at the Horticultural Teaching and Research Center, Michigan State University, East Lansing, Michigan. Seeds were extracted, air dried and stored at room temperature (21C) until used.

14C-GA12-aldehyde (specific activity 210 μCi/umol) and 14C-GA12 were prepared by Drs F. G. Dennis, Jr., J. Ozga and M. Brenner at University of Minnesota, St. Paul, Minnesota, by incubating liquid endosperm of pumpkin

(Cucurbita maxima) with 14C-Mevalonic acid, according to the method described by Birnberg et al. (1986).

Seed treatments

Seeds were soaked in distilled water for 48 hours, then stratified at 5C on moist filter paper in Petri dishes for 0, 4, or 8 weeks. Seeds held under similar conditions at 20C for 4 and 8 weeks were included as controls.

Incubation with 14C-GA-aldehyde.

Embryos were excised from the seeds and rinsed four times with sterile deionized water, then dissected and placed in Petri dishes lined with Whatmann No.1 filter paper moistened with sterile distilled water prior to treatment. Three tissue types were used, a) half embryos (axis plus one cotyledon), b) cotyledon only and c) embryonic axis. Each replicate consisted of either five half embryos, five cotyledons or 50 embryonic axes.

The 14C-GA12-aldehyde in 95% ethanol was diluted to 80% aqueous ethanol for half embryos and cotyledons, or 50% for embryonic axes to yield 50,000 dpm per 15 µl. The 14C-GA12-aldehyde (15 µl) was applied to the adaxial surface of 5 cotyledons or half embryos or was distributed among 50 embryonic axes. Thereafter the tissues were incubated at 20C for 6 to 96 h. At the end of the incubation period the tissues were chopped with a razor blade and transferred to

20 ml scintillation vials, which were immersed in liquid nitrogen. These were stored at -20C prior to extraction.

A preliminary study was designed to determine appropriate incubation periods. 14C-GA12 aldehyde was incubated with embryos for 0, 1, 3, 6, 12, 24, 48, and 96 h at 20C prior to extraction.

Extraction.

The frozen samples were homogenized in a hand-held glass homogenizer in 4 ml of ice-cold methanol (80%), the homogenate was transferred to an ice bath and the homogenizer was rinsed twice with two 2-ml volumes of cold 80% methanol. The rinses were added to the homogenate, which was placed at 4C to allow extraction over night with occasional shaking. The following morning the homogenate was centrifuged for 8 minutes and the supernatant was decanted. The pellet was re-suspended in 2 ml of cold 80% methanol, allowed to stand for 1 h at 5C, centrifuged and the supernatant decanted. The pellet was rinsed twice with 2 ml cold 80% methanol, centrifuging between rinses. The supernatants were combined, filtered through 0.45 μm filters and evaporated in a Speedvac centrifuge, (S C 200 Savant, Forma Scientific Inc.) connected to a solvent cold trap and a vacuum pump. When approximately 100 ul remained, the vial was removed and 1% acetic acid added to about 600 ul. The sample was sonicated and centrifuge-filtered through a

0.45 μm filter prior to high performance liquid chromatography (HPLC).

HPLC

A Waters (600 multisolvent delivery system) HPLC was used for sample preparation. A Waters analytical column (300 x 4 mm) packed with Bondapack C18, and a C18 guard-pack, as an in-line pre-column were used.

A discontinuous 40 minute gradient from 1% acetic acid (HOAc) in water to 1% HOAc in 100% acetonitrile was used to separate metabolites as follows: 0-20% acetonitrile in 2 minutes, 20 to 35% in 15 minutes, 35 to 75% in 15 minutes, 75 to 100% in 2 minutes and then 100% in acetonitrile for 6 minutes.

The flow rate was 2 ml per minute and fractions were collected at 1 minute intervals. One quarter of each 1-ml fraction was added to 15 ml of Safety Solve scintillation cocktail and radioactivity was determined with a Packard 1500 Tri-Carb Liquid Scintillation analyzer. Each sample was counted for 1 minute.

Of the 50,000 dpm of 14C-GA12-aldehyde applied to each sample, approximately 10% remained in the pellet and 26% on the filters, 58% was recovered in the HPLC eluates and 6% remained unaccounted for (Appendix Table 1). For each sample the total radioactivity in all HPLC fractions was summed, the background counts were subtracted and the percentage in

each fraction was calculated. The data for each replicate sample were graphed. No radioactivity above background was detected in fractions 1 to 7 and 37 to 40, therefore only data for fractions eluted at 4 to 36 minutes are reported.

Samples of 14C-GA12-aldehyde 14C-GA12, 3H-GA4, and 3H-GA1 were chromatographed to determine their retention times. 3H-GA4 was supplied by Dr. M. Brenner, University of Minnesota, and 3H-GA1 by Dr. J. A. D. Zeevaart, Plant Research Laboratory, Michigan State University. Retention times are indicated in Table 1 and Fig. 1. Retention times of additional GAs in other solvent systems are given in the Appendix Table II. To test the possibility of metabolism of 14C-GA12-aldehyde by microorganisms, dormant, imbibed seeds were placed in a microwave oven for 5 minutes prior to incubation with 14C-GA12 aldehyde for 6 h. The only labelled compound in the extract was 14C-GA12 aldehyde (Fig.1)

Experimental Design

Treatments were arranged factorially in a completely random design with two to three replicates per treatment. An analysis of variance (ANOVA) was performed and standard error of means was used to compare treatment means.

Treatments

Experiment 1. Effect of time of incubation of chilled apple embryos with 14C-GA12-aldehyde on relative quantities and profile of 14C-labelled metabolites. Half-embryos chilled for 10 weeks at 5C were incubated with 14C-GA12-aldehyde for 0, 1, 3, 6, 12, 24, 48, and 96 h.

Experiment 2. The metabolism of GA12 aldehyde by dormant vs chilled half-embryos. This study was designed to determine the effect of dormancy on the metabolism of 14C-GA12-aldehyde by half embryos. Dormant or chilled (8 wk at 5C) half-embryos were incubated with 14C-GA12 aldehyde for 0, 6, 12, 24, 48, and 96 hr.

Experiment 3. The metabolism of GA12-aldehyde by dormant vs. chilled embryos, cotyledons, or axes. To determine the effects of chilling on capacity of the different parts of the embryo to metabolize 14C-GA12-aldehyde. Dormant or chilled (8 wk at 5C) embryos were separated into half embryos, cotyledons and axes. The half embryos and cotyledons were incubated with 14C-GA12-aldehyde for 6, 12, 24, 48, and 96 h. while axes were incubated for 6, 12, and 24 h.

Experiment 4. The effect of chilling on the pattern of metabolism of 14C-GA12-aldehyde by embryos, cotyledons and axes. The objective of this experiment was to compare the metabolic activity of chilled embryos with those held for similar periods of time at 20C. Seeds were held at 5C for 0, 4, or 8 weeks. A control treatment consisted of moist seeds held at 20C for 8 weeks. At the end of each period, the embryos were excised and separated into half embryos, cotyledons, and axes. The half embryos and cotyledons were incubated with 14C-GA12-aldehyde for 6, 12, 24, 48, and 96 h while axes were incubated for 6, 12, and 24 h. Percent of recovered radioactivity in each fraction is given in Appendix, Fig. 1.

Results

Experiment 1. Effect of time of incubation of chilled embryos with 14C-GA12-aldehyde on relative quantities and profile of 14-labelled metabolites. At 0 time all radioactivity was confined to the retention time (33 min) of 14C-GA12-aldehyde. An incubation period of 1 h yielded some 14C-label in fraction 30 (GA 12). When the incubation period was extended to 3 h, more 14C was detected in F30 as well as some in F26. As the incubation period was extended to 96 h, radioactivity shifted from less polar to more polar compounds (Appendix Fig.?.) presumably GA's and their conjugates. Incubation periods of 0, 6, 12, 24, 48, and 96 h

were thus used for subsquent experiments.

Experiment 2. The metabolism of GA12-aldehyde by dormant vs. chilled half-embryos. During the 96 h incubation, 6 metabolites were prominent at retention times of 30, 26, 21, 18, 12 and 9 minutes. For convenience, these metabolites were designated F30 (GA12), F26, F21, F18, F12, and F9 (F = fraction number, which also reflects the retention time in minutes) (Table 1). As GA12-aldehyde was metabolized by dormant embryos, a small peak appeared at 30 min. (putative GA12) and a large one at 26 min. (Fig. 2). The latter peak declined in amount as more polar compounds appeared sequentially at 21, 18, 12, and 9 min. Metabolism by chilled embryos paralleled that of dormant ones, except that the metabolite F21 was absent from dormant embryos while the metabolite F18 was absent from chilled embryos (Fig. 3).

Data for F33, F26, F12, and F9 for all 3 replicates for both chilled and non-chilled embryos were averaged (Fig. 4). Metabolism of GA12-aldehyde (F33) was much more rapid in chilled than in non-chilled embryos, and the relative amount of F26 was higher after 24 h.

Experiment 3 The metabolism of GA12-aldehyde by dormant vs. chilled embryos, cotyledon or axes. Metabolism by half-embryos was similar to that observed in Experiment 2,

therefore HPLC profiles are given only for cotyledons and embryonic axes (Fig. 5-8). Metabolism by isolated cotyledons (Fig. 5 and 6) resembled that of half embryos. The same 6 metabolites occurred in extracts of embryonic axes but the pattern of change differed (Figs. 7 and 8). Putative GA12 (F30) was much more prominent at 6 hr, and F26 was not metabolized as rapidly as in cotyledons. The metabolite F18 occurred in both dormant and chilled axes and was more prominent than in both half-embryos and cotyledons, where F18 occurred only in the dormant tissues. Metabolism of GA12-aldehyde was considerably more rapid in chilled than dormant tissues, and in axes than in cotyledons (Fig. 9). An average of only 6% was present in chilled tissues after 6 h, whereas 15 to 22% remained in dormant tissues.

The metabolite, F26, contained a larger percentage of the total radioactivity in chilled than in dormant embryos during the first 12 h of incubation (Fig.10), whereas F18 generally contained considerably less (Fig 11). Percentages of the F12 were little affected by chilling (Fig. 12) although a somewhat greater percentage occurred in cotyledons after 2-3 h incubation. Percentages of the metabolite F9 increased with time of incubation, but chilling had no consistent effect (Fig.13) .

Experiment 4. The effect of chilling on the pattern of metabolism of 14C-GA12-aldehyde by embryos, cotyledons, or axes. Metabolism of GA12-aldehyde was much more rapid in chilled than in dormant half embryos and axes (Fig. 14), although there was little difference between 4 and 10 weeks of chilling. However, the differences were much less pronounced in cotyledons.

Discussion

Both dormant and chilled embryos, cotyledons and axes had an active system for metabolizing 14C-GA12-aldehyde to more polar gibberellins (GA) and presumably GA conjugates. The rate of metabolism of 14C-GA12-aldehyde by chilled embryos was faster than that of dormant ones; however the difference was relatively small and probably cannot account for the differences in germination capacity. The results do not support the hypothesis by Ross (1983) and Bradbeer (1968) that chilling removes a block to GA synthesis, for dormant embryos also metabolized GA12-aldehyde. However, the blockage could occur in the biosynthetic pathway from Mevalonic acid (MVA) to GA12-aldehyde. Sinska and Lewak (1977) reported little effect of chilling on ability of apple embryos to convert MVA to GA4, although activity declined as stratification was prolonged. The activity paralleled embryo GA4 content ; however, the ratios were very different, i.e., 5,000:1 in GA4 content for 30 days vs 0 days of chilling as compared to 4:1 in rate of conversion

of MVA to GA4.

Lewak et al. (1977) have proposed that the catabolic phase of after-ripening is triggered by a high concentration of GA4 which is correlated with an increase in the activity of acid lipase (Zarska-Maciejewska et al. 1980). Halinska et al. (1987) suggested that the GA4-controlled events take place in the embryonic axes rather than in the cotyledons. In this study no free GA4 was detected. Dennis, et al. (1991) also detected little or no free GA4 following incubation of immature apple embryos with 14C-GA12-aldehyde.

These data therefore do not provide support for Lewak's hypothesis. GA4 could be metabolized or conjugated so rapidly that it would not accumulate in sufficient quantities to be detectable. However, Halinska and Lewak (1978) identified GA4 in extracts of apple seeds. Similary, Sinska and Lewak (1977) reported the incorporation of 14C-MVA into putative GA4. Therefore, GA4 should have occurred in sufficient quantities to appear in the HPLC profile. No explanation can be offered at this time for the failure to detect 14C-GA4.

Even though GA12 is the first oxidative product of GA12-aldehyde, levels were generally low, indicating rapid metabolism to more polar compounds. However, putative GA12 was relatively abundant in extracts of embryonic axes.

Compounds more polar than GA12 appeared in order of polarity (least to greatest), suggesting progressive

conversion of less polar to more polar gibberellins and finally to glycosides. The 6 metabolites may be free or conjugated GA's or a mixture of both. None of these compounds was identified and hence further work is required to determine their identity and the pathway for GA metabolism followed. Compounds that have similar retention times need not be identical. Therefore chilled and non-chilled embryos could be producing quite different metabolites.

Conjugated GAs occur in apple seeds (Halinska and Lewak 1978, 1985; Isaia and Bulard 1978) and a reversible release of free GA's from their conjugates may control free hormone levels in mature apple seeds (Halinska and Lewak 1987). Conjugated GA's can serve as reserve and/or transport forms of physiologically active GA's (Sembdner et al. 1970). Halinska et al. (1987) attributed the increase in free GA's during stratification to the hydrolysis of conjugates supplemented by de novo synthesis of GA.

Table 1. Retention times on HPLC of GA1, GA4, GA12 and GA12-aldehyde standards and also GA12-aldehyde metabolites in apple embryos.

Metabolite	Fraction No or Retention Time (minutes)
GA12-aldehyde	33
GA12	30
F26	26
GA4	23/24
F21	21
F18	18
F12	12
GA1	10/11/12
F9	9

Figure 1. High performance liquid chromatography (HPLC) of 14C-GA12 aldehyde with and without extract of killed embryos and of 14C-GA12, 3H-GA4 and 3H-GA1. (Discontinuous gradient elution with 0 to 100% acetonitrile in water at 2 ml per minute. Both solvents contained 1% acetic acid. All samples were corrected for bacground; no radioactivity occurred in fractions 1-4 and 37-40 when corrected for background).

Figure 1.

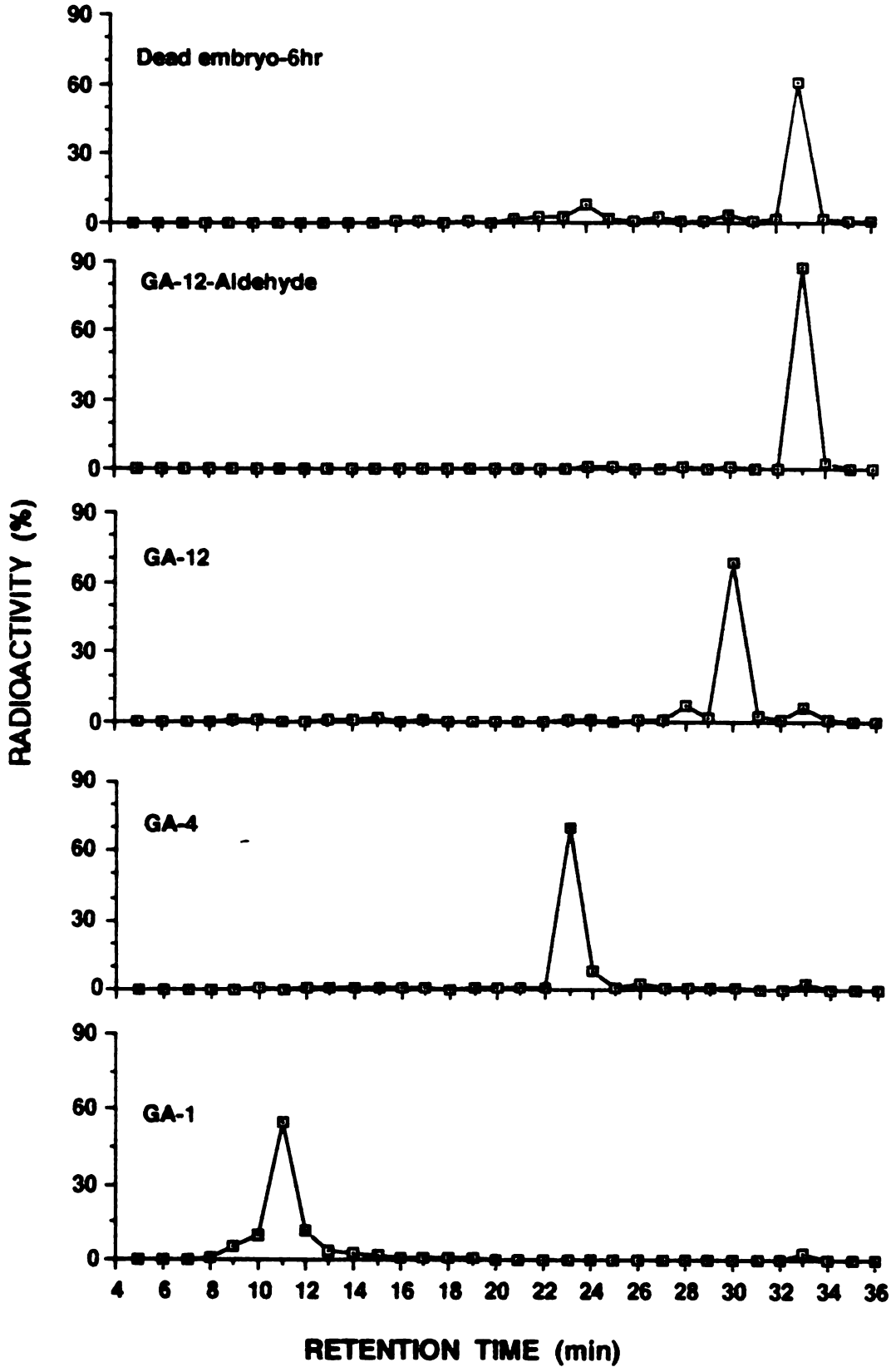

Dead embryo-6hr
GA-12-Aldehyde
GA-12
GA-4
GA-1
RADIOACTIVITY (%)
90
60
30
0
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
RETENTION TIME (min)

Figure 2. HPLC of 14-C-labelled metabolites in dormant apple embryos following 6 to 96 hours incubation with 14C-GA12-aldehyde at 20C. (see Fig. 1 for conditions; seeds held at 5C for 10 weeks; Expt. 2).

Figure 2.

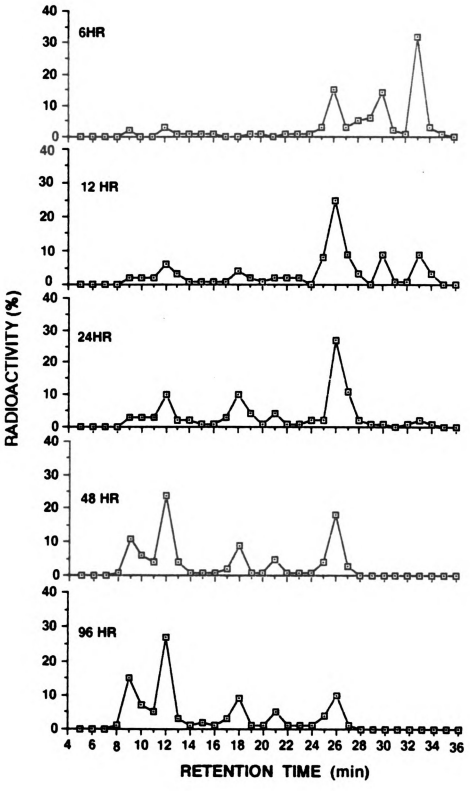

6HR
12 HR
24HR
48 HR
96 HR
RADIOACTIVITY (%)
RETENTION TIME (min)
0
10
20
30
40
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Figure 3. HPLC of 14C-labelled metabolites in chilled apple embryos following 6 to 96 hours incubation with 14C-GA12-aldehyde at 20C. (see Fig. 1 for conditions; seeds held at 5C for 10 weeks; Expt. 2).

Figure 3.

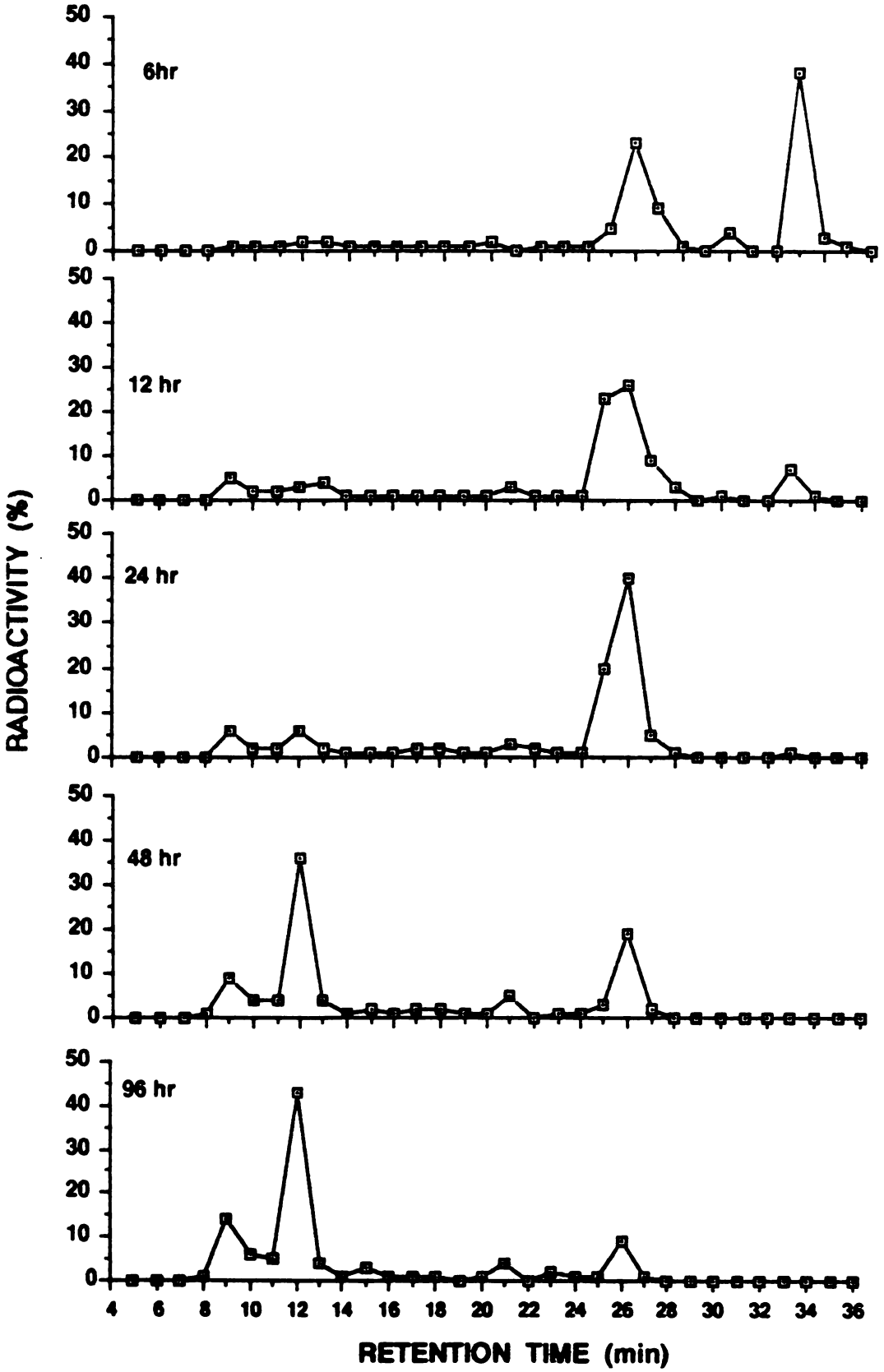

6hr
12 hr
24 hr
48 hr
96 hr
RADIOACTIVITY (%)
RETENTION TIME (min)
0
10
20
30
40
50
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Figure 4. Relative quantities and profile of major 14C-labelled metabolites in eluates from HPLC column of extracts following 6 to 96 hours incubation of dormant and fully chilled apple embryos with 14C-GA12-aldehyde. [Embryos excised from seeds chilled for 0 (dormant) or 10 weeks at 5C prior to incubation at 20C. Values calculated from data in figs. 2 & 3 plus additional replicate samples; Expt. 2].

Figure 4.

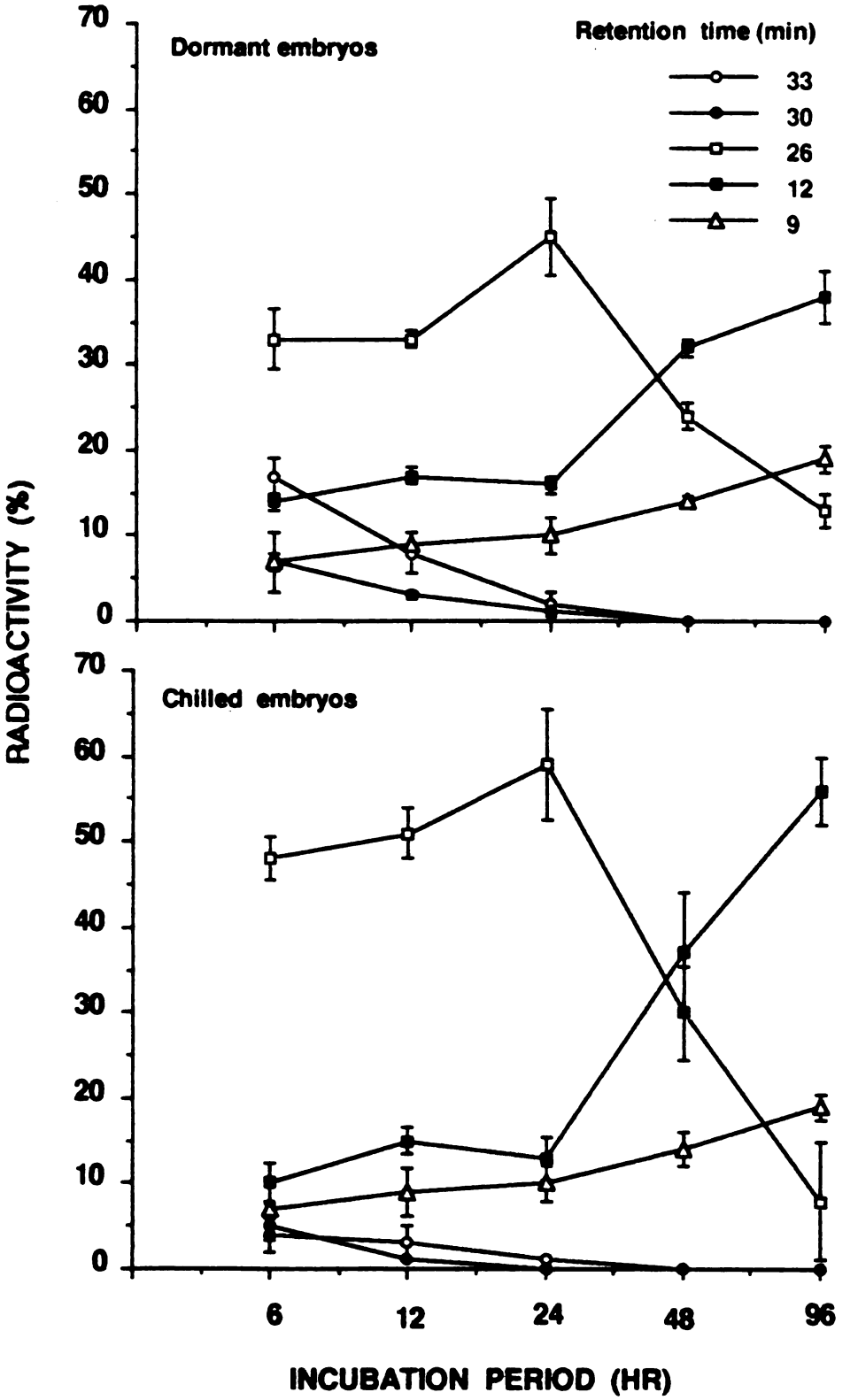

Dormant embryos
Retention time (min)
33
30
26
12
9
Chilled embryos
RADIOACTIVITY (%)
0
10
20
30
40
50
60
70
6
12
24
48
96
INCUBATION PERIOD (HR)

Figure 5. Relative amounts of 14C-labelled metabolites in dormant apple cotyledons following 6 to 96 hour incubation with 14C-GA12-aldehyde at 20C. (see Fig. 1 for conditions; Expt. 3).

Figure 5.

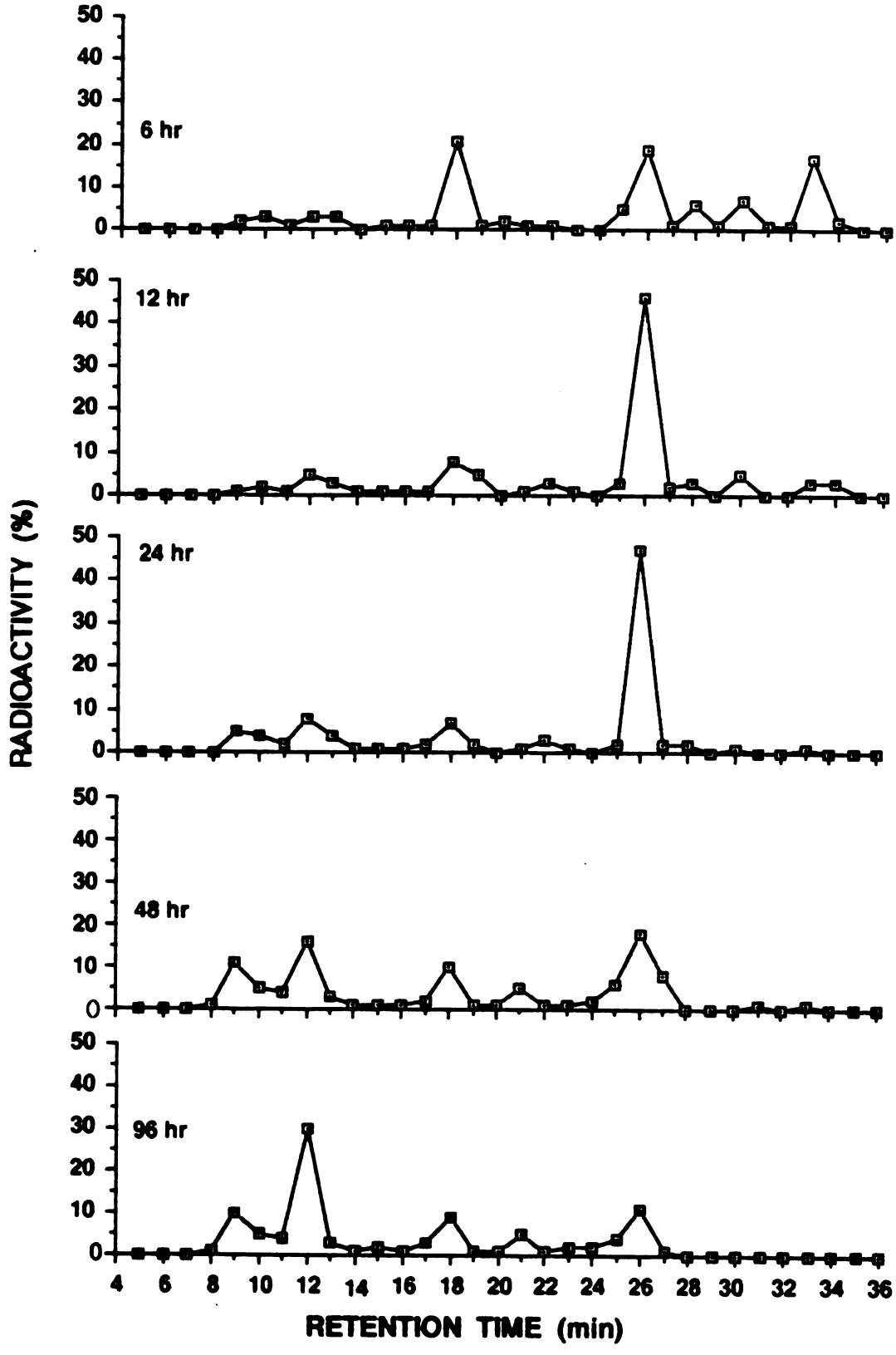

6 hr
12 hr
24 hr
48 hr
96 hr
RADIOACTIVITY (%)
RETENTION TIME (min)
0
10
20
30
40
50
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Figure 6. Relative amounts of 14C-labelled metabolites in chilled apple cotyledons following 6 to 96 hour incubation with 14C-GA12-aldehyde at 20C. (see Fig. 1 for conditions; seeds held at 5C for 8 wk; Expt. 3).

Figure 6

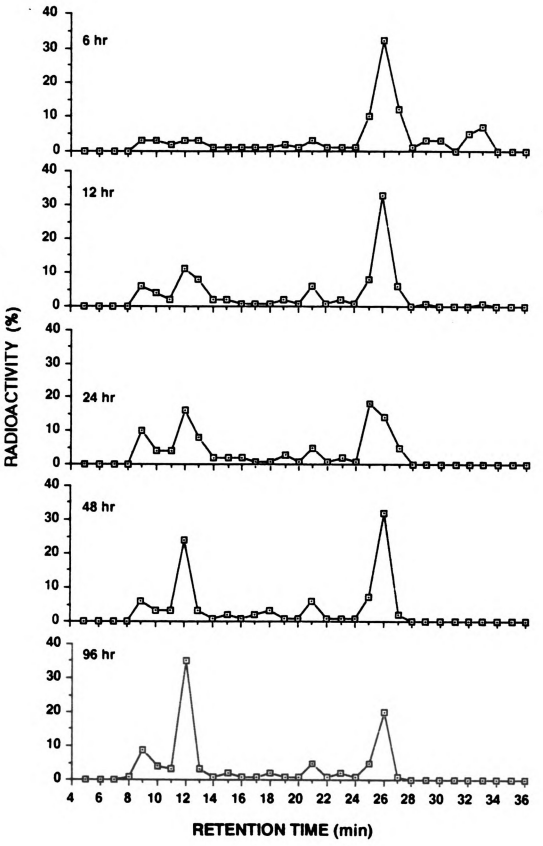

6 hr
12 hr
24 hr
48 hr
96 hr
RADIOACTIVITY (%)
RETENTION TIME (min)
0
10
20
30
40
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Figure 7. Relative amounts of 14C-labelled metabolites in dormant apple embryonic axes following 6 to 24 hours incubation with 14C-GA12-aldehyde at 20C. (see Fig. 1 for conditions; Expt. 3).

Figure 7.

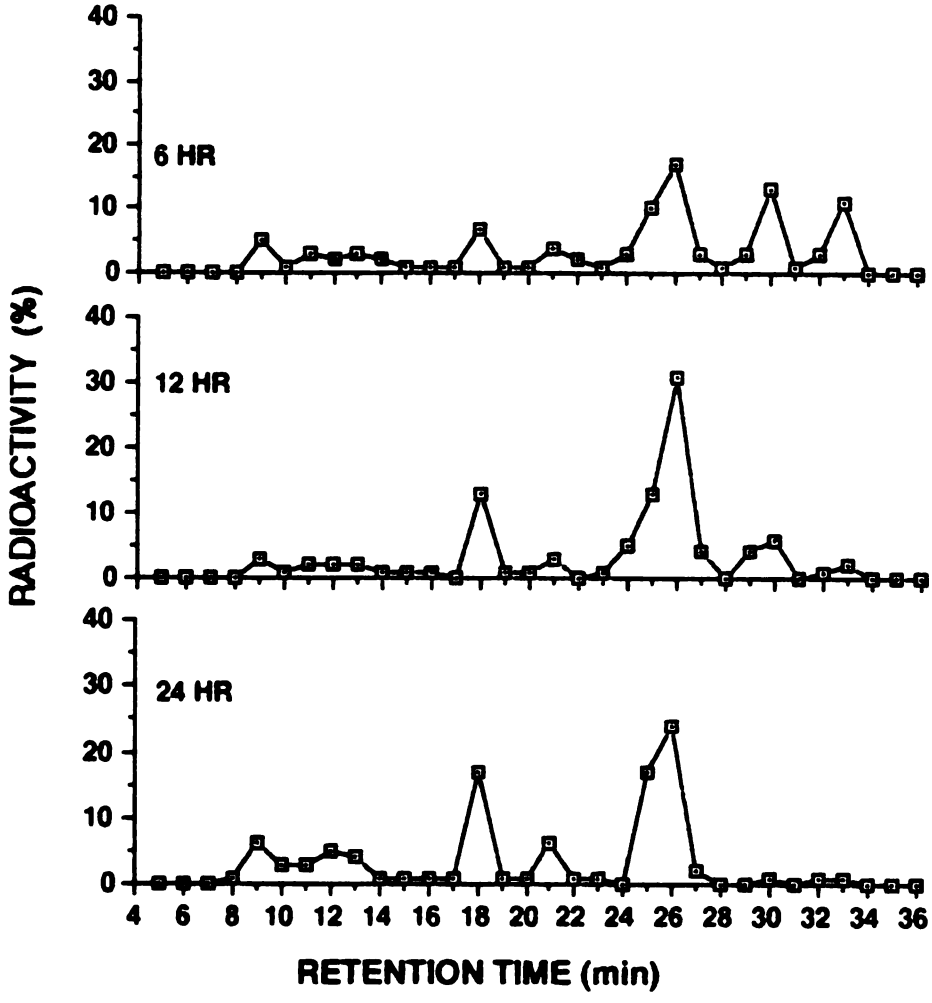

6 HR
12 HR
24 HR
RADIOACTIVITY (%)
RETENTION TIME (min)
0
10
20
30
40
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36

Figure 8. Relative amounts of 14C-labelled metabolites in chilled apple embryonic axes 6 to 24 hours incubation with 14C-GA12-aldehyde at 20C. (see Fig. 1 for conditions; seeds held at 5C for 8 wk.; Expt. 3).

Figure 8.

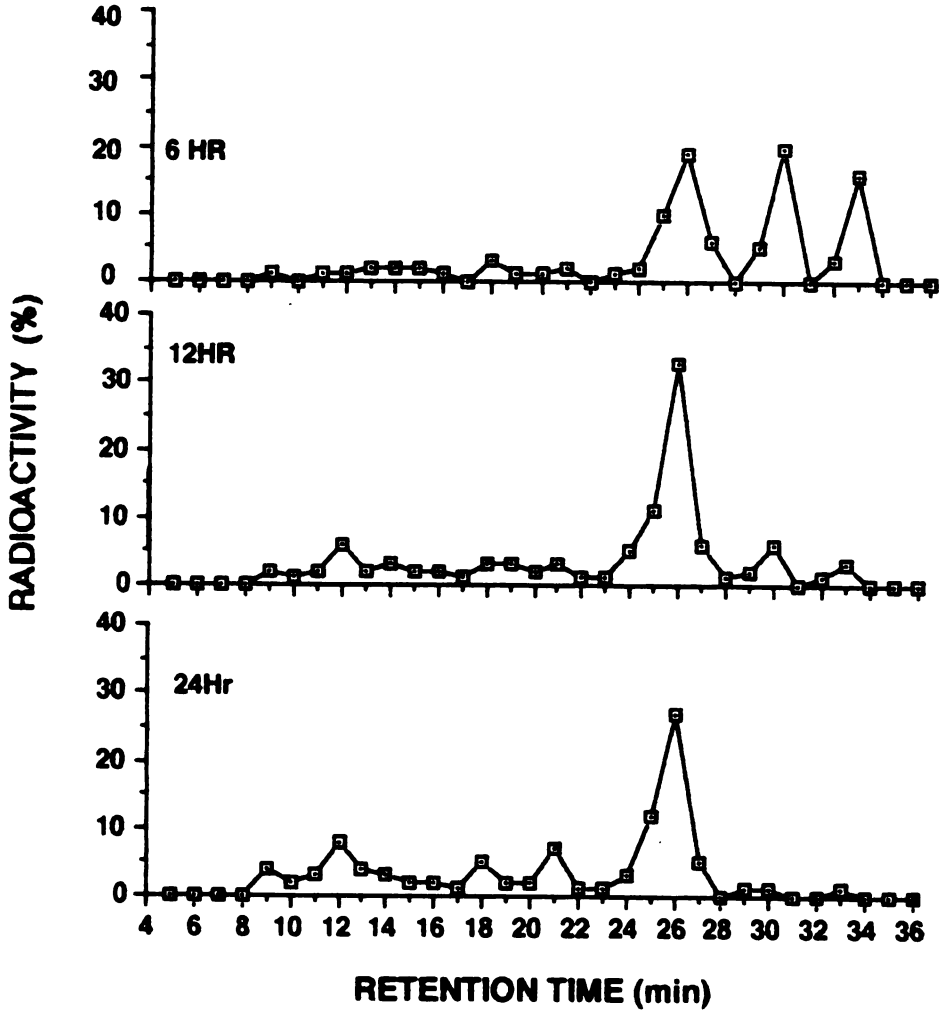

40
30
20
10
0
6 HR
40
30
20
10
0
12HR
40
30
20
10
0
24Hr
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
RADIOACTIVITY (%)
RETENTION TIME (min)

Figure 9. Relative quantities of 14C-GA12-aldehyde retained in eluates from HPLC column of extracts following 6 to 96 hours incubation of embryos, cotyledons and embryonic axes excised from dormant and fully chilled seeds with 14C-GA12-aldehyde. [Embryos excised from seeds chilled for 0 (dormant) or 8 weeks at 5C prior to incubation at 20C.; Expt. 3].

Figure 9.

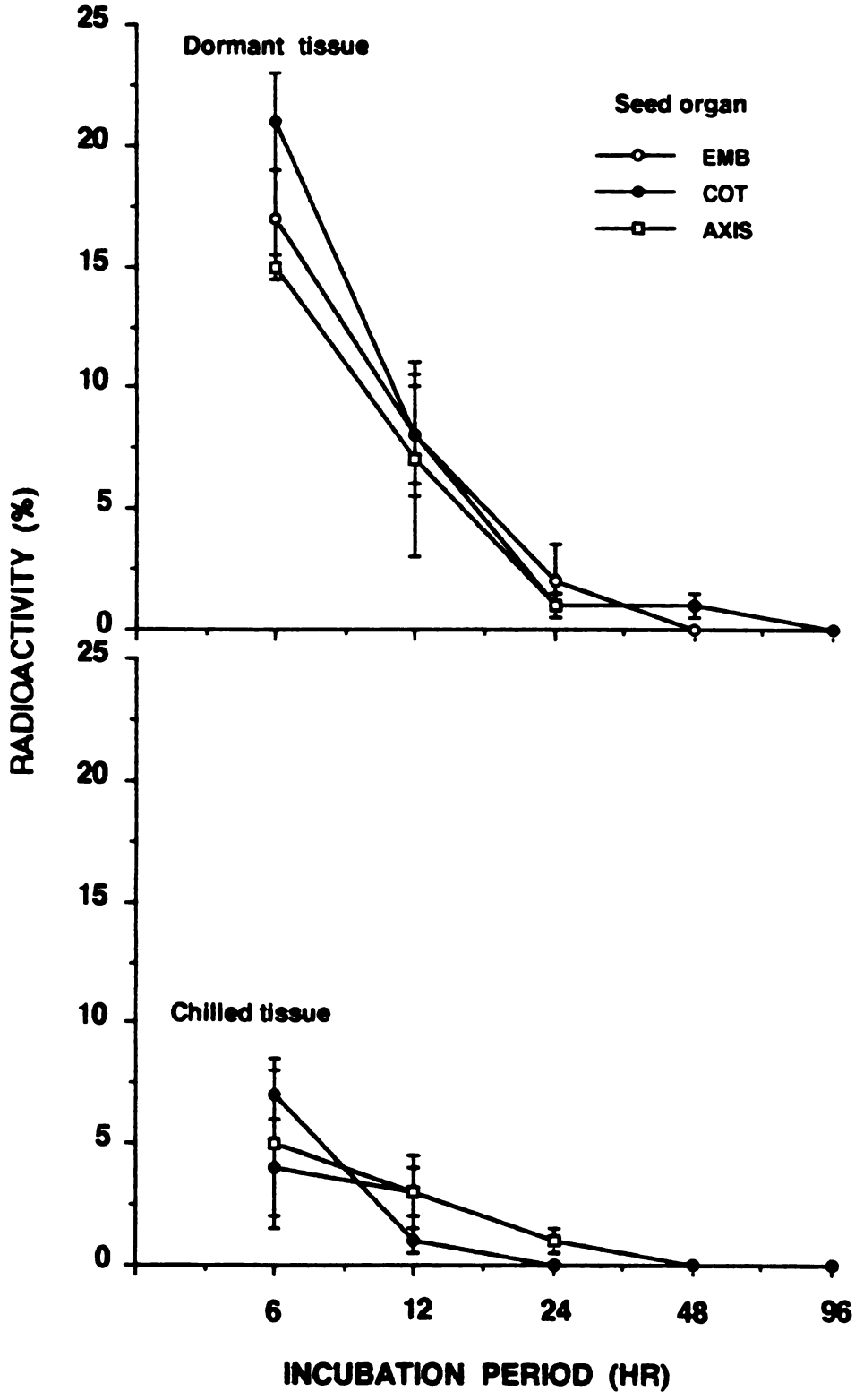

Dormant tissue
Seed organ
EMB
COT
AXIS
Chilled tissue
RADIOACTIVITY (%)
25
20
15
10
5
0
6
12
24
48
96
INCUBATION PERIOD (HR)

Figure 10. Relative quantities and profile of a 14C-labelled metabolite with retention time of 26 minutes in eluate from HPLC column of extracts following 6 to 96 hours incubation with 14C-GA aldehyde of embryos, cotyledons and embryonic axes excised from seeds chilled for 0 and 10 weeks at 5C.

Figure 10.

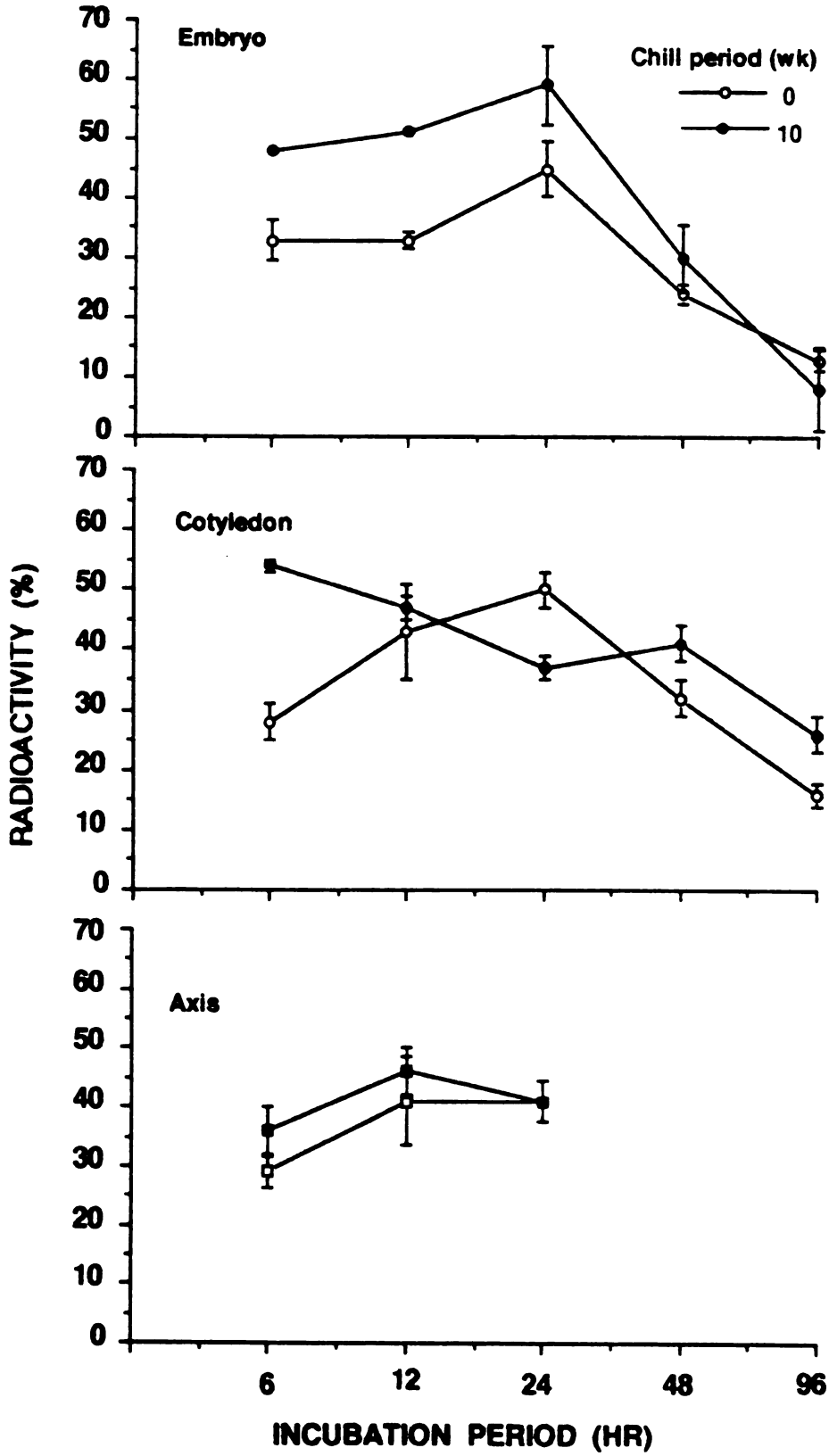

Embryo
Chill period (wk)
0
10
Cotyledon
Axis
RADIOACTIVITY (%)
0
10
20
30
40
50
60
70
6
12
24
48
96
INCUBATION PERIOD (HR)

Figure 11. Relative quantities of a 14C-labelled metabolite with retention time of 18 min. in eluates from HPLC column of extracts following 6 to 96 hours incubation with 14C-GA12-aldehyde of embryos, cotyledons, and embryonic axes excised from seeds chilled for 0 or 8 weeks at 5C. (Expt. 3).

Figure 11.

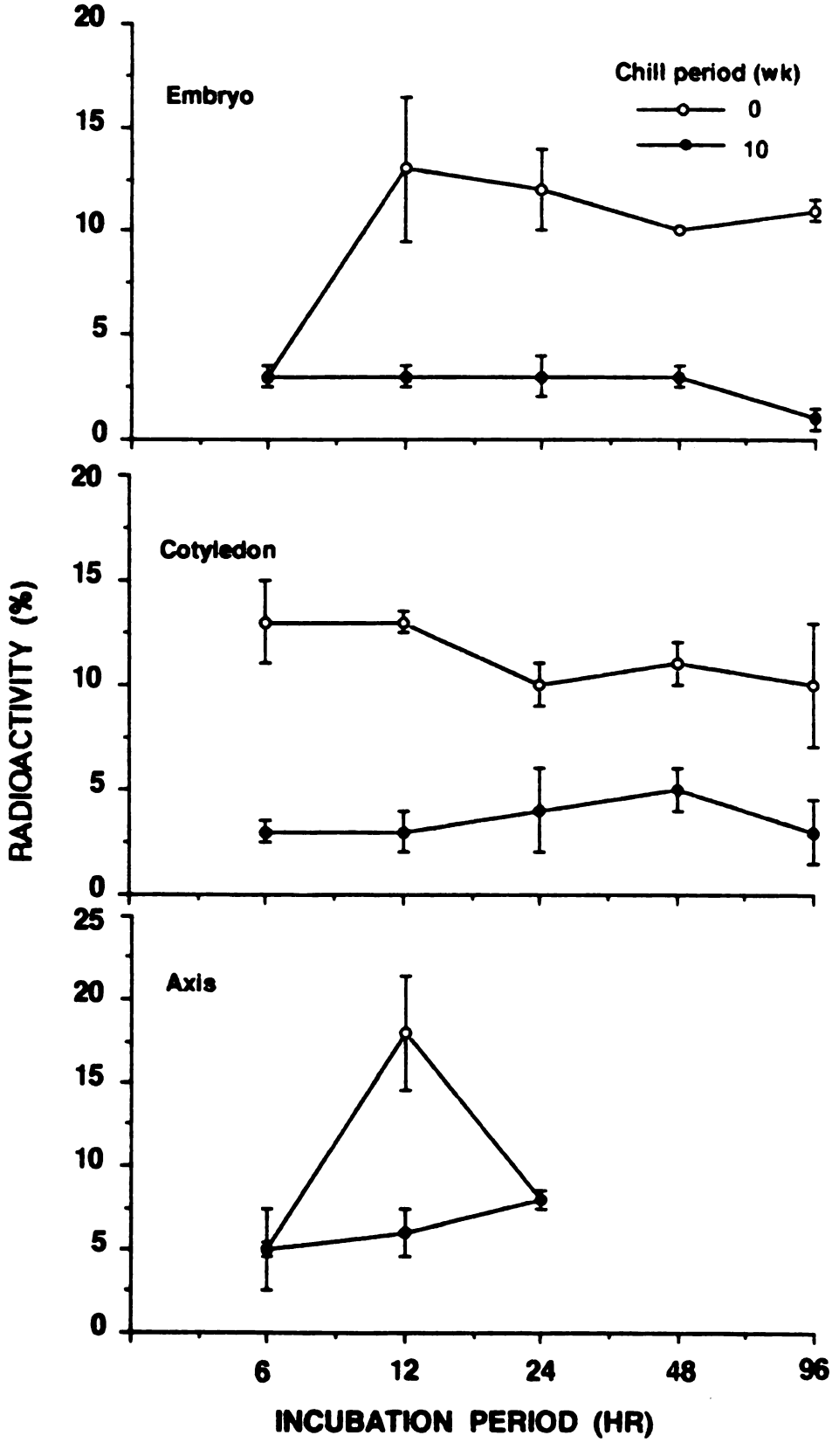

Embryo
Chill period (wk)
0
10
Cotyledon
Axis
RADIOACTIVITY (%)
INCUBATION PERIOD (HR)
6
12
24
48
96

Figure 12. Relative quantities of a 14C-labelled metabolite with retention time of 12 minutes in eluates from HPLC column of extracts following 6 to 96 hour incubation with 14C-GA12 aldehyde of embryos, cotyledons and embryonic axes axcised from seeds chilled for 0 or 8 weeks at 5C. (Expt. 3).

Figure 12.

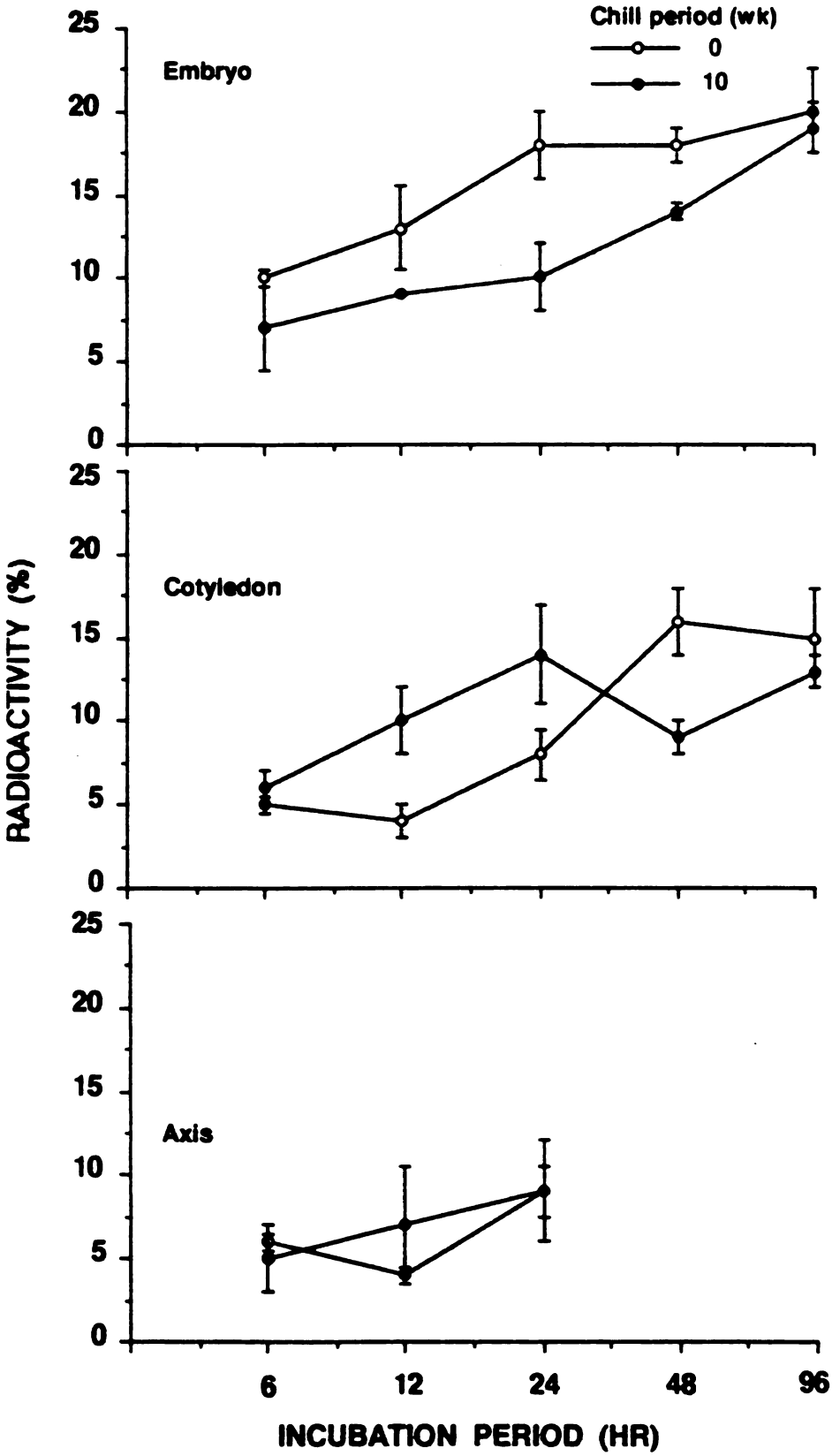

Chill period (wk)
0
10
Embryo
Cotyledon
Axis
RADIOACTIVITY (%)
25
20
15
10
5
0
6
12
24
48
96
INCUBATION PERIOD (HR)

Figure 13. Relative quantities of a 14C-labelled metabolite with retention time of 9 minutes in eluates from HPLC column of extracts following 6 to 96 hours incubation with 14C-GA12 aldehde of embryos, cotyledons and embryonic axes excised from seeds chilled for 0 to 8 weeks at 5C. (Expt 3).

Figure 13

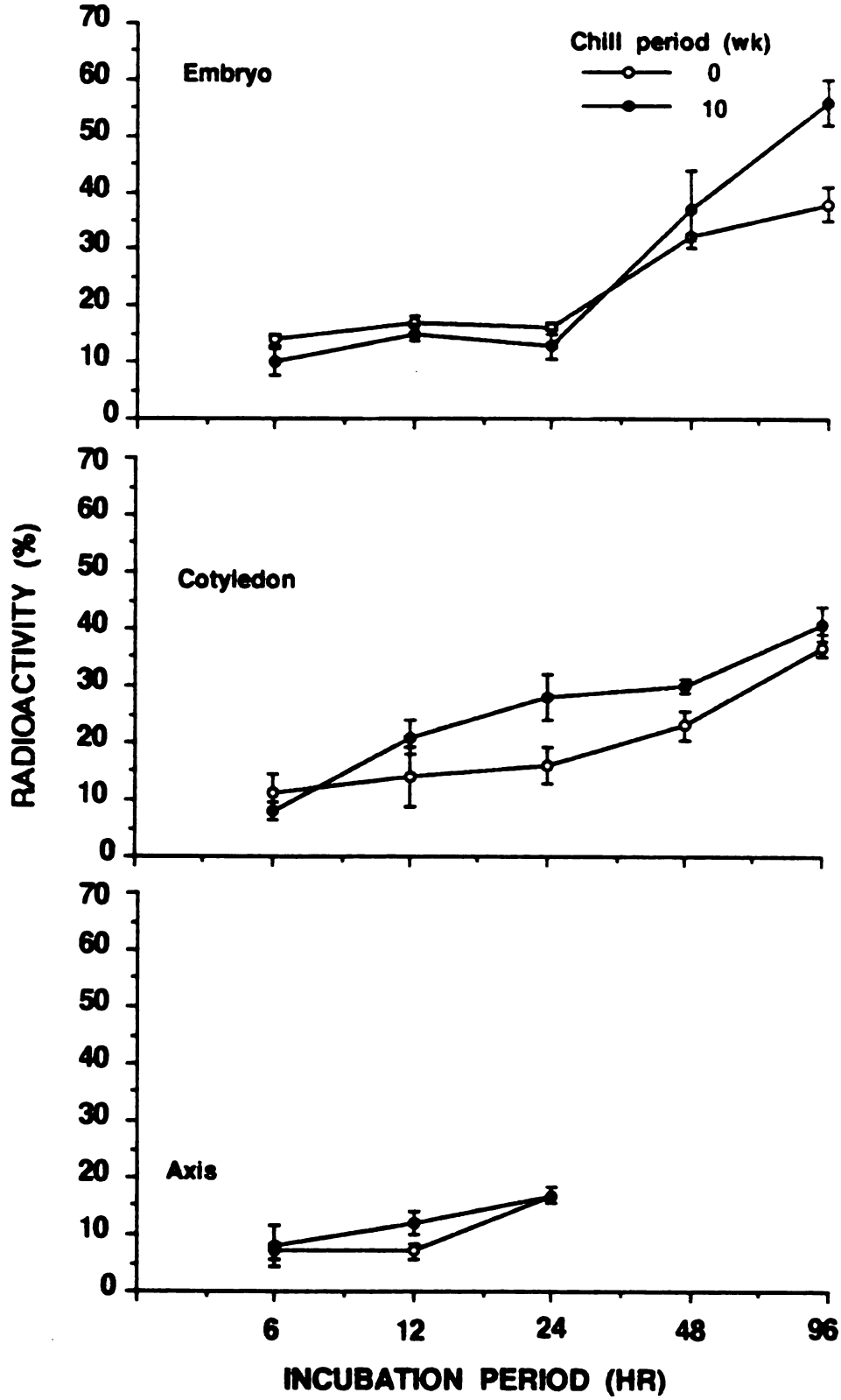

Chill period (wk)
0
10
Embryo
Cotyledon
Axis
RADIOACTIVITY (%)
INCUBATION PERIOD (HR)
0
10
20
30
40
50
60
70
6
12
24
48
96

Figure 14. Relative quantities of 14C-GA12-aldehyde remaining in eluate from HPLC column of extracts following 6 to 96 h incubation with 14C-GA12-aldehyde of dormant vs. chilled (4 or 8 wk) embryos, cotyledons and embryonic axes (Expt.4).

Figure 14.

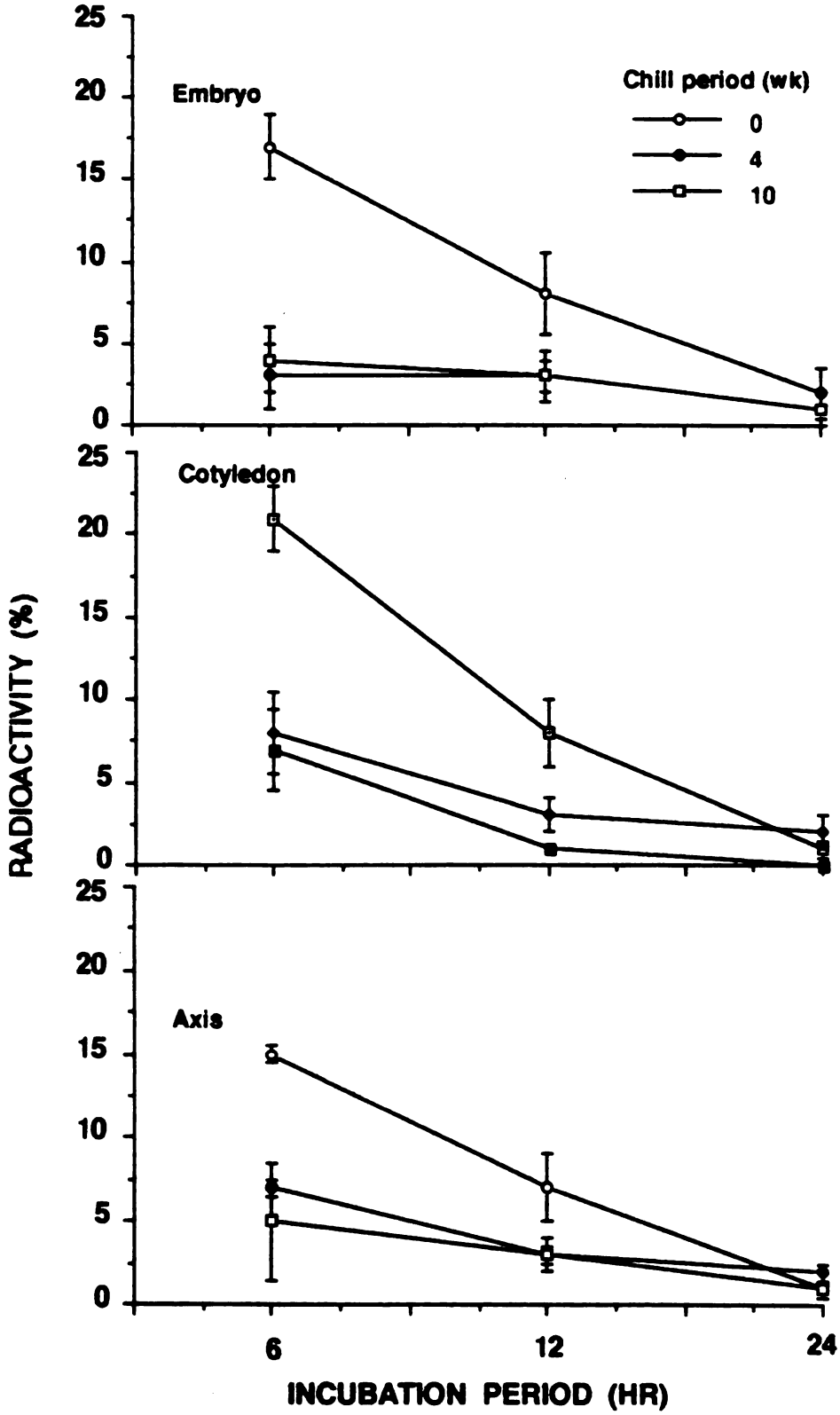
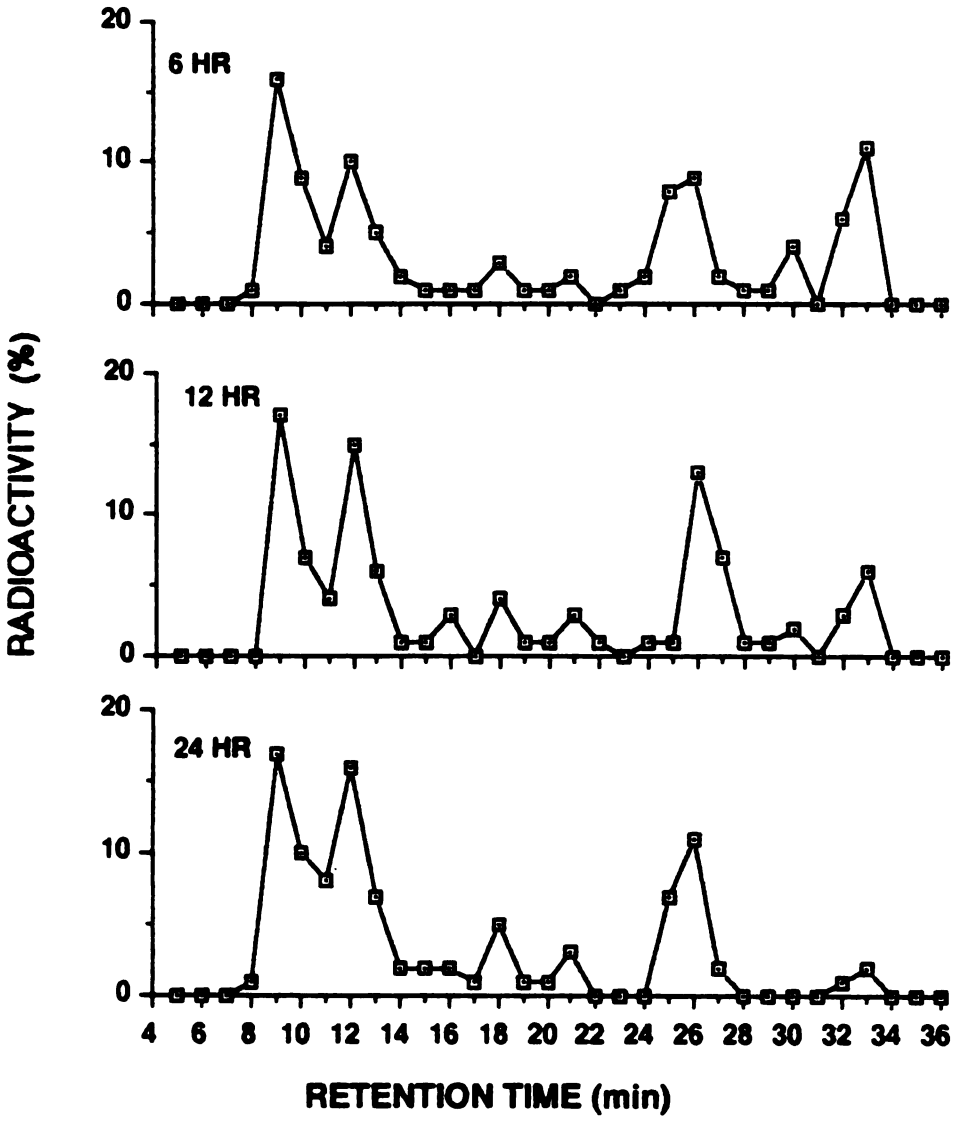

Embryo
Cotyledon
Axis
Chill period (wk)
0
4
10
RADIOACTIVITY (%)
INCUBATION PERIOD (HR)
6
12
24
0
5
10
15
20
25

Figure 15. HPLC of 14C-labelled metabolites in apple embryos, excised from seeds held moist at 20C for 8 wk, following 6, 12, and 24 h incubation with 14C-GA12-aldehyde at 20C (see Fig. 1 for conditions; Expt.4).

Figure 15.


6 HR
12 HR
24 HR
20
10
0
RADIOACTIVITY (%)
4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
RETENTION TIME (min)

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SUMMARY AND CONCLUSIONS

About 20 to 35% of embryos excised from non-chilled seeds at harvest time were able to germinate, thus not all embryos were completely dormant. The optimum constant temperature for stratification of seeds to break embryo dormancy was between 2.5 and 7C; six weeks of chilling were required at these temperatures. Temperatures of 0 and -2.5C had little effect, 15C was ineffective and 20 or 25C were inhibitory. The germination capacity of embryos excised from seeds chilled within fruit vs. in Petri dishes were similar but the former germinated more slowly. All intact seeds were dormant at harvest time. They started germinating only after 6 weeks of stratification and reached maximum germination in 10 to 12 weeks at constant 5C. Seeds stratified within fruit started to germinate after 10 weeks of chilling and only about 40% germinated after 15 weeks. Hence the chilling period required to break the dormancy of seeds is twice as long as that of embryos, indicating that whole seed dormancy is in part attributable to the presence of the seed coat and presumably the inhibitory chemicals contained in it.

The chilling effect of 5C on embryos was enhanced when cycled with 10C in both daily and longer cycles. However, this enhancement was due to the additive effects of the two temperatures. Cycling 15C with 5C had no effect while 20 and 25C had inhibitory effects. The degree of negation by

high temperature was dependent more on temperature than on cycle length. Alternating 5C with 10C partially inhibited seed germination, while cycling 5C with 15, 20, or 25C totally inhibited germination of the seeds. Thus, apple seeds have a narrower optimum temperature range for stratification and also were more sensitive to induction of secondary dormancy by high temperature than embryos.

Exposure to high temperature (30C) for 1 to 2 weeks reduced the germination capacity of seeds held in petri dishes or in fruit at 2.5C for 2-6 wk as well as embryos excised from them. Interruption of the chilling period by temperatures higher than 15C partially negated the previous chilling effect of 5C for both seeds and embryos excised from them but the effect diminished as stratification at 5C was prolonged. This agrees with the hypothesis that secondary dormancy can be induced by exposure to high temperature or ABA when the growth potential is low.

Anaerobiosis created by holding seeds under water at 20C failed to break embryo dormancy despite sparging the water with nitrogen gas for 1h prior to treatment. However, embryos from seeds held under water at 2.5C germinated as well as those chilled in Petri dishes, indicating that oxygen is not necessary for the release of dormancy by cold temperature.

Gibberellin A4+7, benzyladenine (BA), promalin (GA 4+7 + BA) and Dormex (Cyanamide) all significantly stimulated

the germination of embryos excised from non-chilled seeds but not of seeds themselves. The latter fact plus the germination percentages obtained for embryos (70 to 90%) reflect the failure of dormancy-breaking chemicals to completely replace the effects of chilling temperatures. Therefore more is involved in dormancy release than just an increase in growth promoters.

GA 4+7, BA, cyanamide and low temperature, all induced similar cytological changes in the embryonic axes of apple embryos, i.e., degradation of protein bodies, appearance of well-organized, long, rough endoplasmic reticulum and Golgi apparati, and an increase in the volume of the nucleus and numbers of mitochondria. These results suggest that proteolysis, which occurs at 4C but not at 25C, is essential for the breaking of dormancy. This again is dependent on the activation of enzymes that are most active at chilling temperatures, i.e., aminopeptidase and acid lipase. The degradation of reserves is accompanied by an increase in the nucleolar volume of the cells and synthesis of RNA. The amino acids released by hydrolysis are for synthesis of proteins for new enzymes, membranes and organelles. Furthermore, hydrolysis of glycosides could convert hormones such as GA's and cytokinins from their inactive, conjugated forms to active, free forms. The products of both hydrolysis and <u>de novo</u> synthesis are transported from source to destination by the endoplasmic reticulum (ER).

Gibberellins promote the development of the ER and also activate the enzymes associated with it. The degradation of reserve materials results in dissappearance of hydrophobic colloids; this enhances the hydration properties of the cell and the permeability of the membranes to water and solutes, which are essential for germination. There are structural connections between the ER, Golgi apparati and plasma membrane, all of which form a dynamic continuum. The ER proliferates by synthesizing its own macromolecules. After synthesis on the ER, the proteins move to the Golgi apparatus where they are sorted out and directed to their proper destinations. Both GAs and cytokinins apparently activate certain enzymes during the early period of stratification, while cyanamide stimulates RNA and protein synthesis. Chilling temperature is the most effective method of releasing dormancy probably because it promotes the activities of all the enzymes and hormones and their interactions, while simultaneously decreasing the activities of growth inhibitors.

Six 14C-labelled metabolites with retention times of 30, 26, 21, 18, 12 and 9 minutes (metabolite F30, F26, F21, F12 and F9, respectively) were recovered from extracts of apple embryos, cotyledons and axes incubated with 14C-GA12-aldehyde for 6 to 96 h. There were no qualitative differences in metabolism profiles on HPLC between chilled vs. dormant embryos, cotyledons or embryonic axes, except

for F18 which was absent from chilled tissues. The rate of metabolism was higher in chilled than in dormant tissues.

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APPENDIX

Figure 1. Relative quantities and profile of a 14C-labelled metabolite with retention time of 30 minutes (GA12) in eluete from HPLC column following 6 to 96 h incubation with 14C-GA12-aldehyde of dormant vs. chilled embryos, cotyledon, and embryonic axes (Expt. 3)

Figure 1.

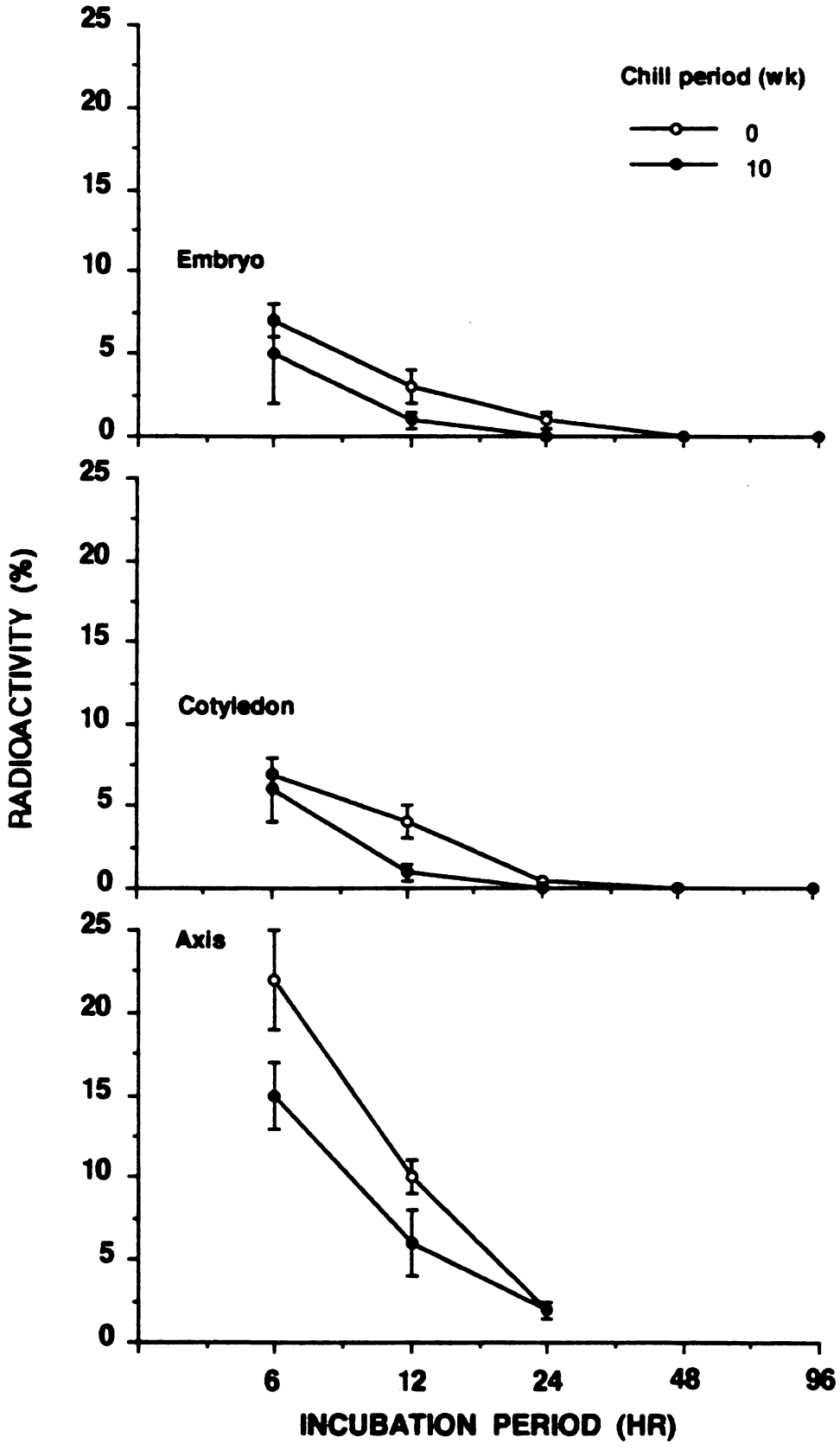
Chill period (wk)
0
10
Embryo
Cotyledon
Axis
RADIOACTIVITY (%)
25
20
15
10
5
0
6
12
24
48
96
INCUBATION PERIOD (HR)

Figure 2. Relative quantities and retention times of 14C-labelled metabolites and standards in eluates from HPLC column of embryos excised from seeds chilled for 8 weeks at 5C.

Figure 2.

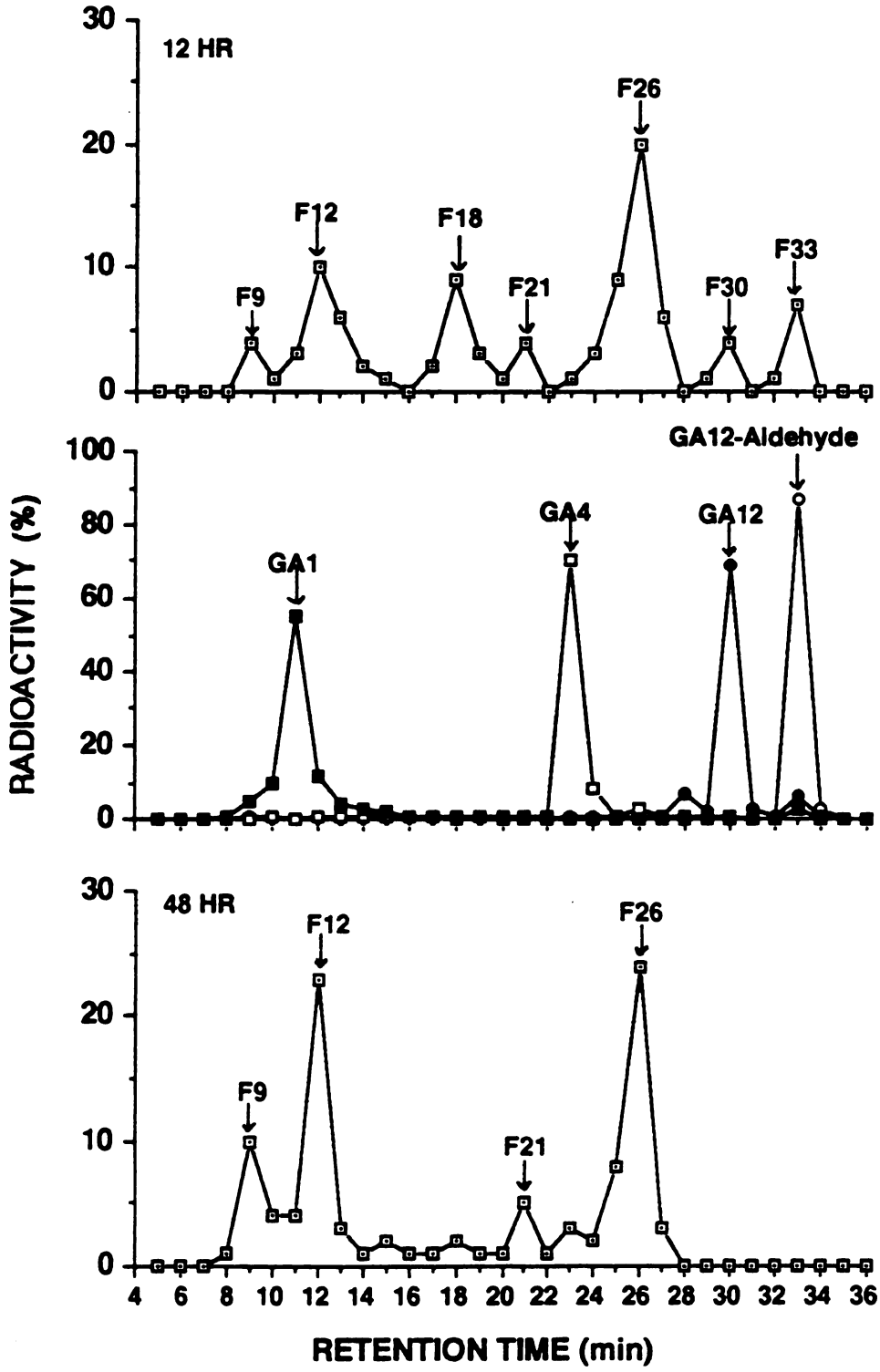
12 HR
F9
F12
F18
F21
F26
F30
F33
GA1
GA4
GA12
GA12-Aldehyde
48 HR
RADIOACTIVITY (%)
RETENTION TIME (min)

Table I Distribution of radioactivity during extraction and purification of apple embryo tissues. (Means for 3 replicates).

Tissue	Incub Period	Chilled embryo		Dormant embryos	
		Pellet	Column	Pellet	Column
Embryo	6	5,981	27,292	846	17,988
	12	5,540	29,668	1,191	18,220
	24	4,015	37,304	1,090	25,548
	48	4,572	40,992	10,736	23,584
	96	5,981	31,688	992	39,740
	Mean	5,218	33,389	2,971	25,016
Cotyledon	6	1,454	22,728	1,472	9,232
	12	1,481	18,588	1,365	19,628
	24	1,334	28,448	1,665	20,668
	48	1,365	36,612	1,444	35,560
	96	1,744	36,892	1,381	41,196
	Mean	1,476	28,654	1,465	25,257
Axis	6	5,515	22,764	4,981	35,484
	12	4,581	36,928	5,972	23,384
	24	4,011	30,596	4,534	28,628
	Mean	4,702	30,096	5,162	29,165

Table 2. Retention times (minutes) of gibberellins in reverse Phase High Performance Liquid Chromatagraphy using u-Bondapak C18 column.

	Reference		
Gibberellin	Ozga[Z]	Brenner[Y]	Talon[X]
1	9.8	15/16	15/16
3	-	13/14	-
4	23.2	26/27	27
5	15.2	-	-
7	-	26/27	26
8	9.6	7/8	7/10
9	28.2	28/29	30/32
12	33	30	33/36
13	-	21	21/24
14	-	28/29	-
15	-	28/29	30/32
17	14.4	-	25/26
19	14.4	-	23/25
20	15.6	22	21/22
23	-	11/22	-
24	-	-	30/32
25	-	-	30/32
29	8.2	9/10	11/12
34	-	24	22/27
36	-	-	21/24
37	-	-	23/24
44	16.0	23	23/24
51	-	26	25/26
53	19.8	-	28/29

Z - discontinuous gradient using acetonitrile solvent; personal communication.

Y - linear gradient using acetonitrile solvent; personal communication.

X - linear gradient using methanol solvent; personal communication.