AN ABSTRACT OF THE THESIS OF

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Title: The Role of Glutathione in the Development and Release of Rest on 'Redhaven' Peaches.

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A rapid and sensitive HPLC method for quantifying both the reduced and oxidized forms of glutathione simultaneously in woody plant tissues was developed. This method was applicable to a variety of woody plant species and different tissues. Total glutathione content in the bark tissue of seven of the woody plant species tested were lower during active plant growth, and higher in the dormant stage. All tested tissues of peach plants contained glutathione. The highest and lowest content was found in the leaves and roots, respectively.

'Redhaven' peach trees acquired the onset of rest on 1 Oct, before any chilling had been experienced. Maximum rest was reached on 1 Nov after the plants were exposed to 320 CU, and 50% of the buds were broken at 860 CU on 1 Dec. Total budbreak and phytotoxicity induced by hydrogen cyanamide applied to 'Redhaven' peach trees was dependent on concentration and time of application. Phytotoxicity was evident at all application dates, but greatest at the highest concentrations. Plants were more resistant to hydrogen cyanamide at maximum rest. Hydrogen cyanamide induced budbreak was best during the post-rest period. Budbreak and phytotoxicity induced by hydrogen cyanamide applied during the quiescent stage were dependent on post-treatment temperature and concentration. All cyanamide treatments inhibited budbreak at all tested temperatures. The inhibition was

greatest at the the lowest temperature and at the highest concentrations. Phytotoxicity was greater at 15C and 35C than at 25C, and increased with increased concentrations.

The content of GSH and GSSG in the buds changed with the development of rest. During the early phases of rest, when rest intensity increased, the content of GSH and GSSG decreased (15 Oct) and then increased at maximum rest (1 Nov). Content of GSH continued to increase and peaked on 1 Dec at 860 CU. On 15 Dec when the buds were quiescent GSH content decreased. In contrast, contents of GSSG did not drop during quiescence and were highest on 15 Dec. GSH content induced by chilling was closely associated with the end of rest.

Hydrogen cyanamide treatments caused a rapid decrease of GSH content within 12 hr followed by a large increase 24 hr after application. The changes of GSH induced were inversely proportional to cyanamide concentration. Non-treated plants showed at relatively constant GSH and GSSG content during this period of time. Hydrogen cyanamide treatments increased GSH contents at all application dates. The extent of GSH changes was dependent on the physiological status of the bud and cyanamide concentration. At maximum rest the plants were more resistant to cyanamide and this coincided with the highest induced GSH content as compared to all application dates.

The Role of Glutathione in the Development and Release of Rest on 'Redhaven' Peaches

by

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1.0 INTRODUCTION

An important and difficult problem for agricultural scientists is the measurement and prediction of rest induction and release in fruiting plants. Rest in deciduous fruit trees and other woody perennials of the temperate zones is a phase of development that occurs annually and enables plants to survive cold winters (Saure, 1985). In its broadest sense, rest covers every meristematic structure in the plant and involves any period when no appreciable growth occurs (Lang et al., 1987). Because of its relation to frost hardiness, horticulturists initially dealt mainly with the induction of rest, especially in those parts of the world where cold winters are common. The release from rest was studied first by those who were engaged in early forcing of ornamentals. It received little attention in deciduous fruit trees until early attempts were made to grow temperate fruits in the subtropics or even in the tropics, where cold winters do not exist. In those regions, prolonged rest became an important obstacle to economic production of temperate crops.

The dynamics of rest induction and release, i.e., the role of the factors involved both within and outside the plants, and their mode of action, are still not yet fully understood, in spite of the countless publications dealing with rest or some of its aspects. Many interesting hypotheses and theories have been suggested, only to be rejected (Doorenbos, 1953; Samish, 1954; Vegis, 1964; Saunders, 1978; Nooden and Weber, 1978). Recently, Fuchigami and Nee (1987) hypothezised that the development of rest and the action of rest breaking agents is related to the effect of glutathione on disulfides.

Glutathione (γ-glutamyl-cysteynl-glycine) is a low molecular weight thiol compound implicated in a wide range of metabolic processes (Meister and Anderson, 1983). It is widely distributed in plants and present predominantly in the reduced form (GSH) (Rennenberg, 1982). Stress-induced glutathione production is a widespread, if not ubiquitous response of plants to unusual growing conditions. These include stresses imposed by low temperature (Kuroda et al., 1990; deKok and Oosterhuis, 1987; Wise and Naylor, 1987), water (Burke et al., 1985; Gamble and Burke, 1984), heavy metals (Rennenberg, 1987), heat shock (Mitchell et al., 1983; Nieto-Sotelo and Ho, 1986), oxygen concentration (Foster and Hess, 1980; 1982), light quality and quantity (Schupp and Rennenberg, 1988), exposure to air pollutants such as ozone and sulfur dioxide (Mehlhorn et al., 1986; Heath, 1987), and herbicides (Lamoureux and Rusness, 1981).

The fact that glutathione biosynthesis is stimulated by such a wide variety of plant stresses, suggest an important role for glutathione in the plant's attempt to cope with diverse conditions. Rest is the result of a highly useful adaptation to the environmental conditions which prevail where the species or varieties originates (Vegis, 1964). Thus, the ability to survive and leave descendents is highly dependent upon optimizing phenological events, and consequently the mechanisms regulating the induction and release of rest are important. The response in alterations of glutathione metabolism induced by biological and physical stresses appears to be of greater survival value as disposal of oxygen toxic species at its expense is necessary to maintain metabolic integrity (Elstner, 1982). Although much is known about stress glutathione biosynthesis, relatively little is known about the role that glutathione may play in the development and release of rest of temperate zone plants.

This research is intended to contribute to our understanding of rest and of the mechanisms involved in overcoming rest, especially in

deciduous fruit trees, and possibly in other woody perennials. The objective of this study was to determine the role of glutathione in the development and release of the rest period in flower and vegetative buds of 'Redhaven' peach trees. For this purpose, we developed a sensitive and accurate HPLC method of quantifying simultaneously both the reduced and oxidized forms of glutathione in woody plant tissues. This method was utilized to study the changes in GSH and GSSG under natural conditions during the development and release of rest of 'Redhaven' peach trees. In addition, glutathione contents induced by the rest breaking chemical, hydrogen cyanamide, were studied throughout the rest period.

A characteristic common to most of the rest breaking treatments is their effectiveness at near-lethal dosages (Fuchigami and Nee, 1987; Erez, 1987). Because of such a narrow effective range, it is not surprising that variable effectiveness in overcoming rest and phytotoxicity are commonly encountered (Dozier et al., 1990; Wolak and Couvillon, 1976). Without an understanding of the physiological status of rest, it is difficult to improve the management of rest release. Thus, a second objective was to characterize the physiological status of the 'Redhaven' peach buds during the winter dormancy period and to examine the effect of hydrogen cyanamide on budbreak and phytotoxicity. The purpose was to establish the relationship between the physiological status of buds and the effective hydrogen cyanamide concentration to break rest.

The post-application temperature has been suggested as one of the factors influencing the variable results in phytotoxicity (Erez, 1987). Thus, a third objective was to study the effect of post-treatment temperature on the efficacy and phytotoxicity of hydrogen cyanamide during the quiescent stage.

2.0 LITERATURE REVIEW

2.1. DORMANCY

2.1.1 Definition. Plant dormancy is a vast topic, and in some ways, constitutes a research 'black box' with respect to plant growth. The understanding of plant growth is certainly far from clear, and yet dormancy is a phenomenon that has been defined in terms of growth, or more specifically, the lack of growth (Romberger, 1963). Thus, in addition to the difficulties associated with the complex process which generate dormancy, more difficulties are imposed by the gaps in our understanding of its antithesis - growth.

What is meant by 'dormancy'? In the broad context, after Doorenbros (1953), Samish (1954), Romberger (1963), Saure (1985), and Lang et al (1987), 'dormancy' is a general term used to indicate the temporary suspension of visible growth of any plant structure containing a meristem.

2.1.2. Phases of Dormancy. Dormancy is not an uniform state within the development of plants, but is rather a phenomena covering a spectrum of different physiological conditions. Thus, bud dormancy of trees is usually classified into three main categories according to the factor that prevents growth. Different nomenclatures for the categories have been used by different authors i.e., winter dormancy, imposed dormancy, and summer dormancy (Doorenbos, 1953); rest, quiescence, and correlative inhibition (Samish, 1954); true dormancy, imposed dormancy, and predormancy (Saure, 1985); endodormancy, ecodormancy, and paradormancy (Lang et al, 1987).

This thesis, with its narrow focus, will use three terms accepted by most horticulturists and physiologists to describe general types of dormancy: 1) Quiescence (Romberger, 1963), which is the state of dormancy regulated by environmental factors - when one or more factors

in the basic growth environment are unsuitable for overall growth metabolism; 2) Correlative inhibition (Romberger, 1963), defined as the state of dormancy which is regulated by physiological factors outside the affected structure - buds remain dormant in a favorable environment, but rapidily resume growth if neighboring organs (leaves, buds) are removed, thus removing the source of inhibitors; and 3) Rest (Romberger, 1963) defined as the state of dormancy regulated by physiological factors inside the affected structure - buds remain dormant for prolonged periods even when the environment is favorable for growth or even when competitive/ inhibitory organs are removed. Such internally inhibited buds normally require prolonged exposure to chilling temperatures before growth resumes.

2.1.3 Evolutionary and Ecological Considerations. Dormancy is the result of a highly useful adaptation to the environmental conditions which prevail where the species or varieties originates (Vegis, 1964). Thus, no consideration of a plant phenomenon should be complete without recognition of the evolutionary strategy associated with it.

Hundred of millions of years ago, as plant life evolved from its aqueous origin in the sea to land-based colonies, a greater diversity of environments and more extreme fluctations of climate were encountered (Axelrod, 1960). As the planet cooled and plant life extended further from sea, continuous growth of pioneer plants became detrimental for survival. Later, the falling sea level during the last few hundred thousand years of the Cretaceous period gave rise to further seasonal extremes of temperature that increased environmental stress on terrestrial plants (Axelrod, 1960). Adaptation to unfavourable weather, and to predation pressures brought on by insect and animal populations whose activities were tied to environmental changes, were required to insure parental survival and/or dispersal of reproductive propagules (Osborne, 1981). Interruption of continued

growth, or dormancy, probably evolved as a specialized mechanism to survive extreme climatic fluctuations.

In time, the adaptation growth mechanisms of plants became as specialized as the diverse environments in which plant growth could be supported, resulting in further diversification of species and establishment of plant communities in ecological niches (Lockhart, 1983). Plants with the greatest genetic capacity to adapt to changing conditions may be more successful in fluctuating environments, while plants with narrow gene expression may be the dominant colonizers in relatively unchanging conditions (Stern and Roche, 1974).

As plant dormancy is closely tied to evolutionary survival strategies associated with the environment, temperate zone bud dormancy is basically the culmination of genetic expression in the plant in anticipation of a future environmental change unfavourable to growth (Levins, 1969). Thus, the ability to survive and leave descendents is highly dependent upon optimizing phenological events, and consequently the mechanisms regulating the induction and release of rest is important.

2.2. ENDOGENOUS CONTROL OF DORMANCY

2.2.1 General Metabolic Changes. Free sugars, Amino acids and Organic Acids. Upon satisfaction of the chilling requirement, there is a rapid and large increase in free sugars, amino acids, and organic acids, with a concomitant depletion of total sugars and starch (Bacheland and Wightman, 1973; Nooden and Weber, 1978; Nowacki et al., 1984; Priestly, 1981; Steffens et al., 1985; Wang et al., 1987a). This has been attributed to the transition of reserves into new growth or to respiration loss (Wang and Faust, 1987). However, the conversion of starch to sugar alone may not be indicative of the breaking of dormancy.

Nucleic Acids and Proteins. The level of total proteins have been reported to decrease as dormancy is alleviated (Bacheland and

Wightman, 1973), though as post-dormancy activity increases, the levels of RNA, DNA, and total protein increase sharply (Bobrysheva and Oknina, 1962; Wang et al., 1985). Torrigan et al. (1978) concluded that in dormant peach flower buds polysomes were numerous and their activities as regards phenylalanine incorporation in a cell-free system were high; in vivo protein synthesis increased 13-fold from dormancy to flowering. The marked increase of these components observed in buds during dormancy breaking may either represent a de novo synthesis or be due to the transport of dissassembling storage protein accumulated in bark tissues during spring growth (Titus and Kang, 1982). Whether the activation of nucleic acids and protein synthesis is involved in the 'trigger mechanism of budbreak' or it is a consequence of dormancy breaking mechanisms, remains to be clarified by further experiments.

Respiration. An increase in O_2 consumption occurring between the early dormant stage in winter and the resumption of growth in spring has been reported in peach and pear flower buds (Cole et al., 1982; Hatch and Walker, 1969). The respiratory quotient, which is high prior to dormancy, decreases during dormancy until the point of chilling satisfaction (Bacheland and Wightman, 1973; Hobson, 1981). Numerous dormancy-breaking treatments have been shown to increase respiration of dormant buds (Shulman et al., 1983). However, treatment with the dormancy breaking chemical, thiourea failed to enhance respiration yet still overcome rest (Shulman et al., 1983).Also, inhibition of respiration with treatments to limit oxygen has been found to stimulate peach bud break (Erez et al., 1980). The potential regulatory role of respiration in dormancy alleviation is not fully understood.

2.2.2. Hormonal Promoters/Inhibitors. The role of hormones controlling dormancy and budbreak is not clear. Interactions of hormones, both inhibitory and promotory, are currently thought to be

involved in the regulation of rest (Borkowska, 1980; Lavee, 1973; Powell, 1987, Walker and Seeley, 1974; Weaver et al., 1968). Generally, promoter concentration is low and inhibitor concentration high during rest and the reverse occur when rest is broken (Lavee, 1973; Samish et al., 1967; Wang et al., 1986; Weaver et al., 1968).

In most cases the inhibitor reported to regulate rest intensity was abscisic acid (ABA) (During and Bachmann, 1975; Powell, 1987). Among the promoters, cytokinins have been reported to accumulate in apple floral buds before bloom and in the buds of pecan during bud swelling (Thomas and Katterman, 1986; Wood, 1983). Exogenous application of cytokinins induced growth of buds that has been at least partially released from dormancy (Nee, 1986; Shaltout and Unrath, 1983a; Weinberger, 1969), however, were more effective in stimulating shoot elongation than in promoting budbreak (Fuchigami and Nee, 1987).

Gibberellins (GA) and Auxins are relatively ineffective in breaking dormancy, but are more effective in advancing bud opening and growth (Brown et al., 1960; Erez et al., 1979). It has been suggested that the GA treatment can substitute for chilling (Hatch and Walker, 1969; Walser et al., 1981). Several other authors observed, however, that GA was able to promote budbreak only after some prior chilling (Brown et al., 1960; Paiva and Robitaille, 1978b; Walker and Donoho, 1959).

2.2.3. Catalase Activity and Glutathione. The changes in catalase activity of grape vines and peach floral buds during dormancy have been investigated by Nir et al. (1984, 1986) and Kaminski and Rom (1974), respectively. Increased catalase activity was associated with the entry of buds into dormancy, and activity subsequently declined until budbreak. Certain dormancy breaking chemicals, such as thiourea and hydrogen cyanamide, have been reported to inhibit catalase in plants (Fuchigami and Nee, 1987; Nir et al., 1986). The decreased

catalase activity is thought to increase peroxide levels in the bud tissue. This, in turn, is hypothesized to activate the pentose-phosphate pathway and subsequently terminate dormancy (Nir et al. 1984; Simmonds and Simpson, 1972).

Conversely, others suggested that an increase of glutathione, not peroxide, is most important in the termination of rest (Amberger, 1984). In plants, Esterbauer and Grill (1978) and Kuroda et al., (1990) have shown that the reduced form of glutathione (GSH) undergoes seasonal changes with higher levels during dormancy and lower levels during active growth. A strong relationship of the status of thiol groups with dormancy was found in the fungus of *Neurospora crassa* (Fahey et al., 1975) and in wheat embryos (Fahey et al, 1980). Oxidized (GSSG) and protein bound glutathione (PSSG) content increased dramatically with dormancy, and rapidly decline during germination. In animal cells, the levels of the oxidized form of glutathione (GGSG) play a role in the regulation of protein synthesis through control of polysome formation (Kosower et al., 1972). In plants, direct evidence that glutathione alone and from yeast extracts breaks rest has been provided in various plant species (Guthrie, 1940; Nee, 1986). Until a better understanding of the complex biochemical and physiological processes involved in rest is achieved, the potential regulatory role of glutathione in rest remains to be demonstrated experimentally.

2.2.4. Polyamines and Ethylene. Polyamines are considered to be potential secondary messengers in the stimulation and regulation of nucleic acids and proteins, and are generally abundant in meristematic and growing tissues (Galston, 1983). Polyamine synthesis have been suggested to have a role in the breaking of dormancy (Fuchigami and Nee, 1987), though experimental evidence is still scarce. Polyamines have been shown to increase following thidiazuron-induction of budbreak, and are present throughout the season in cherry buds (Wang et al., 1985). However, evidence for its direct role in rest

regulation is still lacking.

Doorenbos (1953) suggested that the breaking of rest may be a function of an increase in membrane permeability during chilling. Ethylene production and electrolyte leakage, as induced by dormancy breaking treatments, have been correlated to the breaking of rest (Fuchigami and Nee, 1987). The presence of ethylene in such cases may be a function of the stress induced increases in membrane permeability which would tend to increase the availability of the ethylene-forming enzyme (EFE). Conversely, Wang et al. (1985) found the transition from dormant to active buds was accompanied by increases in SAM, ACC, ACC conjugated and polyamines. Although, exogenous application of ethylene has been reported to break dormancy (Hosoki, 1985; Iwasaki, 1980; Sinska and Gladon, 1984), it is questionable whether sub-lethal stress-induced ethylene has a direct regulatory role in budbreak. Paiva and Robitaille (1978) and Zimmerman et al., (1977) found no evidence for the involvement of ethylene in the emergence from dormancy of apple and tea crabapple buds. Ethylene production followed budbreak rather than preceeding it.

2.2.5. Phase Change Phenomenon. Doorenbos (1953) suggested that the breaking of dormancy may be a function of an increase in membrane permeability during chilling. It is well known that the membranes of chill sensitive plants undergo a physical-phase transition from a flexible liquid-crystalline to a solid gel structure following exposure to chilling temperatures, imparting changes in permeability as well as in enzyme activity (Lyons, 1973; Steponkus, 1984). In apple shoot buds, changes in the fatty acids and sterol content indicative of alteration in membrane permeability and physiological function have been characterized by Wang and Faust (1988). They, also showed that treatment with a dormancy breaking chemical, thidiazuron, induced accumulation of unsaturated polar membrane fatty acids and caused a shift in the levels and composition of sterols.

2.2.6. Gene Expression. Vegis (1964) reported that RNA content in dormant structures is low until after dormancy completion. Tuan and Bonner (1964) demonstrated in potato buds that some dormancy breaking chemicals increase RNA and DNA precursor incorporation and RNA template activity of chromatin over that of dormant buds. This indicates that the genetic material in dormant buds is repressed and that breaking of dormancy is concomitant to derepression of the genetic material.

Callaway and Couvillon (1987), using dormant peach and cherry seeds, have begun investigation of differential gene expression resulting from warm and cold stratification treatments. The identification of apparent control of bud dormancy by a single recessive gene in filbert, *Corylus avellana* (Thompson et al, 1985) may be a major breakthrough for the investigation of dormancy and gene expression.

2.3. CHILLING REQUIREMENT

2.3.1 Effective Temperatures. It is generally accepted that "chilling" temperatures are required for overcoming rest in dormant buds (Doorenbos, 1953, Samish, 1954; Wareing, 1969). Investigations of chilling temperature requirements for the rest release of fruit tree buds began more than 50 years ago (Chandler and Tufts, 1934). Weinberger (1950) coined the term "chilling hours" (CH) for winter air temperatures less than 7°C and correlated CH to the amount of chilling required by fruit trees to break rest and exhibit normal bud growth. The CH became known as the chilling requirement, a valuable attempt used by researchers and the industry to quantify the amount of chilling required to break rest and to establish the suitability of new cultivars for a given environment.

The effective temperature and duration of this temperature to overcome rest is dependent on the genetic makeup of the plant (Bennet, 1950; Doorenbos, 1953; Erez et al., 1979; Gurdian and Biggs, 1964;

Samish, 1954). In most cases, 5°C is thought to be the optimum temperature for satisfy the chilling requirement of peaches (Erez and Lavee, 1971; Perry, 1971). Warm temperatures (Bennett, 1950; Erez and Lavee, 1971; Perry, 1971) or too low temperatures (Coville, 1920; Kobayashi et al., 1982; Nooden and Weaver, 1978; Samish, 1954) are generally not as effective in overcoming rest.

Fluctuating temperatures seem to be more effective than constant temperatures in breaking rest (Samish, 1954). In peach, daily fluctations betwwen 6° and 15°C were more effective in breaking rest than either temperature alone (Erez et al., 1979).

The effective temperature to overcome rest is temperature dependent (Erez and Lavee, 1971; Kobayashi et al; 1983; Vegis, 1956). In peach, moderate temperature during November and December negated chilling (Weinberger, 1967). Also, short periods of high temperature during a daily cycle can negate chilling. Erez et al. (1979a) reported that exposure of dormant peach to 6 hr at 21° to 24°C negated 18 hr of 6°C. In contrast Kobayashi et al (1982) reported that the effective temperature to overcome rest and the rate of rest development changes with growth stages and temperature. Temperature as high as 20°C was also capable of enhancing rest development.

In contrast to the "normal" slow method of breaking rest by chilling temperatures, exposing resting buds to both sublethal freezing and high temperatures and other sublethal treatments for a short period of time can overcome rest relatively quick (Nee, 1986; Orffer and Goussard, 1980; Thomas and Wilkinson, 1964; Sparks et al., 1976).

2.3.2. Calculation of Chilling Requirement. Most of the methods developed to calculate the chilling requirement involve the determination of the number of hours at temperatures of 7°C and below required to break rest (Saure, 1985). They differ in the formulae used, in the assessment of the period to be selected for these

calculations, and obviously in the results obtained in different regions, as disscused by Muñoz (1969) and del Real-Laborde and Gonzalez-Cepeda (1982).

To predict rest release as a function of temperature, Richardson et al. (1974) developed a mathematical model, the so-called 'Utah chill-unit model' that assigns chilling units to various winter temperatures. One 'chilling unit' (CU) was defined as one hour of optimum chilling temperature. Non-optimal temperatures, higher and lower than the optimum temperature were assigned fractional CU values. Once a certain level of CU has accumulated, a certain amount of exposure to warm temperatures above 4.5°C, termed Growing Degree Hours (GDH) or heat unit requirement, was required for budbreak (Richardson et al., 1974).

The Utah model has been criticized for inaccurate predictions of plant response, especially in areas with mild winter conditions (Buchanan et al., 1977; Erez et al., 1979; Shaltout and Unrath, 1983b; Walser et al., 1981). Nevertheless, models based on the Utah model have been developed recently for other fruit species including apple (Shaltout and Unrath, 1983b), blueberry (Norvell and Moore, 1982), and sour cherry (Felker and Robataille, 1985). Seeley and Del Real-Laborde (1987) working in stratification with apple seeds at various temperatures reported that consideration should be given not only to optimum chilling temperatures, but also to how temperature optima may change over the dormant period as tissue sensitivy changes.

A conceptual numerical model, the Degree Growth Stage (°GS) model, has been developed and utilized to describe the annual cycle of plant bud development (Fuchigami et al., 1982; Fuchigami and Nee, 1987). This model takes into account the changing physiology of the plant during the dormant period, denoting as 'point events' the end of dormancy/spring budbreak (360 and 0°GS), the maturity induction point (90°GS), the vegetative maturity point/onset rest (180°GS), and the

point of maximum rest (270°GS).

A two-step cycled temperature model have been suggested by Erez and Couvillon (1987) to explain the effects of alternating warm and chill temperatures on dormancy release. They proposed that the first step of dormancy release was the reversible formation-destruction of a thermally unstable precursor substance, and the second step was the fixation of this substance once it reached a critical level. This hypothesis was formalized in a mathematical model by Fishman et al., (1987).

2.4. AGRICULTURAL MANIPULATION OF DORMANCY

Physiological manipulation of plant development to overcome rest and enhance shoot elongation through chemical applications and cultural practices is important in warm climates where insufficient chilling results in delayed and erratic foliation and bloom. On the other hand, physiological manipulation of plant development to delay budbreak and avoid spring frost through chemical treatments and cultural practices is important to protect the crop.

2.4.1. Delay of Budbreak. Delay of budbreak by use of chemicals have been accomplished by autumn-applied sprays of gibberellic acid (Bowen and Derickson, 1978) and ethylene releasing compounds (Proebsting and Mills, 1972), though results have been variable and dependent upon subsequent winter temperatures (Gianfagna, et al, 1986). Fall application of aminoethoxyvinylglycine (AVG), a inhibitor of ethylene synthesis delayed budbreak in blueberries and peaches (DeKazos, 1979, 1981).

Manipulation of cultural practices have also been reported to delay budbreak. Defoliation (Janick, 1974; Fuchigami et al., 1982), wounding (Samish, 1954), and stimulation of growth late in the fall (Chandler and Tufts, 1934) generally lead to later budbreak. Overhead sprinkling of cold water on days when temperatures exceeded 18°C, after the chilling has been satisfied was also demonstrated to delay spring

bloom due to evaporative cooling (Buchanan et al., 1977). However, substantial bud abscission was observed in some cultivars.

2.4.2 Promotion of Budbreak. Many attempts have been made to overcome the problem caused by insufficient chilling of deciduous fruit trees growing under warm climates (Edwards, 1987). In the tropics, preventing plants from entering rest by cultural practices such as defoliation and witholding irrigation are the methods of choice (Edwards, 1987; Janick, 1974; Sherman and Lyrene, 1984). In the subtropics, hastening budbreak after plants have already entered rest by a combination of cultural practices and application of dormancy breaking chemicals is the main approach utilized (Erez, 1987; Erez and Lavee, 1974; Saure, 1985).

Although many chemicals and physical treatments are known to break rest (Doorenbos, 1953), only a few are applicable under field conditions (Erez, 1987). A characteristic common to most of the rest breaking treatments is their effectiveness at near-lethal dosages (Erez, 1987; Fuchigami and Nee, 1987). Because of such a narrow effective range, it is not surprising that variable effectiveness of overcoming rest and toxicity are reported (Wolak and Couvillon, 1977). The concentration and time of application (Wolak and Couvillon, 1976; Fuchigami and Nee, 1987), the physiological stage of the bud (Fuchigami and Nee, 1987), the nutritional status of the plants (Terblanche and Strydom, 1973), the post-application temperature and variations between seasons in chilling temperature accumulation (Erez, 1979, 1987), are probably factors influencing the observed results.

Chemicals which have been used commercially to break dormancy in various places of the world are: mineral oils, dinitro compounds, potassium nitrate, thiourea, cyanamides, and a mixture of N-(phenylmethyl)-1H-purin-6-amine (BA) and GA_{4+7} (Promalin) (Erez, 1987). Among them, hydrogen cyanamide, has been found to be especially valuable in overcoming rest of several species of deciduous fruit

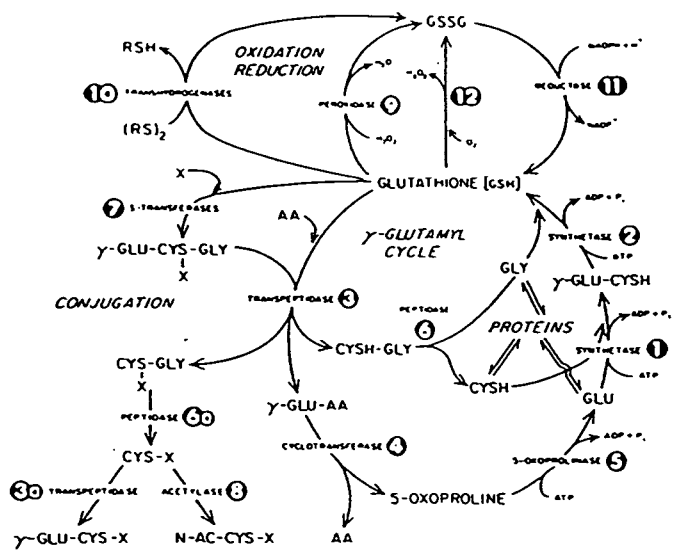
trees (Erez, 1987; Nee, 1986; Shulman et al., 1983; Snir, 1983; Snir and Erez, 1988).

The effective concentration and timing of hydrogen cyanamide to break rest varies with time, physiological stage of the bud, and genotype (Bracho et al, 1984; Erez, 1987; Nee, 1986; Fuchigami and Nee, 1987). This situation is further complicated by the interaction of the chemical with the climatic conditions at and after the treatment. It seems that cool conditions may enhance damage in peaches and plums (Erez, 1987). Presently, the relation between the developmental stage of the buds and their response to hydrogen cyanamide is not clear in most crops. In grapevines, a considerable amount of information on timing hydrogen cyanamide application is available. Thus, the material is commercially used in some countries such as Israel and United States (Lavee et al., 1984; Shulman et al., 1983; Bracho et al., 1984; Jensen and Bettiger, 1984). However, these data are not based on physiological development of the buds which were found critical for the response to dormancy breaking agents (Fuchigami and Nee, 1987; Nee, 1986). Thus, frequent unfavorable or inactive results were reported (Jensen and Bettiger, 1984).

2.5. GLUTATHIONE/STRESS RESPONSE

2.5.1. Metabolism. Glutathione is synthesized intracellularly (Figure 1) by the consecutive actions of γ-glutamylcysteine synthetase (Reaction 1) and GSH synthetase (Reaction 2). Reaction 1 is feedback inhibited by GSH (Meister and Anderson, 1983). The degradation of glutathione is catalized by γ-glutamyl transpeptidase, which catalizes transfer of the γ-glutamyl moiety to acceptors-amino acids, e.g. cystine, glutamine, and methionine, certain dipeptides, water and GSH itself- (Reaction 3). The γ-glutamyl amino acids are substrates of γ-glutamyl cyclotransferase (Reaction 4), which converts these compounds

into the corresponding amino acids and 5-oxo-L-proline. The ATP dependent conversion of 5-oxo-L-proline to L-glutamate is catalized by the intracellular enzyme 5-oxo-prolinase (Reaction 5). The cysteinylglycine formed in the transpeptidase reaction is split by dipeptidase (Reaction 6). These six reactions constitute the γ-glutamyl cycle, which thus accounts for the synthesis and degradation of glutathione (Meister and Anderson, 1983).



Overall summary of glutathione metabolism (see text): Reaction 1, γ-GLU-CYSH synthetase; Reaction 2, GSH synthetase; Reaction 3 and 3a, glutamyl transpeptidase; Reaction 4, γ-glutamyl cyclotransferase; Reaction 5, 5-oxoprolinase; Reactions 6 and 6a, dipeptidase; Reaction 7, GSH S-transferase; Reaction 8, *N*-acetylase; Reaction 9, GSH peroxidase; Reaction 10, transhydrogenases; Reaction 11, GSSG reductase; Reaction 12, oxidation of GSH by O_2; conversion of GSH to GSSG is also mediated by free radicals.

Fig. 2.1 The pathway of glutathione synthesis and degradation. (Meister and Anderson)

Two of the enzymes of the cycle also function in the metabolism of S-substituted GSH derivatives, which may be formed nonenzymatically by reaction of GSH with certain electrophilic compounds or by GSH-S-transferases (Reaction 7). The γ-glutamyl moiety of such conjugates is removed by the action of γ-glutamyl transpeptidase (Reaction 3), a reaction facilitated by γ-glutamyl amino acid formation. The resulting S-substituted cysteinylglycines are cleaved by dipeptidase (Reaction 6a) to yield the corresponding S-substituted cysteines, which may undergo N-acylation (Reaction 8) or an additional transpeptidation reaction to form the corresponding γ-glutamyl derivative (Reaction 3a).

Intracellular GSH is converted to GSSG by selenium-containing GSH peroxidase, which catalyzes the reduction of H_2O_2 and other peroxides (Reaction 9). GSH is also converted to GSSG by transhydrogenation (Reaction 10). Reduction of GSSG to GSH is mediated by the widely distributed enzyme glutathione reductase which uses NADPH (Reaction 11). Extracellular conversion of GSH to GSSG has also been reported, the overall reaction requires O_2 and leads to formation of H_2O_2 (Reaction 12). GSSG is also formed by reaction of GSH with free radicals.

2.5.2 Functions. Glutathione (γ-L-glutamyl-L- cysteinyl-glycine) is a low molecular weight (MW) thiol compound implicated in a wide range of metabolic processes (Meister and Anderson, 1983). Functions proposed for glutathione in higher plants include: storage and transport of reduced sulfur; protein reductant; destruction of H_2O_2 in chloroplasts, and detoxification of xenobiotics including certain herbicides and pesticides (Rennenberg, 1982). It is also implicated in the adaptation of plants to environmental stresses such as low temperature and drought (deKok and Oosterhuis, 1983; Gamble et al., 1984). Overall, glutathione appears to play a key role in protection against a oxidative damage arising from a number of stresses.

It is reasonable to assume that chloroplasts are a primary site for cellular lipid degradation, because, in these organelles, approximately one half of the thylakoid dry weight is composed of lipids and lipid soluble compounds (Park and Pon, 1963). Not surprisingly, green plants possess quenching mechanisms, concentrated in the chloroplast, that can reduce the potentially destructive effects of these toxic oxygen species. About 30 to 40% of the cellular ascorbate (Foyer et al., 1983; Gillham and Dodge, 1986), 10 to 50 % of the glutathione (Smith et al., 1985), all of the α tocopherol (Breke, 1968; Grumbach, 1983), and all of the B carotene (Grumbach, 1983) are localized in the chloroplast. Ascorbate and glutathione are both water-soluble antioxidants involved in the removal of such photosynthetically generated oxidants as $O_2^{\cdot}$ and H_2O_2 (Halliwell, 1984). α Tocopherol and B carotene are located within the membrane and, as such, probably directly quench lipid-peroxy radicals, the Chl triplet state, or O_2 in the thylakoid membrane (Grumbach, 1983; Foote et al., 1978).

One of the principal mechanisms by which oxidants are consumed in plant cells depends on reduced glutathione (Rennenberg, 1982; Smith et al., 1984; Schmidt and Kunert, 1986). For this reason, interest in how glutathione metabolism is altered by different stresses has increased. The information released, suggest that glutathione may play a role in mediating the response of plant cells to biological as well as physical stress.

2.5.3. Analysis. The widespread distribution of glutathione and its apparent involvement in a multitude of biological functions (Alscher, 1989; Meister and Anderson, 1983; Rennenberg, 1982) have generated a continual interest in methods of analysis of this cellular component ever since its discovery and isolation (Hopkins, 1929).

Plant cells contain considerable amounts of free, low MW thiol, and its generally assumed that GSH is a major component of this

fraction; however, most of the early investigations performed relied upon thiol titration of deproteinized extracts in which only the free thiol content was measured, although the determination of GSH was claimed. An unequivocal proof by rigorous chemical methods that GSH is indeed the component determined has only been achieved for a few plant species (Kasai and Larson, 1980).

In contrast, accurate measurement of GSSG levels (oxidized form of glutathione) have proved more difficult, both because of the much lower amounts of this form normally present within the cells and because of the absence of a convenient chemical feature such as that possessed by the reduced peptide. Procedures for the estimation of the oxidized form have, therefore, relied generally on its estimation as GSH following its chemical (Woodward and Fry, 1939), electrolytic (Dohan and Woodward, 1939), or enzymic (Tiezte, 1969) reduction.

An HPLC method for thiols and disulfides which utilizes a dual electrochemical detection was recently validated in biological samples, but, no woody plant material was included (Richie and Lang, 1987). This procedure gave a higher detector response for thiols than disulfides and the life span of the dual electrode was limited to 200 - 500 samples depending on the total SH/SS analyzed.

A rapid and sensitive HPLC method for nanomole levels of glutathione, glutathione disulfide, and cysteine-glutathione mixed disulfides was described for biological samples (Reed et al., 1980). Recently, a modification of this method was evaluated in animal tissues (Fariss and Reed, 1987). This procedure utilized S-carboxy methylation of compounds followed by derivatization of amino groups with Sanger's reagent. The method has been applied for evaluation of thiols and disulfides in corn roots subjected to heat stress (Nieto-Sotelo and Ho, 1986); and in callus cultures exposed to heavy metals (Podhradsky et al., 1990). The possibility of applying a similar method to woody plant tissues is not known.

2.5.4. Responses to stresses. Heat Shock. Organisms respond to heat stress by inducing or enhancing the expression of a set of genes encoding heat shock proteins (hsp) (Schlesinger, et al., 1982). A correlation between the induction of hsp synthesis and increased resistance to supraoptimal temperatures has been found in various organisms (Atkinson and Walden, 1985). Another feature of the heat shock response is that it can also be induced by chemicals such as arsenite, arsenate, mercury ions, and other sulfhydryl reagents (Ashburner and Bonner, 1979; Lin et al., 1984; Schlesinger, 1985). These observations suggest that thiols may play a significant role during the heat shock response.

Recently, Mitchell et al (1983) presented evidence that the tripeptide glutathione increased during heat shock and may be important in the early response to thermal stress. They demostrated that Chinese hamster V79 cells exposed to buthionine sulfoximine, a specific inhibitor of GSH synthesis, or diethylmaleate, an agent that binds GSH, reduced the intracellular GSH levels and increased the thermal sensitivity of the cells at heat shock temperatures.

In plants, it has been shown that the levels of GSH in maize roots increase during heat stress (Nieto-Sotelo and Ho, 1986). Treatment of maize roots to heat shock temperatures of 40^{0}C resulted in a decrease of cysteine levels and an increase of GSH levels. Based on labeling experiments using [^{35}S]cysteine, they demostrated that the elevation of GSH levels is due, at least in part, to an increased rate of synthesis of GSH during heat shock.

The possibility exists that GSH elevation during heat shock reflects an accumulation of H_2O_2 or related oxygen radicals. Heat shock has been proposed to be an oxidative stress (Lee et al., 1983). Recently, it was suggested that the possible role of GSH under heat shock may be the protection against $O_2^{\cdot}$ damage (Freeman et al., 1985), therefore, the elevated synthesis of GSH may be related to the cells

capacity to cope with heat stress conditions (Nieto-Sotelo and Ho, 1986).

Low Temperature. Sulfhydryl groups of proteins and peptides have been suggested as a factor in tolerance of freezing stress of plants (Levitt et al., 1961; Schmuetz et al., 1961). Levitt (1972) proposed that freezing injury was caused by conformational changes of protein due to oxidation of protein sulfhydryl groups by freeze-dehydration. In frost tolerant tissue intermolecular SS bonding would be prevented. It has been suggested that a sulfhydryl compound such as glutathione may increase freezing tolerance of plants by protection against such oxidation (Levitt, 1972; Asahira and Tanno, 1963).

Alterations in glutathione metabolism with large increases in the glutathione content in plant tissues exposed to low temperatures have been demostrated (deKok and Oostethuis, 1983; Esterbauer and Grill, 1978; Guy and Carter, 1982). The increase was most dramatic for reduced glutathione (GSH), but was also observed for oxidized glutathione (GSSG) and protein-bound glutathione (PSSG). In addition, increases in glutathione reductase (GR) activity of crude extracts from plant tissues exposed to low temperatures were demostrated (deKok and Oosterhuis, 1983; Esterbauer and Grill, 1978; Guy and Carter, 1984).

In spruce needles the glutathione content and the glutathione reductase activity undergoes seasonal changes with higher concentrations in the winter and early spring and low concentrations during the summer (Esterbauer and Grill, 1978).

Enhanced content of water soluble non-protein sulfhydryl compounds was observed in frost-hardened spinach leaves (deKok and Oosterhuis, 1983). The enhancement was due to higher levels of GSH as well as to other non-protein -bound sulfhydryl compounds. Also, increased activity of glutathione reductase (GR) upon hardening have been reported (Guy and Carter, 1984). GR from hardened spinach has

greater stability against freezing and a higher affinity for substrates at low temperature than does GR from non hardened spinach (Guy and Carter, 1984). The large changes in GR activity and GSH concentrations at low temperatures suggest that cold acclimation may alter the enzyme so as to make it function more efficiently with greater stability at low temperature (Guy and carter, 1984).

Studies in which the GSH content was evaluated in relation to freezing tolerance showed that an increased GSH content did not result in a higher frost-tolerance. Evidence presented indicates that GSH accumulates in plant tissues exposed to low temperatures but that GSH accumulation is not associated with freezing tolerance (Guy et al., 1984; deKok et al., 1981).

Heavy metals. In both animal and plant cells the presence of metal-binding proteins has unequivocally been proven (Robinson and Jackson, 1986). These compounds have been named metallothioneins (MT) and are characterized by their low MW, their heat stability, an unusually high cysteine content, and a high affinity for certain metal ions.

Recently, a new class of MTs, the so-called phytochelatins (PCs), a group of peptides capable of heavy metal ion detoxification, have been discovered in plants (Grill et al., 1985). The structure of these compounds, i.e. (γ-glutamyl- cysteinyl)n -glycine, strongly suggest that these peptides are not encoded by structural genes, but are the product of a biosynthetic pathway in which glutathione and/or its metabolites are involved.

In cells of tomato suspension cultures, selected for Cd^{2+} resistance, phytochelatins accumulated in the presence of Cd^{2+}. Buthionine sulfoximine, a specific inhibitor of γ-glutamyl-cysteine synthetase, prevented accumulation of PCs and caused Cd^{2+} sensitivity of the resistance cell line (Steffens et al., 1986). These data clearly showed that PCs can represent the primary mechanism for metal

detoxification in plant cells. In addition they showed, that GSH or γ-glutamyl-cysteine synthetase are essential precursors of PCs. This was also indicated by the observation of a transient decline of the GSH content during the initial synthesis of PCs upon addition of metal ions. During prolonged exposure to metal ions, the GSH content recovered (Steffens et al., 1987).

Oxygen Concentration. In higher plants, O_2 is a product of photosynthesis, a necessary reactant in physiological process, and the terminal electron acceptor in light independent mitochondrial respiration. Metabolism of O_2 is initiated by a univalent reduction to superoxide free radical; dismutation of superoxide anions catalyzed by superoxide dismutase results in formation of H_2O_2 and O_2 (Asada et al., 1977; McCord and Fridovich, 1969), and concludes with the ultimate reduction of H_2O_2 (Asada et al., 1977; Foyer and Halliwell, 1976; Groden and Beck, 1979). This cycling of O_2 leads to no net evolution or uptake but results in a transport of electrons or oxygen intermediates to locations removed from the immediate site of potential oxidative damage.

Intermediate reduction states of O_2 are associated with respiratory chain activity and glycolate metabolism and may impair cellular metabolism through inactivation of enzymes by oxidation of critical thiol groups, production of other reactive O_2 species which may react with cellular components, or diversion of normal metabolic pathways (Halliwell, 1982).

The ability of photosynthetic cells to tolerate these potentially toxic effects of oxidative damage has been proposed to depend upon the properties of various chemicals which are found in these organisms, including glutathione, ascorbate, pigments, and enzymes (Foyer and Halliwell, 1976; Groden and Beck, 1979; Malstrom et al., 1975; Wolosiuk and Buchanan, 1977).

Evaluations in leaf tissue from intact cotton plants which were

exposed to 75% O_2, and 350 microliters per liter of CO_2 for 48 hours demonstrated that glutathione reductase activity was 2 to 3 fold higher compared with cotton leaf tissue under normal conditions of 21% O_2 (Foster and Hess, 1980). The observed increase in glutathione reductase may reflect increased GSH turnover in tissue exposed to 75% O_2. Thus, the flux of H_2O_2, a product of superoxide dismutation, could be more readily accommodated in the stressed plant. Three other enzymes namely superoxide dismutase, glycolate oxidase, and catalase did not change in response to hyperoxic conditions (Foster and Hess, 1980). In leaves from corn plants exposed to high oxygen concentrations increased activity in glutathione reductase was also reported (Foster and Hess, 1982). The authors proposed that increases in glutathione reductase activity in response to elevated oxygen concentrations suggest a promiment role for this enzyme in the protection of leaf tissue against oxidative damage.

Water Stress. The midday closure of stomates in response to water stress may optimize the water use efficiency of the plant on a daily basis (Cowan and Farguhar, 1977), yet result in a reduction in CO_2 assimilation at times when peak irradiances are commonly encountered (Turner et al., 1978). Under this condition of reduced CO_2 fixation, oxygen can function as an alternative electron acceptor (Elstner et al., 1978).

Although this pseudocyclic pathway for electron transport provides additional ATP, it can result in the production of superoxide and H_2O_2. Superoxide and H_2O_2 can react with chloroplast components to form even more reactive oxygen products such as the hydroxyl radical, and singlet oxygen (Elstner et al., 1978; Takahama, 1979). These toxic products produced within the chloroplast must be effectively removed to prevent lipid peroxidation, inhibition of CO_2-fixation, and the photooxidation of chloroplast pigments.

Studies with Vigna seedlings subjected to increased degrees of

water stress showed an approximately proportional increase in glycolate oxidase activity, hydrogen peroxide and proline content, but a decrease in catalase activity, ascorbic acid and protein content (Mukherjee and Choudhuri, 1983). Pretreatment with L-cysteine and reduced glutathione, slightly improved the water status of leaves stressed for 2 days. The authors suggested that treatment with antioxidants made the plant more tolerant to water stress by modulating the endogenous levels of H_2O_2 in stressed tissue.

The effect of water stress on glutathione reductase and catalase activity and the importance of canopy position on the activity of these enzymes were evaluated in leaf blades of field grown winter wheat (Gamble and Burke, 1984). Wheat was sown at 2 seeding rates under both irrigated and dryland conditions. Flag leaves from dryland plants at the lower density showed no change in either enzyme content per unit leaf area, while leaves from the basal portion of the canopy exhibited at 273% and 60% increase in GR and catalase activity, respectively. GR activity in dryland plants seeded at the higher density increased 25% in flag leaves and 225% in basal leaves. No change in catalase activity was observed in either flag or basal leaves of the same plants. The authors suggested that the increases in GR activity in water stressed wheat plants may protect the chloroplast against damaging oxygen products (Gamble and Burke, 1984). This increase in enzyme activity may constitute an adaptive response of wheat plants to low water potentials, either by ensuring the availability of NADP to accept electrons derived from photosynthetic electron transport, thereby directing electrons away from oxygen and minimizing the production of superoxide (Foster and Hess, 1982), or by functioning in the removal of H_2O_2 generated within the chloroplasts via an ascorbate-glutathione cycle in which ascorbate peroxidase and glutathione reductase are key enzymes (Foyer and Halliwell, 1976; Halliwell, 1982).

Sulfur Dioxide. In the field plants may be exposed over a long period to an atmosphere contaminated with sublethal concentrations of SO_2. The mechanism by which extended exposure to SO_2 exerts phytotoxicity on the biochemical level is poorly understood (Ziegler, 1975). However, recent studies on the mechanism of SO_2 injury in plants indicates damage of plasma membrane (Shu-wen et al., 1982). They concluded that injury by SO_2 is closely related to the peroxidation of membrane lipids.

It is known that the SO_2 taken up through the stomata is oxidized in the plant tissue to sulphate which is finally utilized by the normal sulfur metabolism (Rennenberg, 1984). Peroxidation of membrane lipids could be induced by free radicals which may be generated during the oxidation of sulphite to sulphate (Shu-wen et al., 1982).

When sulfur is present in excess, the glutathione pool(s) of leaf cells can be expanded (Rennenberg, 1982; 1984). Needles of *Picea abies* growing in SO_2-polluted areas contain twice to five times as much glutathione as needles of trees in relatively unpolluted areas (Grill and Esterbauer, 1973). In SO_2-affected leaves of *Picea, Pinus, Larix and Betula* evaluated for total water extractable SH compounds, increase by a factor of 4 to 6 with increasing contamination of the air from SO_2 was found, with glutathione as the most abundant SH compound (Grill et al., 1979). They assumed that the chronic increase in the level of GSH in SO_2 affected leaves changes the natural balance of the SH/SS in the cell and thereby induces a general disruption of fundamental cellular processes.

As a measure of the effect of GSH on metabolism, the relationship between cellular GSH levels and respiration rates on *Picea abies* indicated that the rate of respiration was affected by the level of foliar GSH (Pfeifhofer et al., 1986). At 37^0 the rate of respiration increased with increasing water-soluble sulfhydryl, whereaS AT 25^0C no relationship was observed. Higher thiol

concentrations inhibited the rate of respiration. They concluded that the mechanism by which SO_2 affect plants is temperature dependent.

Ozone. In areas of high motor-traffic density, intense sunlight and stagnant air, the primary pollutants released from exhausts are photochemically converted to an array of new compounds. The principal components of the oxidants in the mixture are ozone and peroxyacyl. Plant damage by these oxidants is very well documented (Mudd, 1982).

What is not known is the mechanism by which these pollutants cause lesions on leaves. The literature on exposure of biological materials to ozone frequently mentions peroxidation as responsible for the damage observed. Evidence that ozone induce lipid peroxidation has been presented (Punyor et al., 1981). Although the reaction of ozone with unsaturated lipids as a basis for ozone toxicity is appealing, the body of evidence does not clearly support such a hypothesis.

One of the most susceptible enzymes to ozone is glyceraldehyde-3-phosphate dehydrogenase (G3PD), an enzyme well known for its susceptibility to reagents which react with thiols. The decrease in the catalytic activity of this enzyme after exposure correlates well with the loss of thiol groups. The active-site thiol of G3PD is more susceptible to ozone than the thiol of GSH (Mudd, 1982).

Oxidation of GSH by ozone generates mainly disulphide, and biological systems could readily re-reduce such a bond, e.g. with excess thiol. In mammalian red blood cells, treatment of erythrocytes with ozone did not change the activities of several intracellular enzymes such as glucose-6-phosphate dehydrogenase, glutathione reductase and 6-phosphogluconate dehydrogenase. However, the activity of the pentose phospate pathway was stimulated several-fold (Freeman et al., 1979). Assay of GSH showed that it was oxidized by the ozone exposure. The authors concluded that the increase in pentose phosphate pathway activity was a result of the oxidation of GSH.

Antioxidants such as GSH and ascorbate have been considered as naturally occurring compounds which might prevent or reverse the effects of ozone (Barnes, 1972). Resistance of petunia plants to ozone damage was correlated with ascorbate (Hanson et al., 1971), but this was not with the resistance of tobbaco plants (Menser, 1964). Chang (1971) has shown the analogy between ozone induced breakdown of chloroplast polysomes and that caused by p-mercuribenzoate, implying that the effect of ozone is mediated by oxidation of sulphydryl groups. If proteins thiols are oxidized in the plastid after exposure of the leaf to ozone, one would expect that some of the GSH would be oxidized.

Light Quality and Quantity. Light provides the fundamental source of energy which directly or indirectly drives the life processes of nearly every living organism. Light quality, intensity, and duration are all vital and exert marked formative effects on plant growth, development, differentiation, and reproduction (Larcher, 1975). In the plant, radiation acts as the source of energy for photochemical reactions and as the stimulus regulating development, but it can also cause injury.

At high light intensities and when the supply of CO_2 is limiting superoxide and peroxide can be formed by the reduction of molecular oxygen in the Mehler reaction within the chloroplast (Halliwell, 1982). Recent evidence suggest that a pathway operates in illuminated chloroplast in which H_2O_2 is detoxified by reduction with donation of electrons from reduced ferredoxin to $NADPH_2$ to GSH/GSSG to dehydroascorbate/ascorbate to H_2O_2/H_2O (Halliwell, 1984). The enzymes involved are ferredoxin-NADP reductase, glutathione reductase, dehydroascorbate reductase, and ascorbate peroxidase.

The changes in GSH/GSSG ratio on illumination, indicate an active participation of GSH in redox reactions in illuminated chloroplasts, presumably related to ascorbate-mediated reduction of peroxide (Foyer

et al., 1983). Because GSH is required at high light intensities, it may be assumed that the GSH content of leaf cells is likely to undergo diurnal changes. In spruce needles, the glutathione content undergoes a light dependent but temperature independent diurnal rhythm with high concentrations during midday and low concentrations during the night (Schupp and Rennenberg, 1988). The authors concluded that the diurnal variation in the GSH content of spruce needles was due to changes in the export of GSH out of the cell. In Tomato seedlings, levels of reduced glutathione were found to vary depending on the time of the day. The level was lowest around the middle of the dark period, and highest around the middle of the light period (Koike and Patterson, 1988). The possibility of a link between changes in reduced GSH and reported diurnal variation in herbicide action was discussed.

If GSH shows diurnal rhythm, it is reasonably to assume that different parts of the plant will show the same trend. In pea plants, total GSH levels were highest in apex and expanding leaves, and lower in older leaves and roots (Bielawsky and Joy, 1986). The state of reduction of GSH, e.g., highest in the apex; may be necessary to permit active protein synthesis, which appears to be inhibited by GSSG (Fahey et al., 1980).

High levels of GR on illumination, indicate an active participation of GSH in redox reactions in illuminated chloroplasts, presumably related to ascorbate-mediated reduction of peroxide (Foyer and Halliwell, 1976). Changes in light quality and quantity within a plant canopy may affect the level of glutathione reductase activity. Light quality and quantity have been shown to influence the level and distribution of glutathione reductase in wheat (Gamble and Burke, 1983) and mustard seedlings (Drumm and Mohr, 1973). Increases in GR activity from the base of the primary blade to the tip of the blade in greenhouse grown plants and etiolated plants have been reported (Gamble and Burke, 1983).

Detoxification of Xenobiotics. Many plants including important agronomic, horiculture, and weed species form conjugates of glutathione with a broad range of intracellularly produced or extracellularly applied electrophilic substances. In the living cells, this process appears to be catalyzed by a group of enzymes named glutathione-S-transferases (GST) (EC 2.5.1.18) (Lamoureux and Rusness, 1987). The reactions catalyzed by this group of enzymes can apparently prevent toxic effects of many pesticides in plants.

Conjugation of glutathione with atrazine in atrazine resistant in contrast to atrazine susceptible species is one of the best documented examples of herbicide selectivity in plants based on metabolic differences (Rennenberg, 1987). Atrazine, an inhibitor of photosynthetic electron transport, inhibits photosynthesis in both resistant (corn, sorghum, sugar cane) and susceptible (barley, oats, pea, wheat) species, but photosynthesis is restored in resistant species upon conjugation of glutathione with atrazine. High atrazine metabolism occurs in resistant species containing high GST activity; little atrazine metabolism is observed in susceptible species containig low GST activity. Also the selectivity of several other herbicides was found to be due to rapid conjugation with glutathione (Lamoureux and Rusness, 1987).

Many herbicides can cause injury to a crop at dosages needed to sufficiently control weeds. This case of selectivity can in some cases be overcome by the addition of compounds called antidotes, protectants, or safeners. These compounds do not reduce herbicide injury to weeds, while decreasing herbicide injury to crops. Thus selectivity is transferred from the herbicide to the mode of action of an antidote. Analysis of the mechanisms responsible for the protection of plants from herbicide injury by antidotes showed many examples of an antidote elevated glutathione-S-transferase activity (Rennenberg, 1982; Lamoureux and Rusness, 1987; Zama and Hatzios, 1986). On the

other hand, synergestic effects in pesticide toxicity could be attributed to an inhibition of glutathione conjugation at the level of glutathione-S-transferase (Lamoureux and Rusness, 1987).

In order to stimulate conjugation of glutathione with herbicides, enhanced glutathione synthesis is needed in addition to enhanced glutathione-S-transferase activity. As glutathione appears to be a feedback inhibitor of its own synthesis (Rennenberg, 1982; Meister and Anderson, 1983) it might be assumed that its removal by conjugation will sufficiently enhance its production. Studies with several antidotes, however, did not shown only an enhanced glutathione level of the antidote treated cells, but also an enhanced activity of glutathione synthesis in vitro (Lamoureux and Rusness, 1987).

Conclusions. Evidence is accumulating that glutathione plays an important role in antioxidant protection mechanisms in plant cells. Its participation in oxyradical scavening in the chloroplast is well characterized, although is importance in the cytosol and in heterotrophic tissue is becoming increassingly apparent. The capacity of plant tissue to respond to an oxidative stimulus with the production of more protective molecules such as reduced glutathione is known to be an important part of metabolic stress resistance. Taken together these observations suggests that glutathione may play a role in mediating the response of plant cells to biological as well as physical stresses. The recent discovery of the role of reduced glutathione as an elicitor of transcriptional events associated with stress resistance mechanisms (Wingate et al., 1988) opens up a new an intriguing connection between antioxidant function and the control of gene expression. Combined efforts of plant physiologists and plant molecular biologists are needed to have a better understanding of the physiological role of glutathione in plant cells.

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3.0 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF REDUCED AND OXIDIZED GLUTATHIONE IN WOODY PLANT TISSUES.

3.1 ABSTRACT

A rapid, and sensitive high performance liquid chromatography method for quantifying both the reduced and oxidized forms of glutathione simultaneously in woody plant tissues is reported. Samples were extracted in 10% perchloric acid with 1 mM bathophenanthroline disulfonic acid, homogenized, and centrifuged. The supernatant was carboxy-methylated with iodoacetic acid, and derivatized with 2,4-dinitro-1-fluorobenzene. A 3-aminopropyl-Spherisorb column was used for separations. Dinitrophenol derivatives were detected at 365 nm. The retention times were 13.9 min and 15.1 min and the lowest limits of detection were 10 pmol and 5 pmol for reduced and oxidized glutathione, respectively.

This HPLC method was applicable to a variety of woody plant species and different tissues. Total glutathione content in the bark tissue of seven of the woody plant species tested were lower during the period of active plant growth, and higher during the dormant stage. Such change was not observed in grape. Reduced glutathione always represented a high percentage of the total glutathione. All tested tissues of peach plants contained glutathione. The highest and lowest levels were found in the leaves and roots, respectively. Both cherry and sugar pine seeds contained high levels of reduced glutathione.

3.2 INTRODUCTION

Glutathione (γ-glutamyl-cysteinyl-glycine) is a tripeptide product of primary metabolism. Most living cells from microorganisms to man contain this tripeptide (Kosower 1976, Meister and Tate 1976, Meister and Anderson 1983, Waley 1966). Glutathione is present as a reduced (GSH) and oxidized (GSSG) form. Its widespread distribution and apparent involvement in a multitude of biological functions (Alscher 1989, Rennenberg 1987, Rennenberg 1982) generate continual interest. Methods of analysis of this cellular component keep improving.

The concentration of glutathione in plants cells is modified by developmental and environmental factors (Earnshaw and Johnson 1987, Esterbauer and Grill 1978, Nieto-Sotelo and Ho 1986, Schupp and Rennenberg 1988, Smith 1985, Smith et al. 1984). Roles of thiol containing compounds on regulation of dormancy status in woody plants have been proposed (Fuchigami and Nee 1987). However, the role of sulfhydryl compounds in the growth and development of plants is not well understood.

Quantification of both reduced and oxidized glutathione is difficult. Early investigations measured reduced glutathione by thiol titration of deproteinized extracts (Tietze 1969). Determination of GSH was then inferred. It is generally assumed that GSH is a major component of non-protein thiols in plant tissues (Rennenberg 1982). An unequivocal proof that GSH is the component determined is shown in only a few plant species (Kasai and Larson 1980). The measurement of GSSG levels is even more difficult, because this form is normally less concentrated in the cells. Methods for direct measurement of GSSG are cumbersome. Procedures for the estimation of the oxidized form, require converting GSSG to GSH by chemical (Woodward and Fry 1932),

electrolytic (Dohan and Woodward 1939), or enzymatic (Tietze 1969) reduction.

An HPLC method, specific for both thiols and disulfides by dual electrochemical detection, was described and validated in biological samples (Richie and Lang 1987). However, this procedure gave a higher detection response for thiols than disulfides and the life span of the dual electrode was limited to 200-500 samples depending on the total thiol/disulfide analyzed (Richie and Lang 1987).

A rapid and sensitive HPLC method for detecting nanomole levels of GSH, GSSG, and cysteine-glutathione mixed disulfides was described in biological samples (Reed et al. 1980). Recently, this method was evaluated in animal tissues (Fariss and Reed 1987). This procedure utilized S-carboxy methylation of compounds followed by derivatization of amino groups with Sanger's reagent. This method has been applied to the evaluation of thiols and disulfides in corn roots subjected to heat stress (Nieto-Sotelo and Ho 1986); and callus cultures exposed to heavy metals (Podhradsky et al. 1990). The possibility of using a similar method to determine thiols and disulfides in woody plant tissues is not known.

The objectives of the study included: (i) to develop an HPLC method to quantify simultaneously GSH and GSSG in woody plant tissues, (ii) to quantify GSH and GSSG in different woody plant species at different times of the year and in different plant tissues.

3.3 MATERIALS AND METHODS

Plant material. The plant species used were: peach (*Prunus persica* L. cv. Redhaven); cherry (*Prunus avium* L. cv. Bing); prune (*Prunus domestica* L. cv. Brooks); apple (*Malus domestica* L. cv. Golden Delicious); pear (*Pyrus communis* L. cv. Bartlett); grape (*Vitis vinifera* L. cv. Pinot Noir); hazelnut (*Corylus avellana* L. cv. Barcelona); and blueberry (*Vaccinium corymbosum* L. cv. Stanley). Shoots from one-year old growth of field grown plants were harvested on June 13, and on Dec 13, 1990. One gram fr wt of bark tissue from each shoot (4 replicates) was collected for HPLC analysis. An additional 1.0 gram fr wt of bark tissue from each was collected for dry wt determinations.

One year old peach plants (*Prunus persica* L. cv. Lovell), grown outside in one gallon containers with a soil mixture of loam soil, washed cement sand, canadian peat moss, and white pumice rock (1:1:1:2 v/v) were used to study glutathione levels in several tissues. Samples (1.0 gram fr wt) of leaves, bark, buds, and roots were collected on Aug 27, 1989.

Other plant materials included in the study were seeds of cherry (*Prunus avium* L. cv. Lambert), and sugar pine (*Pinus lambertiana* L.). Seed coats were removed. The coatless seeds were soaked in double distilled water for 24 hrs at 23 ± 2^0 C in the dark. Seeds were blotted dry with paper towels, weighed, immediately immersed in liquid nitrogen and then stored in a -70^{0}C freezer.

Reagents. GSH, GSSG, cysteine, γ-glu-glu, bathophenanthroline disulfonic acid (BPDS), iodoacetic acid, iodoacetamide, KOH, $KHCO_3$, m-cresol -purple and 2,4-dinitro-1-fluorobenzene (DNFB) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); glacial acetic acid (HPLC grade), and perchloric acid (PCA) were from J.T. Baker Inc.

(Phillipsburg, New Jersey, U.S.A.). HPLC-grade methanol was from Mallinckrodt, Inc. (Paris, Kentucky, U.S.A.). Only double-glass-distilled, deionized water was used.

Extraction. Samples were removed from the freezer, placed in liquid nitrogen, pulverized to a fine powder with a mortar and pestle, and quickly transferred to 4 ml of an aqueous solution containing 10% PCA and 1 mM of BPDS. BPDS is a water soluble phenanthroline derivative with excellent iron and copper chelating properties. BPDS prevents thiol oxidation and thiol disulfide interchange and does not interfere with the quantification of the oxidized form of glutathione (Fariss and Reed 1987). The extracts were homogenized in a Teckmar Tissumizer (output set to 60) with 3 pulses of 20 sec each. The homogenates were centrifuged at 7,800 xg for 20 min at 4^0C, and the supernatant collected. The pellet was re-suspended in 4 ml of 10% PCA containing 1 mM BPDS, homogenized, centrifuged and the supernatant collected as above. The procedure was repeated once more and the supernatants combined. The final volume of the PCA extract was measured and used as a dilution factor in the calculations.

Derivatization. A portion of the resulting PCA extract (0.5 ml) was added to 50 μl of 0.5 mM γ-glu-glu, an internal standard. The samples were then carboxy-methylated with 50 μl of 100 mM iodoacetic acid dissolved in 0.2 mM m-cresol purple solution. The acidic solution was brought to pH 9-10 by the addition of 0.48 ml of KOH (2 M)-$KHCO_3$ (2.4 M) mixture, and incubated in the dark for 15 min. After complete carboxy-methylation, the samples were derivatized with 1 ml of 1 % DNFB and kept in the dark at 4^0C over night (Fariss and Reed 1987). The following day, the derivatized samples were centrifuged at 13,000 xg for 15 min, and filtered through a 0.2 μm Nylaflo filter (Gelman Sciences Inc., Ann Arbor, Michigan, U.S.A.) before injection.

Preparation of Standards. Stock solutions of standards were prepared as follows: 5 mM GSH, 5 mM GSSG, and 5 mM Cys were added to

10% PCA containing 1 mM BPDS. Stock solution for the internal standard γ-glu-glu was 5 mM in 0.3% PCA. The stock solutions were kept frozen at -20°C. Working standards were prepared on the day of use. Standard curves were obtained for each compound within the range from 1.25 μM to 325 μM (from 10 pmol to 3 nmol per injection). The estimated content of each compound was based on the ratio of the peak area of each standard to the peak area of the internal standard.

Recovery experiments. Recovery experiments were done for each sample that was analyzed. The reliability of each determination was checked by adding a 0.5 nmol GSH standard to a second sample of the plant material prior to extraction. To determine the recovery rate, the level of the GSH standard, plus the level in the plant sample, were substracted from the GSH level in the spiked plant sample.

High-Performance Liquid Chromatography. Separation and quantification were carried out in a Beckman Gold HPLC system consisting of two 110B pumps, an Analog Interface 406, and a programmable UV detector 166, coupled to a Gold system software computer package for controlling the HPLC, data acquisition and integration. Samples were injected thru a model 210 injector valve with a 100 μl loop. A 3-aminopropyl- Spherisorb column (4.6 x 200mm, 5 μm) (Custom, L.C. Inc. Houston, Tx., U.S.A.) was operated at ambient temperature with a flow rate of 1.5 ml/min. Absorbance of the dinitrophenol derivatives was monitored at 365nm.

Elution was carried out according to Fariss and Reed (1987) with minor modifications. The solvents were prepared fresh on the day of use, filtered thru 0.45 μm Nylon-66 filters, and degassed under vacuum. The solvents utilized were as follows: mobile phase A, 80% methanol; mobile phase B, 0.5 M sodium acetate in 64% methanol. The sample (250 μl) was injected into the column and the mobile phase was maintained at 80% A, and 20% B for 5 min, followed by a 5 min linear gradient to 1% A, and 99% B and was held at those conditions until the

final compound GSSG (≈16 min) had eluted. Then, the mobile phase was returned to the initial conditions (80% A, and 20% B) over a 5 min linear gradient , and maintained there for 20 min for re-equilibration of the column before the next injection. Total running time for each sample including re-equilibration was 40 min. Prior to the first injection each day the column was stabilized by running an equilibrium program with the gradients mentioned above.

Quantification and Peak Identification. The amount of GSH and GSSG in woody plant tissues was quantified by an internal standard method. Peak identification was done by comparison of retention times of pure standards and spiking of plant samples, and verified by running a sample without carboxy-methylation resulting in a single peak of all glutathione present in the oxidized form. Additionally, the peak of glutathione was also verified by using another alkylating agent, iodoacetamide, to alter the retention time of the isoindole on the HPLC column (Cooper and Turnell 1987).

3.4 RESULTS

A representative chromatogram of a mixture of N-dinitrophenyl-S-carboxymethyl derivatives of standards CYS, GSH, GSSG, and γ-glu-glu, is shown in figure 1A. The retention times were 11.0, 12.6, 13.9, and 15.1 min for CYS, γ-glu-glu, GSH, and GSSG, respectively (Table 1 and Figure 1A). Retention times, peak areas and ratios of peak area were highly reproducible (Table 1). All compounds were resolved and eluted within 16 min after injection. Modification of the slope in the linear gradient mobile phase from 10 min in the original method (Fariss and Reed 1987) to 5 min improved cysteine resolution and shortened analysis time.

The calibration curves for CYS, GSH, and GSSG obtained are shown in Figure 2, as the plot of the ratio of peak area of the authentic standard to the peak area of the internal standard vs. content (nmol). The method was linear over a 300-fold range from 10 pmol to 3 nmol, for GSH and GSSG. CYS response was linear in a 60-fold range from 0.5 nmol to 3 nmol. The slope of the standards curve for each compound was determined by least-square linear regression (Figure 2). The coefficients of determination (r^2) for all curves were greater than 0.98. The detector response was highest for GSSG and lowest for CYS.

The reproducibility and repeatability of a single sample and variation of multiple analysis of different single samples were determined by using a one nmol content of the three standards. A high degree of precision was demonstrated, with less than 3% variation in peak areas for all the compounds resolved (Table 1). The method using γ-glu-glu as the internal standard accurately corrects for variations in derivatization efficiency and stability, chromatographic conditions, and injection volumes (Fariss and Reed 1987). The internal standard method for quantification had an accuracy greater than 98%

for GSH and GSSG.

The sensitivity of the method is related, in part, to the number of amines available for dinitrophenol derivatization (Fariss and Reed 1987). GSSG, GSH, and CYS were measurable at contents of 5 pmol, 10 pmol and 500 pmol, respectively. At contents higher than 3 nmol, peak area was non linear and indicated column overload.

Since thiols are very susceptible to oxidation by heavy metals and at neutral and basic pH (Fahey and Newton 1987), tissues were extracted in 10 % PCA containing 1 mM of BPDS. A single extraction of peach bark yield 205 and 33 nmol g^{-1} dry wt of GSH and GSSG, respectively (data not shown). While, three extractions yielded 440 and 31 nmol g^{-1} dry wt of GSH and GSSG, respectively. In general, extracting the pellet two (data not shown) or three times and analyzing the combined supernatants, a higher level of reduced glutathione was found over a range of 23 to 75% depending on the sample tested. Blueberry bark tissue, however, showed no improvement when extracting three times. The recovery of GSSG in the samples studied was 100% (data not shown). Percent recovery of GSH was lower for hazelnut and cherry with 63 and 71.6%, respectively. Recoveries of GSH from apple and pear extracts were 100% (Table 2). Therefore, the purification steps and analytical methods were valid for quantitative determination of GSH and GSSG.

This sensitive HPLC method was applied to various tissues of a variety of woody plant species and at different times of the growth cycle. A typical chromatographic profile for apple bark tissue collected on Dec 13, 1990 is shown in Figure 1B. Retention times for the peaks of interest can be compared with the standard chromatogram in Figure 1A. Ninety-four percent of glutathione in apple bark tissue was GSH.

Reduced glutathione was the predominant form for all the species tested at both sampling dates (Table 2). GSH increased two to eight

times from June to December in all species analyzed, except grape. In grape, GSH decreased 60% from one sampling date to the other. GSSG was not detected in the June samples of peach and prune, nor in hazelnut in December. One specie, blueberry, had no detectable levels of GSSG at either sampling date. The ratio of GSH to GSSG increased in all species evaluated, from June to December.

Among different peach tissues tested, leaves had the highest amount of GSH, 50% of the total glutathione (Table 3). No GSSG was detectable in the buds. Bark and root tissues had the lowest amount of GSH, 28% and 35% of the total glutathione, respectively. The ratio of GSH/GSSG was highest in leaves, with a ratio greater than one. Bark and roots had ratios of less than one.

The seeds of cherry and sugar pine had similar levels of GSH representing 84% and 77% of the total glutathione, respectively. The ratio of GSH/GSSG was higher for cherry seeds than for sugar pine seeds, with values above 4.

3.5 DISCUSSION

This HPLC method for analysis of CYS, GSH, and GSSG is reproducible and applicable to diverse woody plant tissues. The major improvements of this method over previous reported methods (Fariss and Reed 1987, Reed et al. 1980, Richie and Lang 1987) are: the sensitivity for glutathione disulfide, the short time for analysis, and the improved resolution for CYS. This is the first time, to our knowledge, that an extensive quantitative analysis of glutathione content of different woody plant species has been reported.

Not all the glutathione present in woody plant tissues is totally removed with one extraction. This was probably because the breaking or disrupting of the woody plant cells is more difficult. Glutathione was still present in the pellet when only one extraction was performed. After extracting the tissue three times, no further increase in the glutathione level was observed.

The GSH values and seasonal changes we observed are similar to those reported in the literature for other species and tissues (Esterbauer and Grill 1978, Guy et al. 1984). For example, the content of GSH in conifer needles has been shown to undergo seasonal changes (Esterbauer and Grill 1978), with relatively low levels in summer and higher levels during winter. Our studies confirmed that several woody plant species tend to increase levels of GSH when the plants are dormant. Increases in GSH, when plants are exposed to low temperatures, had also been reported for the bark tissue of dogwood plants, and citrus leaves (Guy et al. 1984). Leaf tissues of frost hardened spinach also had a two-fold increases in water soluble non-protein SH (de Kok and Oosterhuis 1983).

Concentrations of GSSG are generally low in plant cells, but increases dramatically and rapidly when plants experience oxidative

stress (Schmidt and Kunert 1986, Smith 1985, Smith et al. 1985). Esterbauer and Grill (1978) measured seasonal changes of total soluble SH-groups and GSH in conifer needle extracts, but did not measure GSSG. Our studies show that GSSG levels in woody plant tissues also undergo seasonal changes, with higher concentrations in the winter and lower in the summer. Evidence exists that GSSG increases when plants are exposed to periods of low temperature (Guy and Carter 1984, Guy et al. 1984). Woody plant cells appear to be suffering increased oxidative stress when exposed to low temperatures. Elevation of oxidative processes lead to the accumulation of high levels of GSSG (Smith 1985, Smith et al. 1985, Smith et al. 1984).

Plants usually maintain a high GSH/GSSG ratio due to the action of glutathione reductase (Smith et al. 1989). Increases in the activity of glutathione reductase during the winter in 22 winter hardy evergreen plants, as well as, in plants exposed to low temperatures has been reported (de Kok and Oosterhuis 1983, Esterbauer and Grill 1978, Guy and Carter 1984). Our results showed an increase in the ratio of GSH/GSSG in the dormant plant samples, in addition to the changes in the concentrations of GSH and GSSG. Similar results were observed when plants were exposed to low temperature (Guy and Carter 1984, Guy et al. 1984).

Glutathione levels in peach tree tissues were highest in the leaves, lower in buds, and least in roots. The concentrations found in our study were moderately less than those reported for some other species (Guy and Carter 1984). Perhaps, differences in species, tissue age, sampling time and method of analysis may explain this. The youngest leaves at the apex of spinach plants have higher levels of glutathione than older leaves or roots (Bielawsky and Joy 1986). We found the lowest level in roots of peach trees with 35% of the total glutathione in the reduced form. In contrast, Bielawsky and Joy (1986) showed that 94% of the total glutathione in spinach roots was present

as GSH. We do not know if the difference is due to variation between plant species, or to different growth conditions.

The GSH content of water imbibed cherry and sugar pine seeds was 84% and 77% of the total glutathione, respectively. A dramatic reduction in GSSG levels has been reported in cherry seeds after imbibition in water (Siller-Cepeda et al. 1990). A decrease in GSSG levels appears to be necessary early in germination to obtain optimal protein synthesis during growth and development (Fahey et al. 1980). Stratification of cherry seeds at 4^0C for up to 12 weeks increased the GSH level six-fold and was correlated with the increases in germination. Seeds held at 26^0C did not show the increase in GSH and germination was low (Siller-Cepeda et al. 1990).

Table 3.1. Retention times, peak areas and ratio of peak area of external standard to internal standard.

Compound Analyzed	Retention Times	Peak* Area	Ratio of Peak Area to Int. Standard
CYS	11.00 ± 0.04**	5.62 ± 0.16	0.448 ± 0.014
-Glu-Glu	12.60 ± 0.03	12.61 ± 0.31	1.00
GSH	13.90 ± 0.03	10.18 ± 0.19	0.810 ± 0.009
GSSG	15.18 ± 0.04	19.14 ± 0.46	1.518 ± 0.006

* peak areas correspond to one nmol of each standard.
** values are the means of 16 replicates ± S.E. of the mean.

Table 3.2. Levels of GSH, GSSG, GSH/GSSG ratio, and percent recovery in bark tissue of several woody fruit trees sampled at two times of the year.

Species	Sampling Dates: June 13, 1990 GSH (nmol g^{-1} dry wt)	June 13, 1990 GSSG	June 13, 1990 GSH/GSSG	December 13, 1990 GSH (nmol g^{-1} dry wt)	December 13, 1990 GSSG	December 13, 1990 GSH/GSSG	Recovery (%)
Peach	219 ± 18**	nd*	___	440 ± 42	31 ± 3	13 ± 1	81.8
Cherry	98 ± 15	8 ± 2	13 ± 3	238 ± 24	8 ± 1	30 ± 7	71.6
Prune	82 ± 6	nd	___	271 ± 15	34 ± 11	10 ± 2	87.1
Apple	163 ± 7	12 ± 1	13 ± 1	833 ± 17	60 ± 4	14 ± 1	100.3
Pear	161 ± 25	11 ± 4	20 ± 5	405 ± 17	13 ± 5	44 ± 11	100.1
Grape	431 ± 34	35 ± 5	13 ± 1	170 ± 7	10 ± 3	21 ± 4	95.3
Hazelnut	130 ± 28	23 ± 5	6 ± 1	362 ± 34	nd	___	63.0
Blueberry	54 ± 16	nd	___	413 ± 64	nd	___	89.2

* not detected.
** values are means from four replicates ± standard error. Units are nmol g^{-1} dry wt.

Table 3.3. Levels of GSH, GSSG, and GSH/GSSG ratio in several tissues of peach plants and seeds of cherry and sugar pine.

Specie	Tissue	GSH (nmol g^{-1} dry wt)	GSSG	GSH/GSSG
Peach	Leaf	73 ± 3**	58 ± 3	1.2 ± 0.03
	Bud	50 ± 9	nd*	___
	Bark	18 ± 1	45 ± 4	0.4 ± 0.01
	Root	25 ± 7	46 ± 5	0.5 ± 0.13
Cherry	Seed	403 ± 16	74 ± 19	6.5 ± 1.47
Sugar Pine	Seed	401 ± 75	121 ± 31	4.2 ± 2.03

* not detected.
** values are given in nmol per gram of dry wt, and are the means of three replicates ± standard error.

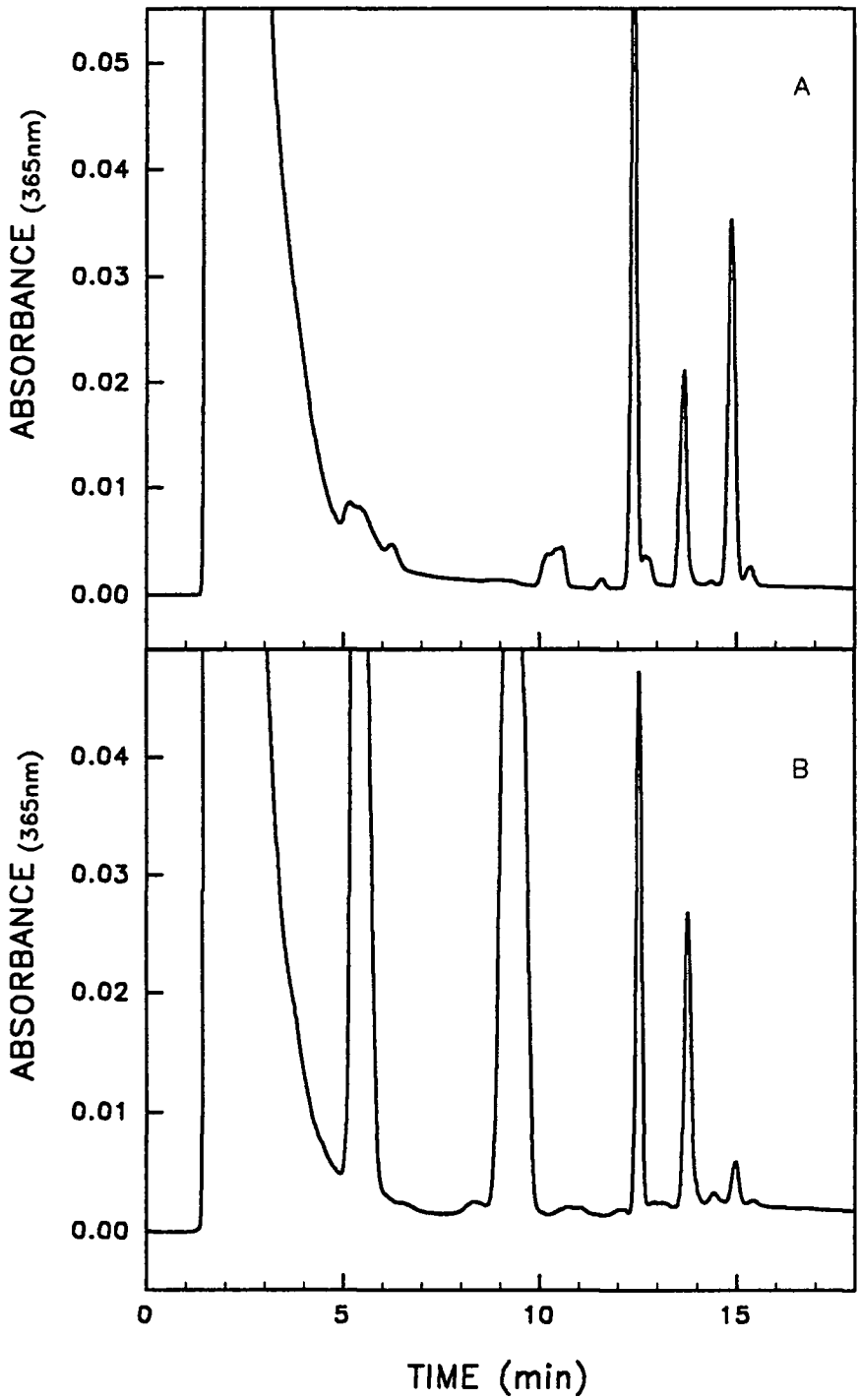

Figure 3.1. Representative chromatograms of N-dinitrophenyl-S-carboxymethyl derivatives for (A): combined standards (0.5 nmol); Retention times are 11.0, 12.6, 13.9, and 15.1 min for CYS, γ-glu-glu, GSH, and GSSG, respectively; and (B) apple bark tissue collected on Dec 13, 1990. (Column and conditions for HPLC as described in Materials and Methods).

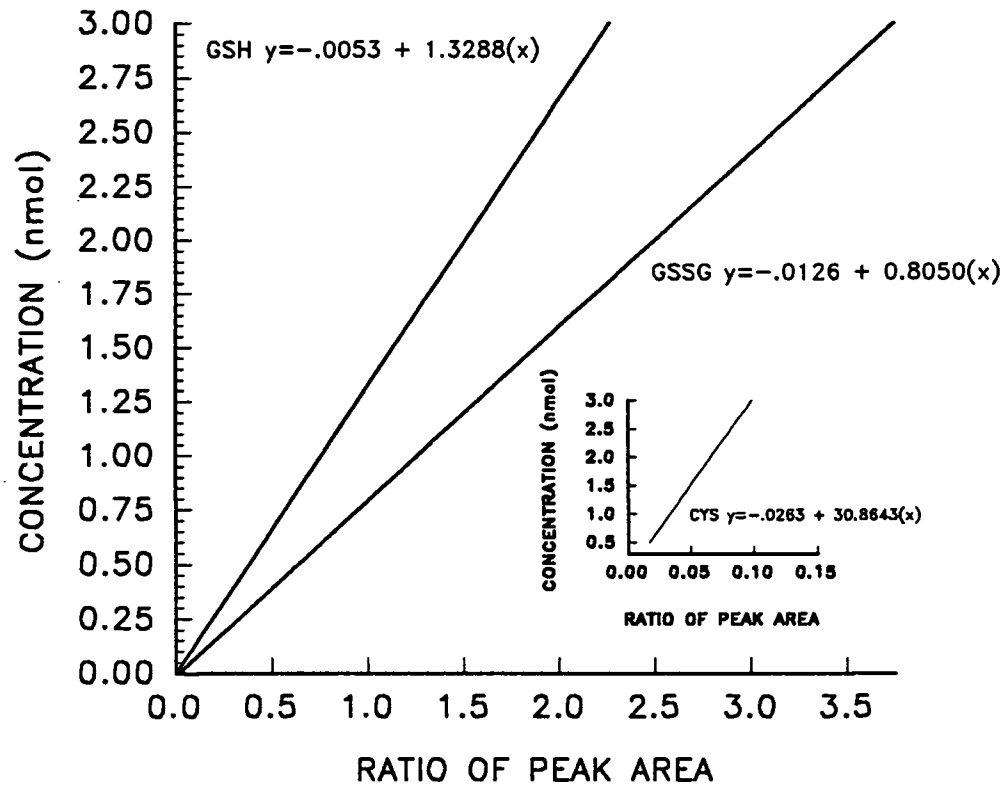

Figure 3.2. Calibration curves for CYS (insert), GSH, and GSSG, obtained by plotting the ratio of peak area of the external standard to the peak area of the internal standard vs. content in nmol. The slopes of the standard curves for each compound were determined by least-square linear regression. The coefficient of determination (r^2) for all curves was greater than 0.98.

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4.0 HYDROGEN CYANAMIDE INDUCED BUDBREAK AND PHYTOTOTOXICITY IN 'REDHAVEN' PEACH BUDS. TIMING AND CONCENTRATION.

4.1 ABSTRACT

Budbreak and phytotoxicity induced by hydrogen cyanamide applied to one year old potted peaches (*Prunus persica* L. cv. Redhaven) over a wide range of concentrations at different rest stages was studied. Plants acquired the onset of rest (180°GS) on 1 Oct. Maximum rest (270°GS) was reached after the plants were exposed to 320 chill units on 1 Nov, and 50% of the buds were broken at 860 chill units on Dec 1. Five concentrations of hydrogen cyanamide (0, 0.125M, 0.25M, 0.5M, and 1.0M) were applied on 1 Oct, 15 Oct, 1 Nov, 15 Nov, 1 Dec, and 15 Dec, 1990. All concentrations were effective in promoting budbreak, however, total budbreak and phytotoxicity were dependent on concentration and timing. Phytotoxicity was evident on all application dates, but greatest at the higher concentrations. Plants were more resistant to hydrogen cyanamide at maximum rest. Hydrogen cyanamide induced budbreak was highest during the post-rest period (295°GS to 315°GS). Treatments applied during the quiescent stage inhibited and delayed budbreak, and damaged the buds and stems.

4.2 INTRODUCTION

Dormancy in deciduous fruit trees and other temperate zone woody perennials is a phase of development that occurs annually (Saure, 1985). Release of rest requires a period of chilling temperatures during winter (Richardson et al, 1974, Kobayashi et al., 1982), followed by a rise in temperature in spring (Richardson et al, 1976, Kobayashi and Fuchigami, 1983). An important obstacle to economic production of deciduous fruit trees in the subtropics and tropics is a deficiency of chilling temperatures. Inadequate chilling may result in poor budbreak and foliage development, delayed foliation and bloom, uneven budbreak, sparse bloom, abnormal flower development, poor fruit set, reduced leaf area, and/or early growth cessation due to secondary dormancy (Erez, 1987; Saure, 1985).

Many attempts have been made to overcome the problem caused by insufficient chilling of deciduous fruit trees grown in warm climates. In the tropics, plants are prevented from entering rest by cultural practices such as defoliation and withholding irrigation (Edwards, 1987; Janick, 1974; Sherman and Lyrene, 1984). In the subtropics, budbreak is hastened after plants have already entered rest by a combination of cultural practices and application of rest breaking chemicals (Erez, 1987; Shulman et al., 1983).

Although many chemical and physical treatments are known to break rest (Doorenbos, 1953), only a few have proven useful under field conditions (Erez, 1987). Chemicals which have been used commercially to overcome rest include cyanamides, cytokinins, dinitro-o-cresol, mineral oils, potassium nitrate, thiourea, and thidiazuron (Diaz et al., 1987; Erez et al., 1971; Saure, 1985; Shulman et al., 1986; Wang et al., 1986).

One characteristic common to most of the rest breaking treatments

is that they are effective only at near-lethal dosages (Fuchigami and Nee, 1987; Erez, 1987). Because of the narrow effective range, variable effectiveness of overcoming rest and phytotoxicity are commonly encountered (Dozier et al., 1990; Wolak and Couvillon, 1976). The time of application (Wolak and Couvillon, 1976; Fuchigami and Nee, 1987), the physiological stage of the bud (Fuchigami and Nee, 1987), the nutritional status of the plants (Terblanche and Strydom, 1973), post-application temperatures (Erez, 1987) amount of chilling accumulated (Erez, 1979), are probably factors which influence the variable results.

A major problem in the application of artificial rest breaking agents is the inability of the grower to know when and at what concentration to apply the chemicals. As a result, the effective rate of the chemicals to promote budbreak cannot be determined objectively. Both the environmental conditions and the physiological status of field grown plants, should be considered when applying rest breaking agents.

The objectives of this study were: a) to examine the effects of hydrogen cyanamide (H_2CN_2) on budbreak and phytotoxicity of 'Redhaven' peach trees at different times during the dormant phase and; b) to establish the relationship between the physiological timing of H_2CN_2 application and the effective concentration of the dormancy breaking treatment.

4.3 MATERIALS AND METHODS

One year-old potted peach trees were used in this study. 'Redhaven' scions of current season's growth were grafted onto prune (*Prunus insititia* cv. St. Julian A) rootstocks in early Feb 1990. The graft union was placed for two weeks in a hot callusing pipe at 26C to promote callus formation. The grafted plants were then transferred into 4 l containers containing loam soil, washed sand, peat moss, and pumice rock (1:1:1:2 v/v). Plants were grown under natural conditions during spring and summer, and pruned to a single stem in July. At the end of the growing period, plants averaged 1.5 m in height and had an average of 150 lateral and 4-8 terminal buds.

To determine the stage of dormancy, after terminal bud set plants were manually defoliated at two week intervals from 29 Aug to 1 Nov (Fuchigami et al.,1982). Defoliated plants were moved to the greenhouse with temperatures of 25 ± 3C during the day and 20 ± 3C at night. Radiation sources in the greenhouse consisted of natural daylight and 400-W high pressure sodium lamps (Energy Technics, York, Pa.) that provided a PAR level of ≈ 400 to 500 $\mu mol \cdot s^{-1} \cdot m^{-2}$ for 16 hr/day. Natural defoliation was completed by the second week of Nov. The total number of buds was counted on each tree before moving them to the greenhouse. Rate of budbreak was monitored every two days, and total percentage budbreak was evaluated after four weeks.

To determine budbreak and phytotoxicity induced by H_2CN_2, trees were sprayed to run-off with distilled water (control) or H_2CN_2 solutions at concentrations of 0.125, 0.25, 0.50 and 1.0 M. Dates of H_2CN_2 application were: 1 Oct, 15 Oct, 1 Nov, 15 Nov, 1 Dec, and 15 Dec. After application the plants were moved into the greenhouse. The total number of buds was determined before application of the treatments.

Rate and total percentage of budbreak were expressed as the percentage of buds which opened out of the total number after four weeks in the greenhouse. Vegetative buds were considered open at stage 2 based on the Guerriero and Scalabrelli (1974) rating scale, corresponding to visible swelling of two leaves. Flower buds were considered open at the green calyx stage (when green coloration was evident in the calyx surface). Phytotoxicity was expressed as the percentage of dead buds at the end of the four week period.

An estimate of the growth stages during rest development was determined and expressed as the degree growth stage (°GS) according to Kobayashi et al (1982) model. Temperature was recorded outside with an hygrothermograph placed in a wooden shelter raised above the ground, next to the plants. Chilling unit accumulation was calculated according to the model of Richardson et al (1974). Chilling was considered satisfied when 50% of the buds had broken after 4 weeks of forcing conditions (Weinberger, 1950).

Treatments were arranged in a completely randomized design with factorial arrangement for time of application and hydrogen cyanamide concentration. Percentage data were transformed into the square root of arcsin percentage before statistical analysis. A computer package was utilized to perform the statistical analysis (SAS Institute, Inc. N.C. 1987). Data is presented as the mean of four replications ± SE.

4.4 RESULTS

Chilling accumulation. According to the Utah chilling accumulation model, chilling started to accumulate in the field on 5 Oct (Fig. 1). Chilling temperatures occurred throughout Oct, Nov, and Dec, and the percentage of budbreak began increasing after 320 CU had been accumulated. Chilling accumulation was rapid during Oct and Nov, and by 1 Dec, release from dormancy was evident.

Degree of dormancy. Plants defoliated on 29 Aug and 15 Sep broke buds approximately 15 days (±7) after forcing in the greenhouse (Fig. 1). The percentage of budbreak from 1 Oct to 1 Nov decreased considerably (Fig. 1). By 1 Oct, rest (180°GS) had already been attained, and by 1 Nov, maximum rest (270°GS) was achieved (Fuchigami et al, 1982). Percent budbreak increased from 23 % on 15 Nov to 92% on 15 Dec corresponding to 536 and 1146 CU, respectively. The number of chilling units associated with 50% budbreak was calculated to be about 860 CU (Fig. 1). Budbreak under natural conditions occurred on 5 Feb.

Response to H_2CN_2. Application of cyanamide to peach plants from 1 Oct to 1 Dec increased budbreak (Fig. 2A to 2E). Budbreak on treated plants was observed 8 to 12 days after application (Fig. 3B). In general, the most effective concentration (greatest budbreak and lowest phytotoxicity) was 0.125M H_2CN_2 on all treatment dates. Budbreak at 0.5 and 1.0M H_2CN_2 was confined to the basal buds due to phytotoxicity of the upper buds.

On 1 Nov, at maximum rest (270°GS), no significant differences among 0.125, 0.25 and 0.5M H_2CN_2 rates were found in budbreak (Fig. 2C). The highest percent of budbreak occurred during the post-rest period (295-315°GS) (Fig. 2D, and 2E). Cyanamide induced earlier and higher budbreak during this period as compared to non treated plants (Fig. 3B). During the quiescent period (315-360°GS), cyanamide

significantly reduced and delayed budbreak at all concentrations tested (Fig. 2F and 3A).

Phytotoxicity. H_2CN_2 was phytotoxic to peach buds at all concentrations tested (Fig. 2A to 2F). The extent of phytotoxicity was dependent on concentration and the stage of development.

H_2CN_2 was less toxic on 1 Nov, when the plants were at maximum rest (270°GS) (Fig. 2C). During the post-rest period (295°GS-315°GS), phytotoxicity induced by H_2CN_2 was variable in all, but the lowest hydrogen cyanamide concentration (0.125M) (Fig. 2D, 2E, 2F).

4.5 DISCUSSION

A highly significant interaction of cyanamide concentration and time of application was found for budbreak and phytotoxicity (Table 1). Thus, percent of budbreak and phytotoxicity of 'Redhaven' peach buds induced by hydrogen cyanamide was dependent on timing and concentration.

Chilling has been shown to stimulate deep rest as well as to overcome rest in several species (Amling and Amling, 1979; Kobayashi, et al., 1982; Hauagge and Cummins, 1991). In our study, 'Redhaven' peaches required ≈ 320 CU from the onset of dormancy to attain maximum rest (Fig. 1). Additional chilling induced earlier budbreak and eventually released the plants from rest.

Based on greenhouse forcing studies, chilling requirement to overcome rest in 'Redhaven' peach buds was fulfilled on ≈ 1 Dec with 860 CU. These results are in close agreement with that reported for the same cultivar by Richardson et al., (1974).

Budbreak of 'Redhaven' peach trees under greenhouse conditions following defoliation on 29 Aug was ≥ 60%, and on 15 Sep ≥ 50% after 21 days (Fig. 1). The rapid rate of budbreak, and high percentage of budbreak of plants defoliated on 29 Aug and 15 Sept suggest that the plants were still under correlative inhibition (90°GS to 180°GS), rather than being in rest before defoliation (Fuchigami et al, 1982; Saure, 1985). Defoliation is a common practice in the tropics to induce budbreak and growth during correlative inhibition, and avoid rest (Erez and Lavi, 1985; Notidimejo et al., 1981; Sherman and Lyrene, 1984).

Rest is generally considered to have started when budbreak does not occur after either artificial defoliation and/or decapitation of the shoots (Fuchigami et al., 1982; Saure, 1985). Defoliation and

greenhouse regrowth tests on 1 Oct, 15 Oct, and 1 Nov showed a significant decrease in the percentage of budbreak (Fig. 1) which demonstrates that the plants had already entered rest (Fuchigami et al., 1982). The same response in reduction on budbreak with successive defoliation dates had been shown for 'Washington' peach plants (Lloyd and Couvillon, 1974).

Comparing the results of previous reports on the effect of rest-breaking chemicals is difficult, mainly because in most of them, the rest stage was not determined. This study and the work of others (Nee, 1986; Bracho et al., 1984; Erez, 1987;) indicates that the effect of H_2CN_2 on budbreak and phytotoxicity are dependent on concentration and timing, physiological stage of the bud, and genotype. Other researchers have not considered the physiological status of the buds, and this may be the reason for the highly variable results (Diaz et al., 1987; Dozier et al., 1990; Erez, 1987a; Jensen and Bettiger, 1984; Wolok and Couvillon, 1977).

At the onset of rest (1 Oct), all concentrations were effective in promoting budbreak. However, due to phytotoxicity the lower rates are preferred. Erez, (1987a) proved that application of 2% (0.5M) cyanamide to peaches in the autumn, before deep rest was attained induced higher phytotoxicity than later application when the plants were in deep rest. In contrast, results on dogwood and crabapple at the onset of rest (Nee, 1986), indicated that as cyanamide concentration was increased a higher percentage of budbreak occurred. Stone fruit species having simple flower buds are more sensitive to chemicals than species with protected flower buds, such as pome fruits, grapes and kiwi (Erez, 1987).

At maximum rest (270°GS), higher H_2CN_2 concentrations were required to stimulate budbreak. Nee (1986) showed that as the depth of rest increased, correspondingly higher concentrations were required to break rest. In our study, we found no differences on budbreak at

maximum rest among 0.125M (39%), 0.25M (42%), and 0.5M (33%) rates. At this stage, even the highest concentration enhanced budbreak, although it only stimulated the breaking of basal buds. In grapes, concentrations ≥ 1M have been shown to stimulate budbreak of basal and latent buds and is used when renewal of old vines with bare cordons is needed (Bracho, et al., 1984; Lavee et al., 1984; Whiting and Coombe, 1984). The greater tolerance of the lower buds to H_2CN_2 is probably because of the rest gradient down the shoot, with lower buds in deeper rest than those above (Paiva and Robitaille, 1978). Erez (1987a), reported from autumn cyanamide applications to peaches a reduction in the level of damage as the trees acquired deep rest.

The best responses in budbreak were found during the post-rest period (295^{oGS}-315°GS). However, phytotoxicity during this period was quite variable at all but the lowest concentration (Fig. 2D and 2E). Fernandez-Escobar and Martin (1987) reported that cyanamide strongly inhibited flower bud development of peaches when applied at concentrations higher than 0.6M. However, cyanamide applied at 0.1M to 0.6M promoted vegetative growth when accumulated chilling hours were less than 474 (Fernandez-Escobar and Martin, 1987). In contrast, Dozier et al (1990) reported that peaches and nectarines responded linearly to cyanamide levels applied on 15 March after 650 CU, with the highest budbreak at the 2% (0.5M) rate. Surprisingly, the control plants showed greatest dieback, while the highest concentration (0.5M) resulted in almost no damage. Since they applied the different cyanamide concentrations to different sectors of the same tree, it is not known if the translocation of the chemical should be considered or not (Amberger, 1984).

Application of cyanamide after chilling satisfaction had no effect in promoting budbreak. Rather than enhancing budbreak, cyanamide reduced and delayed bud growth, and injured the bud and stems. Similar results have been reported for other species when

cyanamide was applied during quiescence (Nee, 1986; Shulman et al., 1983; Whiting and Coombe, 1984). It is likely that most of the variability in budbreak and toxicity found in this study can be attributed to the physiological status of the bud and the amount of chilling accumulated before the application. Furthermore, the variable results of others from field application of hydrogen cyanamide can be partially explained by differences in degree of rest of the plant. It appears that the physiological stage of the bud is a critical parameter in defining the narrow limits between optimum and phytotoxic concentrations of the chemical treatments. The results presented here suggests that: 1) cyanamide is effective in overcoming rest in unchilled as well as partially chilled buds; 2) timing and concentration of cyanamide affect the percentage budbreak; 3) timing is a function of the rest stage of the buds; and 4) concentrations $\geq$ 0.5M are highly phytotoxic to peaches.

Table 4.1. Analysis of variance of the effect of hydrogen cyanamide concentration and time of application on budbreak and phytotoxicity of 'Redhaven' peach buds.

Source of variation	df	Budbreak		Dead Buds	
		MS	F	MS	F
Model	29	1302.7	19.5**	2485.0	47.1**
Hydrogen Cyanamide	4	4098.7	61.3**	15609.7	295.9**
Time of Application	5	677.6	10.1**	788.0	14.9**
Hydrogen Cyanamide X Time of Application	20	832.0	12.4**	212.9	4.0**
Error	80	66.7			

** significant at 0.001 level.

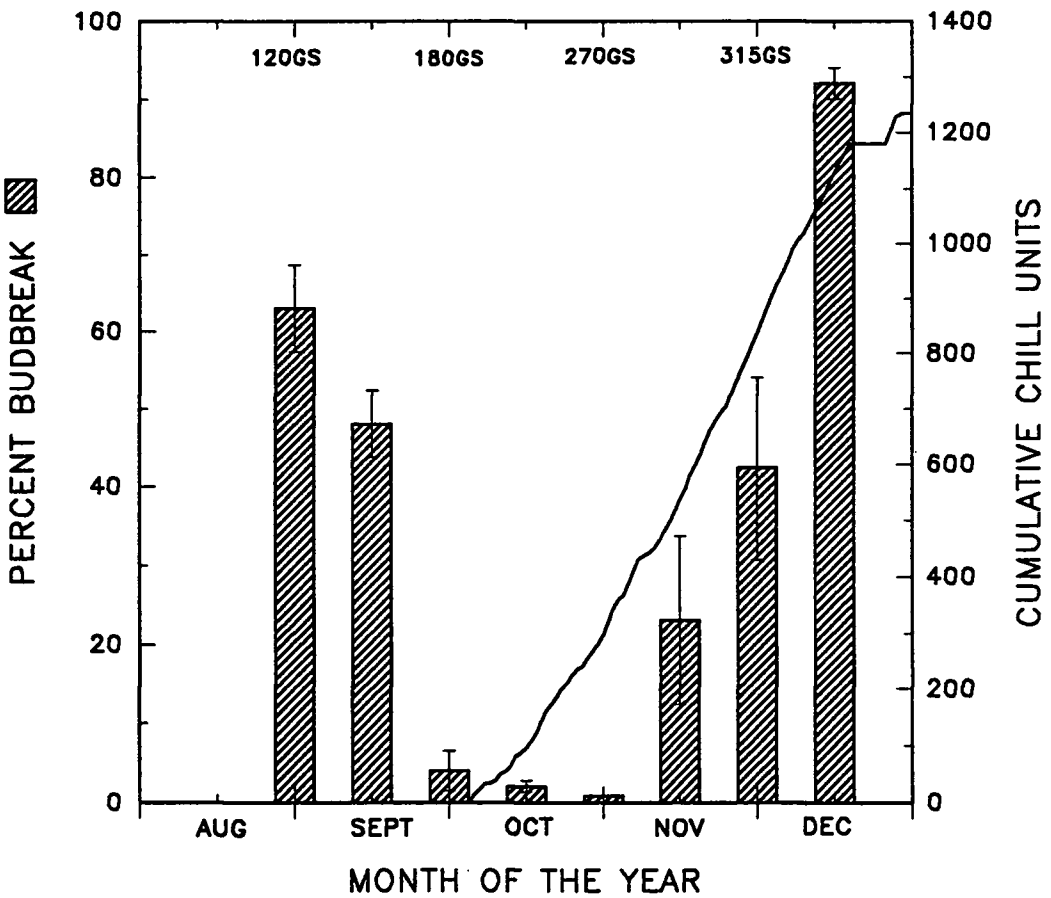

Figure 4.1 Percent of budbreak of 'Redhaven' peach buds (control) after four weeks of forcing conditions, and chilling unit accumulation from October to December 1990.

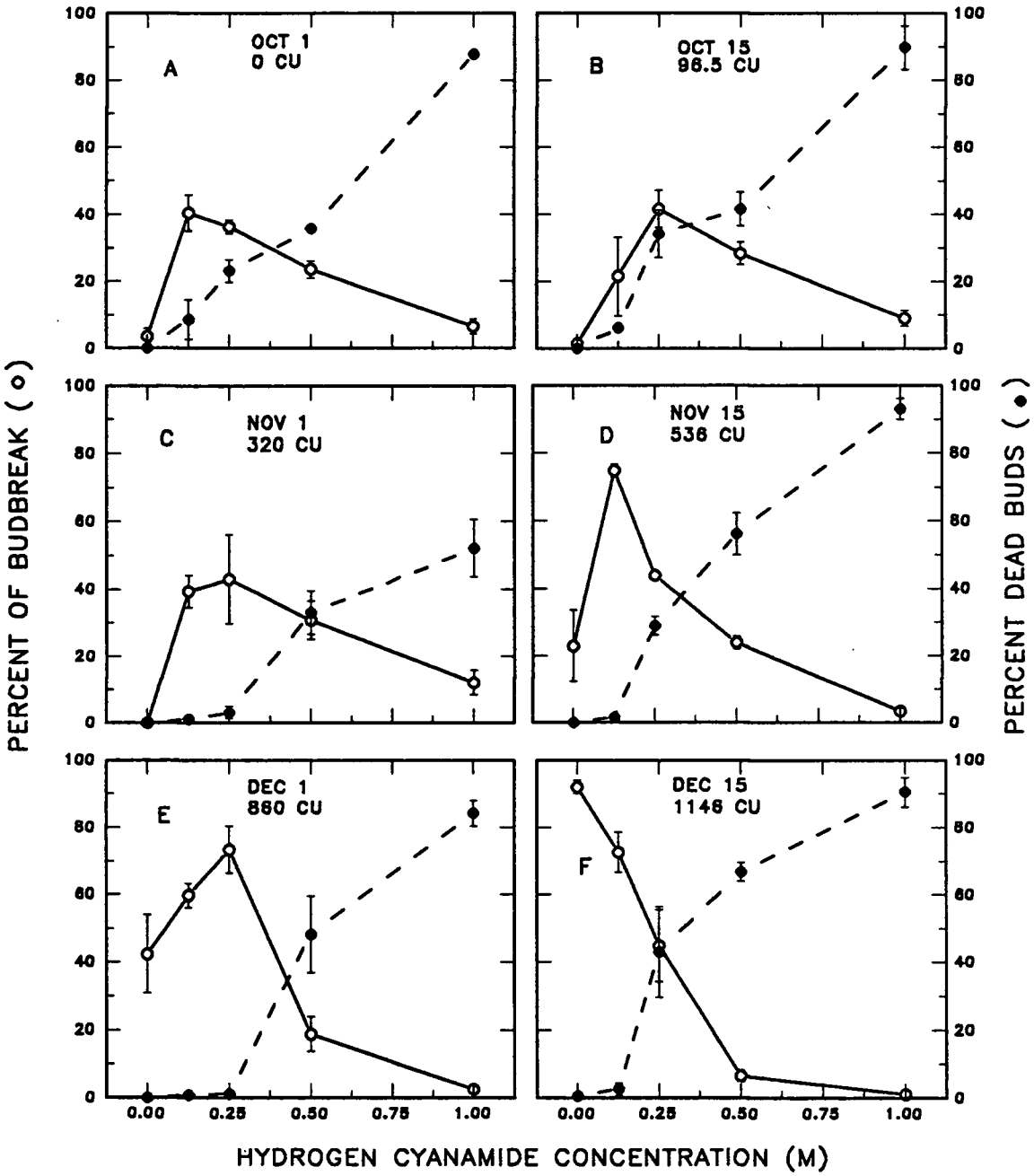

Figure 4.2. Percent of budbreak and percent of dead buds in 'Redhaven' peach trees induced by hydrogen cyanamide sprayed at 0, 0.125, 0.25, 0.5 and 1.0 M concentrations and applied on A) 1 Oct (0 CU); B) 15 Oct (96.5 CU); C) 1 Nov (320 CU); D) 15 Nov (536 CU); E) 1 Dec (860 CU); and F) 15 Dec (1146 CU).

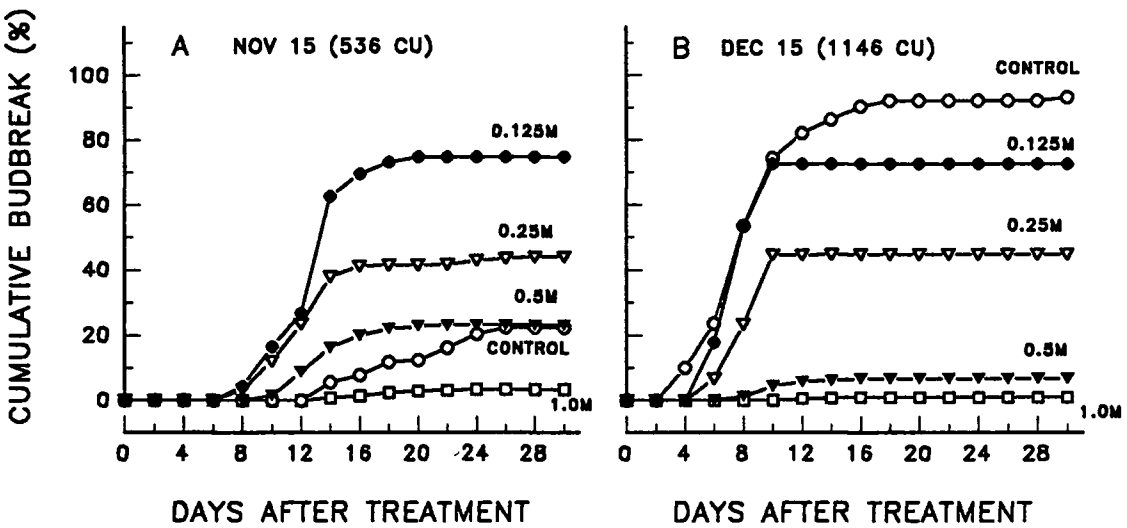

Figure 4.3. Days to budbreak and percent of budbreak of 'Redhaven' peach buds induced by hydrogen cyanamide sprayed at 0, 0.125, 0.25, 0.5, and 1.0 M, and applied on A) 15 Nov (536 CU); and B) 15 Dec (1146 CU).

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5.0 POST-TREATMENT TEMPERATURE ON PHYTOTOXICITY INDUCED BY HYDROGEN CYANAMIDE IN 'REDHAVEN' PEACH BUDS.

5.1 ABSTRACT

The effects of post-treatment temperature and hydrogen cyanamide concentration on budbreak and phytotoxicty applied to one year old potted peach trees (*Prunus persica* L. cv. Redhaven) were studied. Hydrogen cyanamide at 0, 0.125M, 0.25M, 0.5M, and 1.0M rates were applied after chilling satisfaction and moved to growth chambers at either 15°, 25°, or 35°C for two weeks. Budbreak and Phytotoxicity were dependent on post-treatment temperature and concentration. All hydrogen cyanamide treatments inhibited budbreak at all test temperatures. The inhibition was greatest at the low temperature and at the highest concentrations. Daily average rate of development was faster at the high temperature. At 25°C, concentrations of 0.25M or higher delayed and inhibited budbreak. Phytotoxicity was greater at 15° and 35° than at 25°C, and increased with increasing concentrations.

5.2 INTRODUCTION

When temperate zone fruits trees are grown under subtropical conditions that have insufficient low winter temperatures, they experience delayed and prolonged bloom period and foliation, resulting in low yields and delayed harvest (Erez, 1987; Saure, 1985). Availability of low-chilling cultivars, management practices, and rest breaking chemicals have permitted production in these areas (Edwards, 1987).

Numerous compounds have been used to release deciduous fruit tree buds from rest (Bloommaert, 1965; Erez et al, 1971; Fernandez-Escobar and Martin, 1987; Iwasaki and Weaver, 1977; Hosoki, 1983; Shulman et al, 1986; Snir, 1983; Wainwright and Price, 1984). Among them, hydrogen cyanamide has been found to be especially valuable in overcoming rest in several species of deciduous fruit (Erez, 1987). The effective concentration and timing of hydrogen cyanamide to break rest varies with time, physiological stage of the bud, and genotype (Bracho et al, 1984; Erez, 1987; Fuchigami and Nee, 1987; Siller-Cepeda et al, 1991a).

Most of the experiments conducted to elucidate the effect of chemicals in rest release have not considered the role of post-treatment temperature. Such experiments generally have been carried out in greenhouse conditions with controlled temperature in the range from 20 to 25°C (Erez et al, 1971; Fernandez-Escobar and Martin, 1987; Steffens and Stutte, 1989; Shaltout and Unrath, 1983). As a result, researchers often speculate on the interaction of rest breaking chemicals and temperature (Dozier et al, 1990; Erez et al 1971; Snir, 1983).

Although Erez et al (1971) and Erez and Lavee (1974) have discussed the effect of climatic factors on budbreak and phytotoxicity

after application of rest breaking chemicals, little work has been attempted to define these effects. Erez et al (1971) noticed that the efficacy of DNOC + mineral oil increased with increased temperature during the days following treatment. Erez (1979) concluded that the post-treatment environment may be the cause for the unpredictable effects of the rest breaking chemicals.

The interaction between temperature and hydrogen cyanamide is not clear. It seems that cool conditions may enhance damage in peaches and plums (Erez, 1987). Therefore, to clarify this interaction, our study was initiated with the objective of determining the effects of post-treatment temperature on budbreak and phytotoxicty of 'Redhaven' peaches following hydrogen cyanamide application.

5.3 MATERIALS AND METHODS

One year-old potted peach plants were propagated as described previously (Siller-Cepeda et al, 1991a). Plants were grown under natural conditions at Corvallis, OR. During July the plants were pruned to a single stem. At the end of the growing period, plants averaged 1.5 m in height with an average of 150 lateral and 4 to 8 terminal buds.

On 15 Dec, following 1146 CU based on the Utah model (Richardson et al., 1974) plants were sprayed to run-off with hydrogen cyanamide (0, 0.125, 0.25, 0.5, and 1.0M). At this stage the chilling requirement to overcome rest was satisfied (Siller-Cepeda et al., 1991a). After application plants were moved to three growth chambers at either 15°, 25°, or 35° ± 1°C for two weeks. Radiation sources in the growth chambers consisted of flourescent lights of 50 $\mu mol \cdot s^{-1} \cdot m^{-2}$ for 16 hr/day (0500 to 2100HR). After two weeks, plants were transfered to greenhouse conditions with temperatures of 25 ± 1°C during the day and 20 ± 1°C at night. Radiation sources in the greenhouse have been described previously (Siller-Cepeda et al., 1991a).

Total number of buds were determined on each plant before application of the treatments and rate of budbreak was monitored every two days. Total percentage of budbreak was evaluated after four weeks. Rate and total percentage of budbreak were expressed as the percentage of buds which opened out of the total number after four weeks. Vegetative buds were considered open at stage 2 based on the Guerriero and Scalabrelli (1974) rating scale, corresponding to visible swelling of two leaves. Flower buds were considered open at the green calix stage (when green coloration was evident in the calix surface). Phytotoxicity was expressed as the percentage of dead buds at the end of the four weeks period.

Treatments were arranged in a completely randomized design with factorial arrangement for temperature and H_2CN_2 concentrations. The number of days (W) necessary to reach 40% budbreak was determined for each treatment. A reciprocal transformation, 100/W, was used in data analysis. This transformation has been used by Arnold (1959), and Cambell and Sugano (1975) and represents the percentage daily average rate of development (DARD) towards budbreak. Percentage of budbreak and phytotoxicity after four weeks were transformed into the square root of arcsin percentage before statistical analysis. Statistical analysis was done using a computer package (SAS Institute, Inc. NC. 1987). Data are presented as the mean of four replications ± SE.

5.4 RESULTS

A highly significant interaction effect (p=0.001) of hydrogen cyanamide concentration and post-treatment temperature was found for percentage of budbreak and a significant interaction (p=0.005) for phytotoxicity (Table 1).

Daily Average Rate of Development. DARD values for control and cyanamide treated plants were higher with increasing temperature (Figure 1). However, some differences were found by comparing hydrogen cyanamide concentration within a single temperature. At 15C, cyanamide treated plants induced earlier budbreak than the controls, as shown by the higher DARD values (Figure 1). At 35C, the DARD values of control and cyanamide treated plants were similar. At 25C, the rate of budbreak was slower at concentrations of 0.25M hydrogen cyanamide or higher (Figure 1).

Final Budbreak. All H_2CN_2 cyanamide treatments inhibited budbreak as compared to the controls at the three temperatures tested. The inhibition was less pronounced at 35C and at the lowest concentrations (Figure 2). In general, as temperature increased, budbreak decreased in control plants, but have the opposite effect on the H_2CN_2 treated plants (Figure 2). Only plants held for 2 weeks at 15C showed an improvement in total percentage of budbreak after moving them to the greenhouse conditions. The potential of budbreak of plants held at 25C or 35C reached its maximum in the first two weeks and level-off afterwards (data not shown).

Phytotoxicity. The percentage of dead buds increased with increased hydrogen cyanamide concentrations at all test temperatures (Figure 3). The less toxicity was found at the 25C post-treatment temperature (Figure 3).

5.5 DISCUSSION

In general, vegetative growth increases as temperature is raised and attains a maximum in the range of 20 to 35C depending on species (Kozlowski, 1983). In this study budbreak was faster as the temperature was raised. DARD for non-treated plants ranged from 4.1 at 15C to 24.1 at 35C. This values agrees closely with those reported by Werner et al, (1988) for 'Redhaven' peach. DARD values of cyanamide treated plants were dependent on post-treatment temperatures. At 35C, DARD values of 0.125M and 0.25M H_2CN_2 treated plants were similar to controls, while at 25C, concentrations of 0.25M or higher delayed budbreak. In contrast, at 15C H_2CN_2 treated plants had higher DARD values than control plants. Similar observations in inducing early bud opening, but inhibiting final percentage of budbreak by applying hydrogen cyanamide during quiescence have been reported for several deciduous fruit species (Williams and Smith, 1984).

Final budbreak in control plants decrease as temperature increase. Higher temperatures were reported to possibly induce aberrations in the metabolism that, under certain conditions, is expressed as a reduction in growth or yield of the plant (Hale and Orcutt, 1987). In contrast, H_2CN_2 treated plants produced higher budbreak with increasing temperature. Similar results were reported after application of oil+DNOC to break rest in apples (Erez, 1979). This effect of temperature was attributed to changes in respiration rate and the induction of temporary anaerobic conditions (Erez et al, 1980).

When H_2CN_2 treatments were compared within each temperature, no differences in final percent budbreak were found at the higher temperature between control and the lowest cyanamide concentrations. At the lowest temperature, H_2CN_2 had an inhibitory effect on budbreak.

The reason for the differences in budbreak in response to temperature is not known, possibly the higher temperatures enhances the activity of the chemical, as has been shown for the oil+DNOC applications (Erez, 1979). Another possible explanation is faster evaporation of the chemical from the surface of the tissue at the higher temperature allowing less chemical uptake.

The post-treatment temperature interacted with H_2CN_2 and increased the toxicity of peach buds. Interestingly, peach plants were more resistant to H_2CN_2 treatment at the 25C post-treatment condition. Cooler (15C) or warmer temperatures (35C) increased the percentage of dead buds. These data agree with those published by Erez (1987) for 'Redhaven' peach. An explanation of how temperature influences the effect of hydrogen cyanamide treated plants is not clear.

The enzyme, Glutathione reductase (GR) is thought to play an important role in the protection of the plants from both high and low temperature stresses by preventing the oxidation of enzymes and membranes (DeKok and Oosterhuis, 1983; Haliwell and Foyer, 1978; Leshem, 1985; Smith, 1989). It is possible that a temperature-induced decline in GR activity could adversely affect the metabolism of the plant and thereby increase its susceptibility to different stresses. Alterations in glutathione levels induced by low temperatures and after H_2CN_2 treatment has been shown in cherry seeds and peach buds (Siller-Cepeda, 1991). Recently, it has been suggested that the protective activity of GR may be limited to species-specific thermal ranges (Mahan et al, 1990). The apparent K_m of GR of three representative species of cool, moderate, and warm environment was found to vary up to 9-fold between 12.5C and 45C. Such thermal limitations may play a role in the ability of the plant to withstand both low and high temperature stresses.

Thus the variable results found in the applications of hydrogen cyanamide in this study could be partially explained by the post-

treatment temperature. It appears that the physiological stage of the bud (Siller-Cepeda et al., 1991a) as well as the post-treatment environment are decisive parameters in defining the narrow limits between optimum and phytotoxic effect of the chemical treatments. The results presented here suggests that: 1) the effectiveness of hydrogen cyanamide applications depends on the post-treatment environment; 2) higher temperatures increases budbreak; 3) phytotoxicity is less at 25C that either 15C or 35C temperatures.

Table 5.1 Analysis of variance of the effect of hydrogen cyanamide concentration and post-treatment temperature on percent of budbreak, and phytotoxicity of 'Redhaven' peach buds.

		Budbreak (%)		Phytotoxicity (%)	
Source	df	MS	F	MS	F
Cyanamide	4	9228.0	157.2**	12461.0	305.5**
Temp.	2	387.6	6.6**	1165.4	28.5**
Cyanamide X Temp.	8	292.7	4.9**	143.3	3.5*
Error	45	58.6		40.7	

* significant at p=0.005
** significant at p=0.001

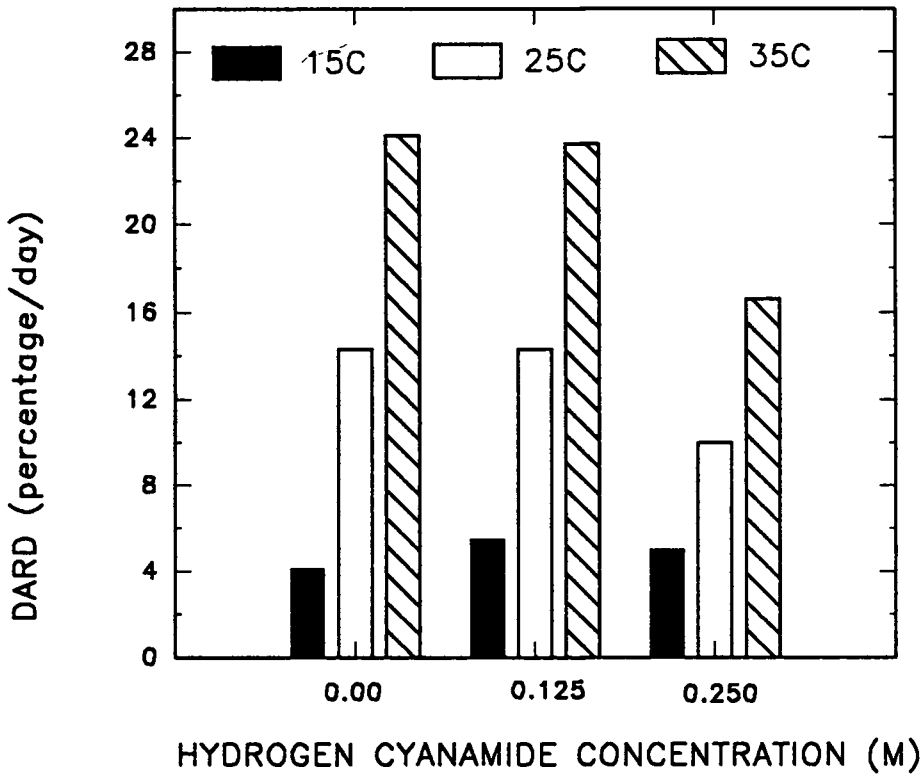

Figure 5.1. Daily average rate of development of 'Redhaven' peach buds as affected by hydrogen cyanamide concentration and post-treatment temperature.

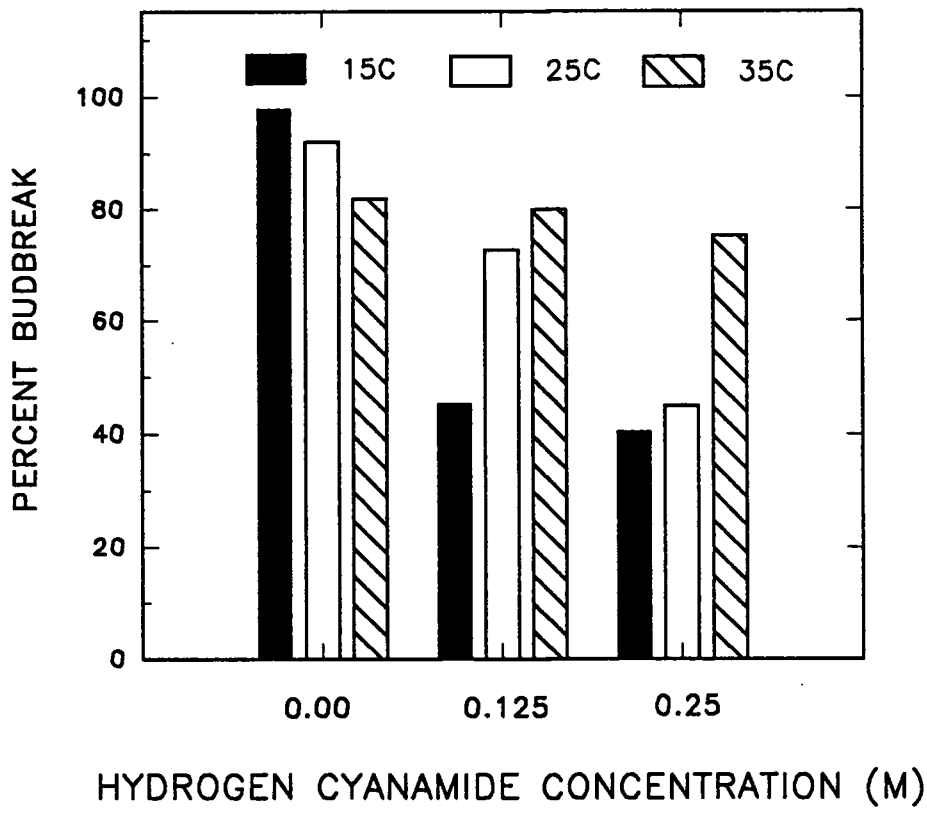

Figure 5.2. Percent of budbreak of 'Redhaven' peach trees as affected by hydrogen cyanamide concentration and post-treatment temperature after four weeks of forcing conditions.

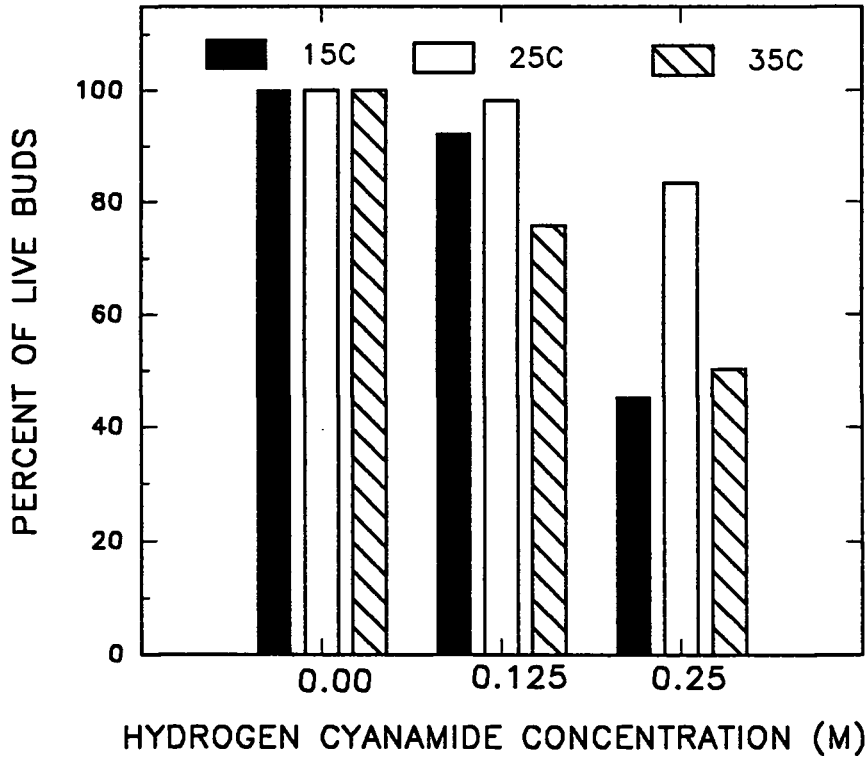

Figure 5.3. Percent of buds alive of 'Redhaven' peach trees as affected by hydrogen cyanamide concentration and post-treatment temperature after four weeks of forcing conditions.

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6.0 GLUTATHIONE CONTENT IN 'REDHAVEN' PEACH BUDS IN RELATION TO DEVELOPMENT AND RELEASE OF REST.

6.1 ABSTRACT

Reduced (GSH) and oxidized (GSSG) glutathione contents were determined in one year old potted peach (*Prunus persica* L.) trees during development and release of rest. The content of GSH and GSSG changed with the accumulation of chilling units. In the early phases of rest, the content of GSH and GSSG decreased, and then increased at maximum rest. Content of GSH continued to increase and peaked on 1 Dec at 860 chill units, and then dropped during the quiescent stage. It appears that the increase of GSH induced by chilling was closely associated with the end of rest. In contrast, GSSG showed a continuous increase from Oct to Dec. Five concentrations of cyanamide (0, 0.125, 0.25, 0.5, and 1.0 M) were applied on 1 Oct, 15 Oct, 1 Nov, 15 Nov, 1 Dec, and 15 Dec, 1990. Cyanamide treatments caused a rapid depletion of GSH within 12 hr followed by a large increase 24 hr after treatment, whereas the non-treated plants showed a relatively constant level of GSH and GSSG during this time. The changes of GSH induced by cyanamide were inversely related to cyanamide concentration applied. It appears that the extent of GSH change was dependent on the physiological status of the bud and cyanamide concentration. At maximum rest the plant were more resistant to cyanamide and this coincided with the highest level of cyanamide induced GSH.

6.2 INTRODUCTION

In temperate deciduous fruit trees, the rest period is overcome by exposure to low "chilling" temperatures (Brown et al, 1967; Samish, 1954; Saure, 1985). Although chilling requirement have been well established for most cultivars, little is known about specific physiological or biochemical events that are involved during the development and release of rest.

In addition to low temperatures, there are numerous chemicals and physical treatments that can overcome rest (Doorenbos, 1954; Erez, 1987; Erez and Lavee, 1974; Hosoki et al., 1986; Fuchigami and Nee, 1987; Wang et al., 1986). Among them, hydrogen cyanamide has been shown to be especially valuable to overcome rest in several species of deciduous fruit trees (Erez, 1987; Nee, 1986; Nir et al., 1986; Shulman et al., 1983; Siller-Cepeda et al., 1991a). However, the mode of action and the effect of hydrogen cyanamide on tree physiology are not well understood, except that near lethal dosages of the chemicals are usually required to obtain the desired response (Erez, 1987; Fuchigami and Nee, 1987).

Taylorson and Hendricks (1977) have shown that many of the rest breaking chemicals inhibit catalase activity in seeds, and allow activation of certain peroxidases, promoting mitochondrial respiration. In grapevine and peach floral buds, the changes in catalase activity induced by low temperatures have been associated with the rest period (Nir et al., 1984, 1986; Kaminski and Romm, 1974). Catalase activity increased when the buds develop rest, and subsequently decreased at the end of the rest period. Nir et al. (1986) hypothezised that the increased peroxide levels in the bud tissue favor a shift from the Embden-Meyerhoff Parnas system (EMP) to the pentose phosphate pathway (PP), leading to an increase in reduced

nucleotide production.

In contrast to Nir et al. (1984) hypothesis, Amberger (1984) believes the resulting accumulation of hydrogen peroxide leads to a higher levels of peroxides and glutathione. This can then affect the development of rest either directly, or indirectly by the activation of peroxidases including more specific oxidases such as ascorbate oxidase which is closely connected with the glutathione redox system. Therefore, glutathione may play an important role in regulation of plant dormancy (Fuchigami and Nee, 1987).

Glutathione (γ-glutamyl-cysteinyl-glycine) is widely distributed in plant cells (Rennenberg, 1982). It is present in the reduced (GSH) and oxidized (GSSG) form. In most living organisms glutathione is present predominantly in the reduced form (GSH) (Rennenberg, 1982). Evidence has been presented that glutathione functions in sulfur storage and transport (Rennenberg, 1982); detoxification of H_2O_2 in the chloroplast (Foyer and Halliwell, 1978); detoxification of xenobiotics such as herbicides (Matringe and Scalla, 1988); of air pollutants such as ozone and sulfur dioxide (Mehlhorn et al., 1986; Heath, 1987; Alscher and Amthor, 1988), and of heavy metals (Rennenberg, 1987). It is also implicated in the adaptation of plants to environmental stresses such as drought and extremes of temperature (Esterbauer and Grill, 1978; Kuroda et al., 1990; deKok and Oosterhuis, 1987; Burke et al., 1985; Wise and Naylor, 1987).

In animal cells, GSSG has been shown to inhibit protein synthesis whereas the amount of GSH present did not affect this process (Kosower and Kosower, 1974). GSSG converts an initiation factor of protein synthesis into an inactive form (Kosower et al., 1972). In plant cells, the GSSG content of dry wheat embryos is high, but declines early during germination (Fahey et al., 1980). A decrease in the GSSG content appears to be necessary early in germination to obtain optimal protein synthesis during growth and development.

In temperate plants, it has been shown that GSH undergoes seasonal changes with higher levels during rest and lower levels during active growth (Esterbauer and Grill, 1978; Kuroda et al., 1990; Siller-Cepeda et al., 1991). Although, direct evidence that glutathione alone and from yeast extracts overcome rest has been provided in various plant species (Guthrie, 1941; Nee, 1986), its role in overcoming rest of deciduous fruit tree buds has not been characterized.

Fuchigami and Nee (1987) speculated that glutathione thiol-disulfides are involved in overcoming rest of temperate crops. They have hypothesized that the ratio of reduced glutathione (GSH) to oxidixed glutathione (GSSG) directly influences the ratio of protein-bound reduced thiols (PSH), to protein oxidized thiol (PSSG), and this is thought to govern the degree or intensity of rest. They also hypothesized that dormancy breaking chemicals cause a conversion of GSSG to GSH, leading to increases in the PSH:PSSG ratio, the activation of protein synthesis and finally budbreak.

The purpose of this study was to determine the relationship between the rest period and glutathione in flower and vegetative buds of peaches. For this purpose, we quantified the changes in GSH and GSSG under natural conditions during the development and release of rest. In addition, glutathione levels induced by the rest breaking chemical, hydrogen cyanamide, were sudied throughout the rest period.

6.3 MATERIALS AND METHODS

One year-old potted peach trees were used in this study. Propagation and growth of the plant material was described previously (Siller-Cepeda et al., 1991a). Plants were grown under natural conditions at Corvallis, OR., and pruned to a single stem in July. At the end of the growing period, plants averaged 1.5 m in height and had an average of 150 lateral and 4-8 branch terminal buds.

At each treatment date, plants were sprayed to run-off with distilled water (control) or H_2CN_2 solutions at concentrations of 0.125, 0.25, 0.50 and 1.0 M. Dates of H_2CN_2 application were: 1 Oct, 15 Oct, 1 Nov, 15 Nov, 1 Dec, and 15 Dec. After application the plants were moved to greenhouse conditions with 25 ± 3C day/20 ± 3C night temperatures. Radiation sources in the greenhouse consisted of natural daylight and 400-W high pressure sodium lamps (Energy Technics, York, Pa.) that provided a PAR level of ≈ 400 to 500 $\mu mol \cdot s^{-1} \cdot m^{-2}$ for 16 hr/day.

Chilling units were calculated according to the model of Richardson et al (1974). Chilling was considered satisfied when 50% of the buds had broken after four weeks of forcing conditions (Weinberger, 1950). At each sampling date, the chilling accumulated was determined, and the degree of rest was expressed as percent budbreak after four weeks of forcing in greenhouse. An estimate of the growth stage during rest development was determined and expressed as degree growth stage (°GS) according to Kobayashi et al (1982) model.

For the determination of glutathione contents, bark tissue (1.0 g fresh weight) and/or buds (0.5 to 1.0 g fresh weight, ≈ 100 to 150 buds) were collected from each plant (3 replications) at two weeks intervals from 1 Oct to 15 Dec. On the first application date (1 Oct), measurements of glutathione after application of hydrogen cyanamide

were determined at 0, 12, 24, and 36 hr after treatment. At the other sampling dates, samples were collected 24 hr after hydrogen cyanamide treatment. The budbreak and phytotoxicity induced by hydrogen cyanamide at several rest stages was described previously (Siller-Cepeda et al., 1991a).

The procedures for the extraction and quantification of glutathione were done as described previously (Siller-Cepeda et al., 1991). Briefly, tissue was homogenized in 4 ml of 10% perchloric acid containing 1mM of BPDS (bathophenanthroline disulfonic acid). The homogenate was centrifuged at 7,800x g for 20 min at 4C. The supernatant was collected, and the tissue homogenized and centrifuged twice more. The supernatants from the three extraction were mixed and a 0.5 ml aliquot was added to 50 ul of 0.5 mM γ-glutamyl-glutamate (an internal standard), carboxymethylated with iodoacetic acid, and derivatized with 2,4-dinitro-1-flourobenzene as described previously (Siller-Cepeda et al., 1991). Separation and quantification of the reduced and oxidized forms of glutathione were carried out in a Beckman Gold HPLC system. A 3-aminopropyl-Spherisorb column (4.6 x 200mm, 5u) (Custom L.C. Inc Houston Tx.) was operated at ambient temperature with a flow rate of 1.5 ml/min. Absorbance of the dinitrophenol derivatives was monitored at 365nm. Elution was carried out as described previously (Siller-Cepeda et al., 1991).
Treatments were arranged in a completely randomized design with factorial arrangement for hydrogen cyanamide concentration and time of application. Data is presented as the mean of three replications ± standard error.

6.4 RESULTS

Budbreak and °GS. The onset of rest, 180°GS (endodormancy) was attained on 1 Oct (Fig. 1). Trees acquired maximum rest, 270°GS, on 1 Nov (320 CU), and chilling satisfaction, 315°GS, occurred on 1 Dec after 860 CU had been accumulated (Fig. 1).

Cyanamide (0.125M) induced earlier and higher budbreak of unchilled and partially chilled trees (Fig. 1). The budbreak effect of cyanamide on unchilled trees (1 Oct, 180°GS) was similar to that of control trees that had satisfied its chilling requirement (1 Dec). The best results of cyanamide induced budbreak was obtained at 536 CU and 860 CU. At 1146 CU (340°GS), when the trees had already satisfied their chilling requirement and were in the quiescent state (ecodormancy), cyanamide had no significant effect on budbreak (Fig. 1).

Glutathione content under natural conditions. Higher contents of GSH were observed in buds collected on 1 Oct (0 CU) than 15 Oct when the plants received 96.5 CU (Fig. 2). After the initial decrease in GSH contents, GSH contents increased six-fold on 1 Nov, and continued to increase sharply until 1 Dec (Fig. 2). GSH contents decreased significantly during the quiescent period (Fig. 2). A similar trend was observed in buds of 'Veteran' peach trees sampled during autumn-winter 1989, in that GSH contents increased with rest development attaining peak values at the end of rest, 315°GS, and decreased during the quiescent period (data not shown).

Contents of GSSG were considerably lower than GSH contents throughout the rest period (Fig. 2). The general trend in GSSG contents were similar to those observed for GSH, except for the 15 Dec treatment period. Rather than decreasing GSSG contents continued to increase on 15 Dec during the quiescent period. The ratio of GSH to GSSG was high on 1 Oct (50:1) and was not calculated for 15 Oct

because GSSG levels were not detected (Table 3). Ratio of GSH to GSSG from 1 Nov to 1 Dec was lower than unchilled trees (≈ 16:1), but remained fairly constant. On 15 Dec, the ratio GSH to GSSG dropped (3:1) and was significantly lower than an any other sampling date (Table 3). In bark tissue, a similar trend was observed in GSH and GSSG levels at all sampling dates, but the contents were significantly lower than in buds (data not shown).

Glutathione content in hydrogen cyanamide treated plants. Hydrogen cyanamide treatments on 1 Oct resulted in a rapid decrease in GSH and GSSG contents of the buds within 12 hr of application (Fig. 3A, B). GSH was depleted completely in all but the lowest cyanamide concentration (0.125M), and no GSSG was detected during this period. Twenty-four hours after application a significant increase in GSH contents was observed. The change was inversely proportional to hydrogen cyanamide concentration (Fig. 3A). GSSG contents increased significantly 24 hr after treatment only in the lowest hydrogen cyanamide concentrations (0.125 and 0.25M) (Fig. 3B). Contents of GSH decreased in all but the lowest cyanamide concentration 36 hrs after application (Fig. 3A). After 36 hr GSSG declined significantly and was only detected in the 0.125M cyanamide treatment (Fig. 3B). Non-treated plants showed a relatively constant content of GSH and GSSG during this period of time. In the bark tissue, a similar pattern was observed (Table 1). GSH and GSSG were completely depleted 12 hr after hydrogen cyanamide application. After 24 hr the contents of GSH and GSSG in the 0.125M cyanamide treated plants were again detectable, however the contents were still below the contents of the control. The contents of GSH and GSSG in the bark tissue of 0.125M cyanamide treated plants continued to increase 36 hr after the treatment reaching contents similar to the non-treated plants.

Hydrogen cyanamide treatments ranging from 0.125 to 0.25 M increased GSH contents in the buds collected 24 hr after treatment at

all application dates (Fig. 4). Hydrogen cyanamide treatments ranging from 0.5 to 1.0 M increased GSH contents only from onset of rest (1 Oct) to maximum rest (1 Nov). Contents of GSSG in the buds of hydrogen cyanamide treated plants were detected only in the lowest concentrations (0.125 and 0.25M) at all treatment dates, but 1 Nov. (data not shown). In general, the higher the cyanamide concentration, the lower the change in GSH contents induced by cyanamide. On 1 Nov (320 CU), the contents of GSH induced by 0.5 and 1.0 M cyanamide was higher than at any other application date (Fig. 4). This treatment date correlated with the time of lowest cyanamide induced phytotoxicy (Table 1). On 1 Dec (860 CU), the lowest cyanamide concentrations (0.125 and 0.25 M) induced the highest GSH contents (Fig. 4). Application of cyanamide on 15 Dec caused a decrease in GSH contents of 0.125 M treated plants as compared to the contents observed when cyanamide was applied at 860 CU (1 Dec).

6.5 DISCUSSION

Overcoming rest. The process of breaking rest in buds of temperate deciduous fruit trees is triggered by chilling temperatures (Samish, 1954; Vegis, 1964). The chilling requirement to overcome rest depends upon the genetic makeup of the plant (Nooden and Weber, 1978; Powell, 1986; Saure, 1985; Samish, 1954). A traditional method of determining the satisfaction of the chilling requirement is when 50% of the buds had broken after four weeks of forcing conditions (Weinberger, 1950). Our data shows that 50% budbreak occurred on 1 Dec when 860 CU had accumulated, and 90% budbreak on 15 Dec after 1146 CU (Fig. 1).

Our studies confirm those of others that hydrogen cyanamide is effective in releasing rest in unchilled and partially chilled peach buds (Erez, 1987; Fernandez-Escobar and Martin, 1987). We demonstrated that lower cyanamide concentrations applied close to chilling satisfaction were more effective in overcoming rest in peaches (Fig. 1) (Siller-Cepeda et al., 1991a).

Glutathione changes during rest development. The contents of GSH and GSSG in the buds changes with the development of rest (Table 3). During the early phases of rest development, when rest intensity increased, the contents of GSH and GSSG decreased (15 Oct) and then increased at maximum rest (1 Nov). Contents of GSH continued to increase and peaked on 1 Dec after 860 CU, decreasing on 15 Dec when the buds were in quiescence. In contrast, contents of GSSG did not drop during quiescence and were higher on 15 Dec.

In plant cells, the GSSG content of dry wheat embryos is high, but declines early during germination (Fahey et al., 1980). GSSG converts an initiation factor of protein synthesis into an inactive form (Kosower et al., 1972). Although a decrease in the GSSG content

appears to be necessary early in germination to obtain optimal protein synthesis during growth and development (Fahey et al., 1980), we did not see the highest GSSG contents when the plants were in deep rest in our study. Our results show that low contents of GSSG were present at maximum rest (270°GS, 1 Nov) and highest contents were detected at the quiescent period (340°GS, 15 Dec)) when plants broke 90% of the buds (15 Dec).

The possibility that chilling overcomes rest by imposing a stress on resting tissues has not been considered previously in temperate deciduous trees. It is known that elevation of oxidative processes lead to the accumulation of high levels of GSSG (Schmidt and Kunert, 1986; Smith et al., 1984, 1985). Evidence exist in woody plants that GSSG levels increases when plants are exposed to periods of low temperature (Guy and Carter, 1984, Guy et al., 1984). Since no temperatures below freezing were observed during the period of this study (Fig. A.3, 4, 5), the slow and continuous increase in GSSG levels as plants acquired more chilling suggests that plant tissues may have experienced oxidative stress. One possible explanation is that the accumulation of chilling units to break rest may be a form of "stress" response in deciduous fruit trees. Similar observations were also observed in the changes in glutathione contents in cherry seeds during stratification (Fig. A.1). Stratification of the cherry seeds at 4C for 8 weeks overcame dormancy and caused the level of GSH and GSSG to increase. In contrast, the seeds held at 20C during the same period of time did not have significant changes in GSH and GSSG (Fig. A.2).

Additional evidence, suggesting that chilling is a stress factor in the breaking of rest, is the changes in GSH content associated with chilling accumulation and cyanamide treatments. The role of GSH as an antioxidant is well documented (Alscher, 1989). GSH participates as the substrate of reactions catalyzed by dehydroascorbate reductase and

glutathione peroxidase, which results in the disposal of peroxides and the formation of GSSG. The GSSG formed in the above mentioned reaction is reduced to GSH by glutathione reductase in an NADPH dependent reaction, so that the removal of H_2O_2 is linked to the activity of the pentose phosphate cycle (Foyer and Halliwell, 1978; Sagisaka and Asada, 1981; Sagisaka, 1982). Recently, Kuroda et al. (1990a) have clearly shown that the process of cold acclimation in apple trees is closely related to increases in the activities of the peroxide-scavening systems of glutathione and ascorbate metabolism, in conjunction with the activities of enzymes in the pentose phosphate cycle. Wise and Naylor (1987) demonstrated the protective role of GSH to cold stress in a chilling-sensitive specie (cucumber) and a chilling-resistant specie (pea). Thus, it appears that the high level of GSH observed as the tissue accumulates more chilling units functions primarily in allowing cells to adjust to stress conditions.

Increases in GSH levels and glutathione reductase activity induced by low temperature have been reported in several species (Esterbauer and Grill, 1978; Guy et al., 1984; Kuroda et al., 1990b; Sagisaka, 1985). However, the association with the rest status of the plants have not been presented. Our results indicate a close relationship between reduced glutathione levels and the chilling accumulation to end rest. Particularly, the peak in GSH in two different peach cultivars coincided with the end of rest (315°GS), yet there was differences in chilling units required to break rest (Brooks and Olmos, 1972; Richardson et al., 1974; Weinberger, 1950).

Our results show that chilling induced significantly higher GSH levels in the bud tissue than in the bark tissue. This information support the idea that the chilling requirement is localized in the bud, and the stimulus is not translocable (Samish, 1954; Perry, 1971).

It is not known how chilling overcomes rest in deciduous fruit trees. Fuchigami and Nee (1987) speculated that glutathione thiol-

disulfides are involved in overcoming rest. They hypothesized that the degree of rest was a function of the ratio of PSG:PSSG and GSH:GSSG. The degree growth stage model was utilized to describe the status of glutathione thiol-disulfides in their studies. Results presented in table 3, shows that the ratio GSH:GSSG is high as plants approached the onset of rest on 1 Oct (≈ 180°GS). The GSH:GSSG ratio was difficult to estimate on 15 Oct (≈ 240°GS) because GSSG levels were below the detection limits. From 1 Nov (≈ 270°GS, maximum rest) when the lowest budbreak was observed to 1 Dec (315°GS, end of rest) when 50% of the buds broke the ratio of GSH:GSSG was high and remained fairly constant. On 15 Dec (≈ 340°GS) the ratio was the lowest and the peach trees broke 92% of the buds . Thus, these results does not support the hypothesis proposed by Fuchigami and Nee (1987).

Glutathione changes after cyanamide applications. The inhibition of catalase by chilling and hydrogen cyanamide treatments observed by Shulman et al (1986) was not associated with increases of peroxidase activity, thus, the plants must rely on another detoxification system to dispose of the peroxides formed. Amberger (1984) believes that the inhibition of catalase by cyanamide results in a higher content of peroxides and also of glutathione contents, which in turn can affect the metabolism involved in rest development. Our results demonstrated that hydrogen cyanamide induced rapid changes in the GSH content of peach buds. The depletion of GSH levels within 12 hr after cyanamide application suggests that the GSH present in the tissues was probably utilized in the detoxification of H_2O_2 formed by the inhibition of catalase as suggested by Amberger (1984).

The pathway of detoxification of H_2O_2 in which glutathione is involved is coupled to the ascorbate-GSH cycle (Foyer and Halliwell, 1976). This pathway involves the reduction of GSSG to to GSH by glutathione reductase in a NADPH dependent reaction (Halliwell and Foyer, 1978). Since the most likely candidate for the compensatory

removal of H_2O_2 is GSH, as demonstrated in houseflies (Allen et al., 1983) and in catalase-deficient barley (Smith et al., 1984), the decrease in levels of GSH observed may be due to an increase in cellular concentrations of H_2O_2 caused by hydrogen cyanamide application.

GSH acts as a feedback inhibitor of its own synthesis, thus removal of GSH will sufficiently enhance its production (Meister and Anderson, 1983; Rennenberg, 1982). The observations of the rapid depletion of GSH levels within 12 hrs of application support the idea that the function of the initial GSH is to detoxify peroxide formation by the way of the ascorbate-GSH cycle. The stimulation of the synthesis of GSH by H_2O_2 has been suggested by Smith et al (1984). Therefore, the release of the feedback inhibition of GSH synthesis through an initial decrease in GSH by H_2O_2 may be responsible for the subsequent increase of GSH levels observed 24 hrs after application (Fig 3A). The increased levels of GSH observed after 24 hrs were inversely proportional to the concentration of hydrogen cyanamide applied. After 36 hr, the levels of GSH in all hydrogen cyanamide concentrations, but 0.125 M started to decline due to toxicity effects (Fig. 3 and Table 2).

It has been suggested that the requirement of different concentrations of cyanamide to break rest at the various growth stages is a direct function of the plants 'resistance to stress' (Fuchigami and Nee, 1987). Reduced glutathione levels increased at all dates after cyanamide application. The extent of GSH changes was dependent on the physiological status of the bud and the cyanamide concentration. At maximum rest, 270°GS, the plants were more resistant to cyanamide and this coincided with the period of highest induced GSH levels as compared to all application dates (Fig. 4 and Table 2). In addition, higher levels of induced GSH were found when cyanamide was applied at 860 CU rather than at 536 CU, when phytotoxicity was

reduced (Siller-Cepeda et al., 1991a). Thus, greater tolerance to cyanamide was found when the level of induced GSH was highest.

The effect of hydrogen cyanamide in ovecoming rest in several species of deciduous fruit trees is well documented (Erez, 1987; Lavee et al., 1984; Nir et al., 1986; Shulman et al., 1983). In peaches lower concentrations (0.125 and 0.25M) of hydrogen cyanamide gave the best results in breaking rest when applied close to chilling satisfaction (Siller-Cepeda et al., 1991a). Our data shows that the highest levels of GSH induced by hydrogen cyanamide were obtained at the lowest cyanamide concentrations (0.125 and 0.25M), near the end of chilling satisfaction (Fig. 4).

The information presented above seems to indicate that glutathione acts as an antioxidant, and/or by activation of metabolic processes in the termination of rest. Furthermore, GSH content and production may also play an important role in the response of plants to environmental and chemical stresses.

In conclusion, the results obtained in our study suggest that: 1) GSH and GSSG levels increases as a result of chilling accumulation; 2) GSH level induced by chilling is closely associated with the end of rest; 3) hydrogen cyanamide applications causes a rapid decrease in GSH levels followed by a large increase in GSH levels and; 4) the induced GSH levels 24 hr after application of hydrogen cyanamide may explain the budbreak and phytotoxicity observed during development and breaking of rest.

Table 6.1. Analysis of variance of the effect of hydrogen cyanamide concentration and time of application on the glutathione content of 'Redhaven' peach buds.

Source	df	MS	F
Cyanamide	4	697957.4	29.22**
Time of Application	5	53877.8	2.26ns
Cyanamide X Time of Application	20	29328.2	1.23ns
Error	60	23882.3	

ns no significant.
** significant at p=0.001

Table 6.2. Changes in GSH and GSSG content induced by hydrogen cyanamide applied on 1 Oct (0 CU) in the bark tissue of 'Redhaven' peach trees.

Time (hrs)	Cyanamide Concentration			
	0.0M		0.125M	
	GSH	GSSG	GSH	GSSG
	(nmol·gram dry wt)		(nmol·gram dry wt)	
0	256.0*	50.1	253.6	49.7
12	253.9	46.4	nd	nd
24	255.2	48.3	172.2	40.86
36	252.1	47.6	322.5	59.71

* values are the means of 3 replications.

Table 6.3. GSH content and phytotoxicity of 'Redhaven' peach buds induced by 0.5 and 1.0 M hydrogen cyanamide at several application dates.

Date and CU	0.5 M		1.0 M	
	GSH*	phytotoxicty**	GSH	phytotoxicity
1 Oct (0 CU)	271.2	35.6	220.3	87.8
15 Oct (96.5 CU)	492.8	41.6	135.9	89.8
1 Nov (320 CU)	586.7	33.0	381.8	52.1
15 Nov (520 CU)	254.2	56.2	250.9	93.1
1 Dec (860 CU)	412.5	48.2	240.0	84.1
15 Dec (1146 CU)	365.6	66.8	232.5	90.5

* GSH levels are expressed in nmol·gram dry wt.
** Phytotoxicity is expressed as the percent of dead buds.

Table 6.4. GSH and GSSG levels and the ratio GSH:GSSG in 'Redhaven' peach buds at different chilling units accumulated in relation to the estimated degree growth stage.

Date	CU*	% Budbreak	Estimated °GS	GSH**	GSSG**	GSH:GSSG***
1 Oct	0	4	180	95.0	1.8	50.3
15 Oct	96.5	2	240	20.3	nd	---
1 Nov	320	1	270	111.2	7.0	16.8
15 Nov	536	23	295	148.4	9.0	16.3
1 Dec	860	42	315	234.7	13.9	15.7
15 Dec	1146	92	340	57.0	18.6	3.0

* CU - Chilling units.
** GSH and GSSG content is expressed in nmol·gram dry wt.
*** GSH/GSSG ratio is the absolute value.

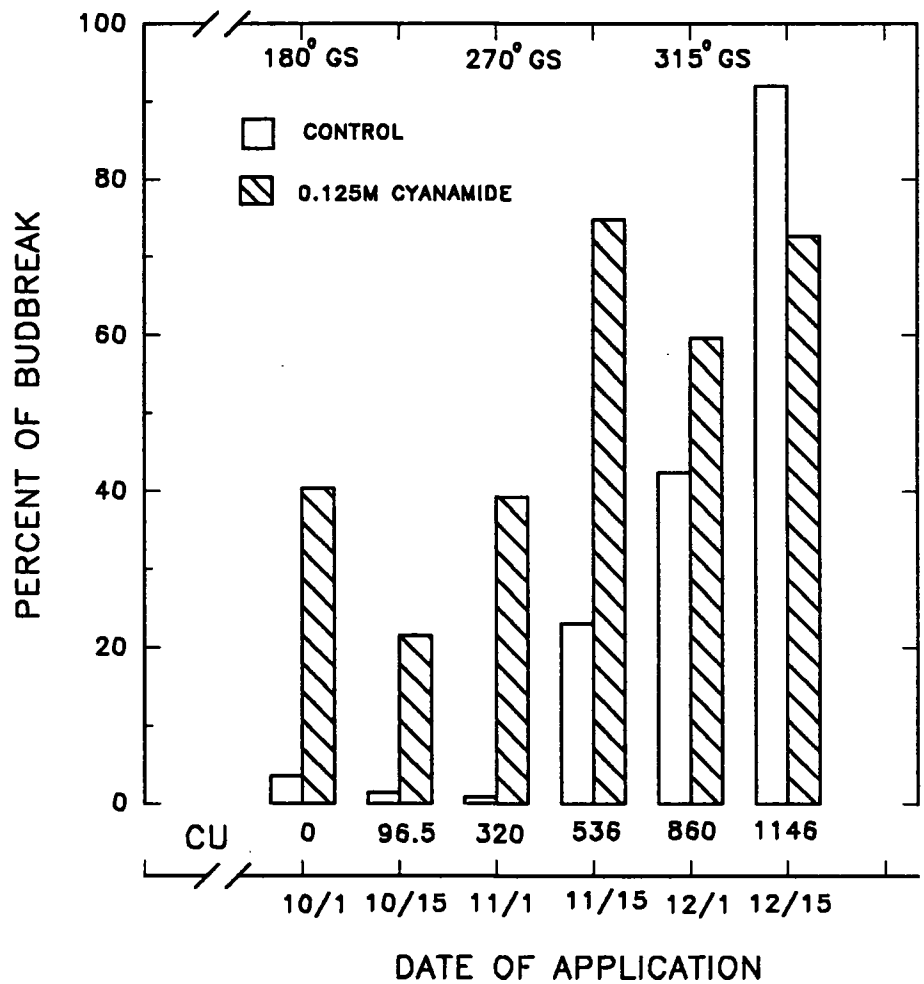

Figure 6.1. Percent budbreak of 'Redhaven' peach buds after different chilling units accumulated, and percent budbreak induced by 0.125 M hydrogen cyanamide.

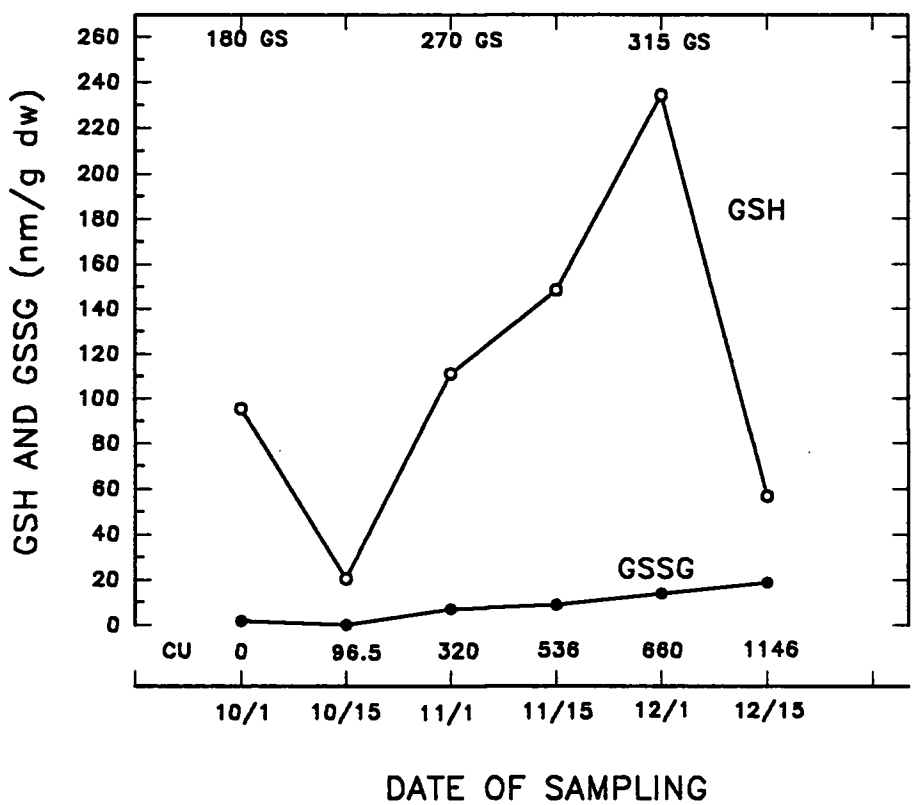

Figure 6.2. GSH and GSSG profile during development and release of rest of 'Redhaven' peach buds.

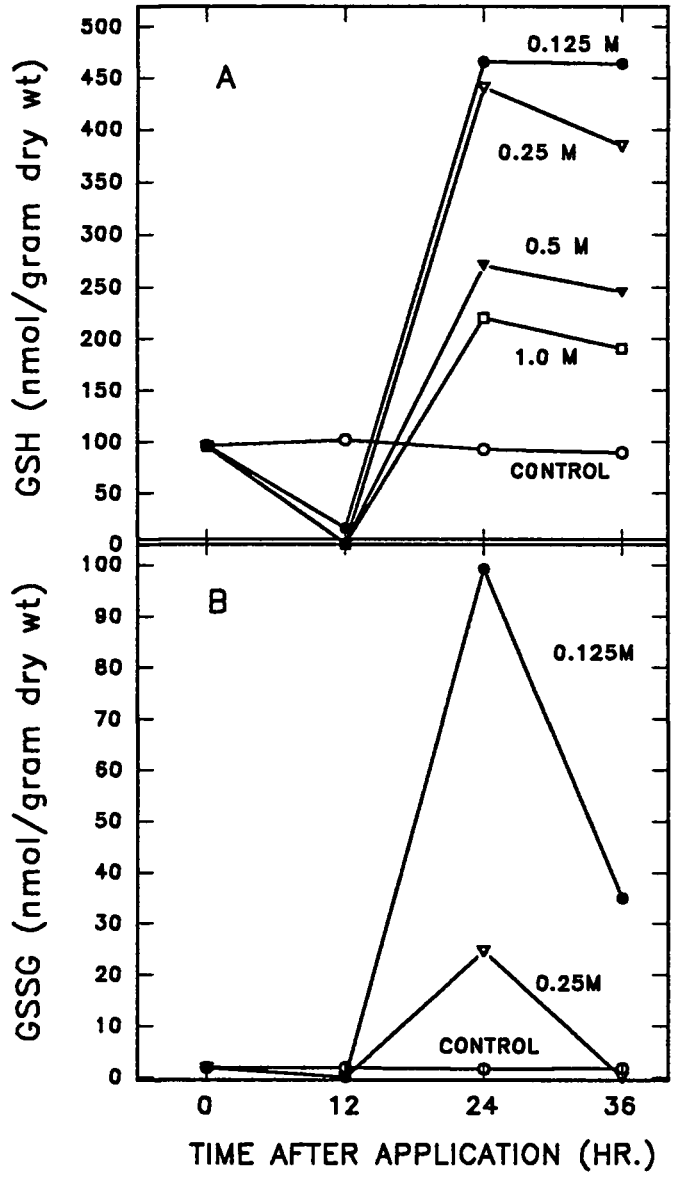

Figure 6.3. Time course of GSH and GSSG levels in the bud tissue as affected by several hydrogen cyanamide concentrations applied on 1 Oct to 'Redhaven' peach trees.

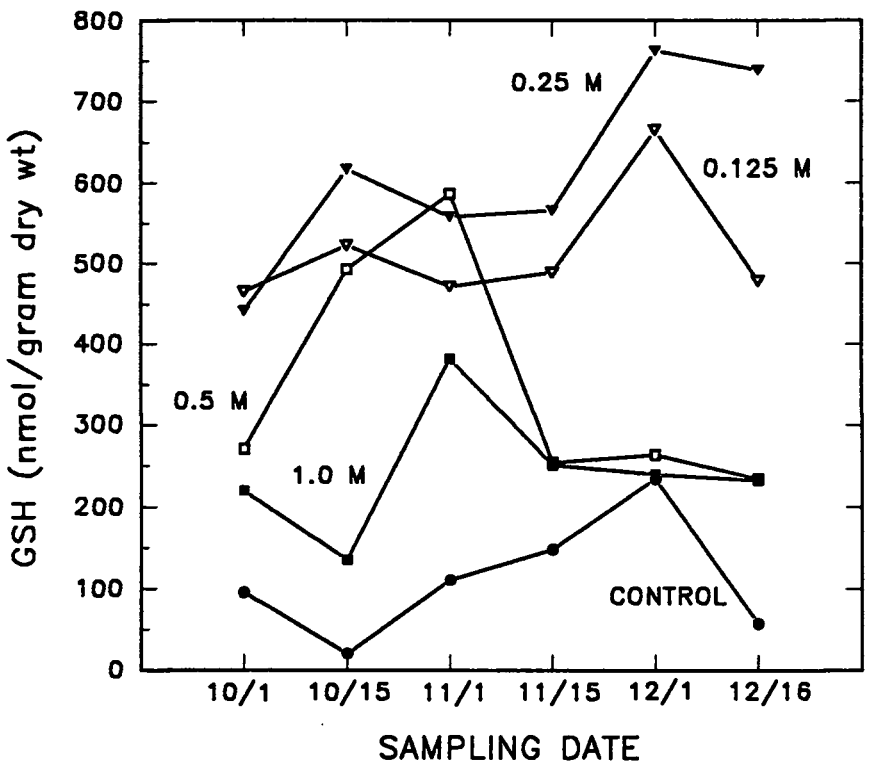

Figure 6.4. Levels of GSH induced by several hydrogen cyanamide concentrations 24 hr after the treatments were applied during development and release of rest.

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7.0. SUMMARY AND CONCLUSIONS

SUMMARY

1. Determination of glutathione by the HPLC Method.
   - i The HPLC method developed is reproducible, accurate, and sensitive to quantify both the reduced (GSH) and oxidized (GSSG) form of glutathione simultaneously.
   - ii This method was applicable to a variety of woody plant species and tissues. Three extractions are needed to remove GSH from woody plant tissues.
   - iii Total glutathione (GSH and GSSG) in the bark tissue of seven woody plant species tested were lower during the period of active plant growth, and higher during the dormant stage. In contrast, this change was not observed in grape. Reduced glutathione always represented a higher percentage of the total glutathione. All tested tissues of peach plants contained glutathione.
2. Rest phases of 'Redhaven' peach trees.
   - i Trees acquired the onset of rest (180°GS) before any chilling acquisition. Maximum rest (270°GS) was achieved after the plants were exposed to 320 CU. End of rest (315°GS) was reached after exposure of the plants to 860 CU, when 50% of the buds broke.
3. Budbreak induced by hydrogen cyanamide at different rest phases.
   - i Hydrogen cyanamide overcame rest of unchilled and partially chilled 'Redhaven' peach buds. Budbreak was dependent on concentration and time of application.
   - ii Application of cyanamide from the onset of rest (180°GS) to end of rest (315°GS) increased budbreak. Cyanamide induced earlier and higher percent budbreak during this period as compared to non treated plants. The most effective concentration (highest budbreak and lowest phytotoxicity) was 0.125M at all

treatment dates. The highest budbreak induced occurred during the post-rest period (295°GS to 315°GS).

iii During the quiescent period (340°GS) budbreak was dependent on post-treatment temperature and concentration. All cyanamide treatments reduced and delayed budbreak at all tested temperatures. This effect was greatest at the lowest temperature (15C) and at the highest cyanamide concentrations (0.5 and 1.0M).

4. Phytotoxicity induced by hydrogen cyanamide at different rest phases.

i Hydrogen cyanamide was phytotoxic to 'Redhaven' peach buds at all concentrations tested. The extent of phytotoxicty was dependent on concentration and stage of development.

ii Hydrogen cyanamide was less toxic when the plants were at maximum rest (270°GS). During the decreasing phase of rest (270°GS to 315°GS) phytotoxicity was variable in all, but the lowest cyanamide concentration (0.125M). Cyanamide concentration ≥ 0.5M was highly phytotoxic to 'Redhaven' peach buds.

iii The post-treatment temperature interacted with cyanamide concentration when applied during the quiescent stage (340°GS) and increased its phytotoxicty of 'Redhaven' peach buds. Cyanamide treatments at the 25C post-treatment condition induced less toxic effects. Cooler (15C) or warmer (35C) post-treatment temperatures increased the percent of dead buds.

5. Glutathione content during rest development.

i The contents of GSH and GSSG of 'Redhaven' peach buds changes with the development of rest. The level of GSH was considerably greater than GSSG throughout the rest period. During the early phases of rest development (180°GS to 240°GS) when rest intensity increased the contents of GSH and GSSG decreased, and then increased at maximum rest (270°GS). Content of GSH continued to

increase and peaked at the end of rest (315°GS), and then decrease when the buds were in quiescent stage (340°GS). In contrast, GSSG did not drop during quiescence and was higher at 340°GS.

ii GSH and GSSG content increases as a result of chilling accumulation. GSH content induced by chilling was closely associated with the end of rest (315°GS) when 50% of the buds broke.

6. Glutathione content in hydrogen cyanamide treated plants.

i Cyanamide treatments applied at the onset of rest (180°GS) depleted GSH content in all but the lowest cyanamide concentration (0.125M) within 12 hr of application. No GSSG was detected during this period. 24 hr after application a significant increase in GSH content 2 to 4 times greater than the initial GSH content was observed. The changes of GSH content induced were inversely proportional to cyanamide concentration. Non-treated plants showed a relatively constant GSH and GSSG contents during this period of time.

ii Cyanamide treatments increased GSH content of 'Redhaven' peach buds at all application dates. The extent of GSH changes was dependent on the physiological status of the buds and cyanamide concentration. In general, the higher the cyanamide concentration the lower the change in GSH contents induced by cyanamide, probably due to the toxicity of cyanamide. At maximum rest (270°GS) the contents of GSH induced by 0.5 and 1.0 M cyanamide were higher than any other application date. This treatment date coincided with the time of lowest cyanamide induced phytotoxicty.

7. Conclusions.

As with most investigations, the research reported in this thesis has generated more questions than it answered. Also some

explanation of the results could not be made because important pieces of information were not available.

Chapter 6 described for the first time the trend in glutathione content in peach buds and in bark tissue during development and release of rest but did not answer the question -- why does the content changes throughout the rest period ? Also, why does hydrogen cyanamide induced higher content of glutathione ? Although there are many reports on the relationship between glutathione and physical, environmental, and biological stresses, we do not know if the increases in glutathione by chilling or hydrogen cyanamide are the cause or an effect. Careful research is needed to test the hypothesis that chilling is a stress factor in deciduous fruit trees.

GSH content and production may play an important role in the response of plants to environmental and chemical stresses. The information presented in this research suggests that glutathione acts as an antioxidant in overcoming the toxic effect of hydrogen cyanamide, and/or by activation of metabolic processes in the termination of rest. Experiments are needed that influence the glutathione content in bud tissue and establish if the plants overcome rest when the glutathione concentration is artificially elevated or lowered.

A single direct approach to establish if glutathione is the cause or an effect of overcoming rest is not possible. Consequently, an approach with multiple experiments is necessary that test the hypothesis from different directions. Much basic and applied research is needed before we have a good knowledge of the metabolism and functions of glutathione in higher plants.

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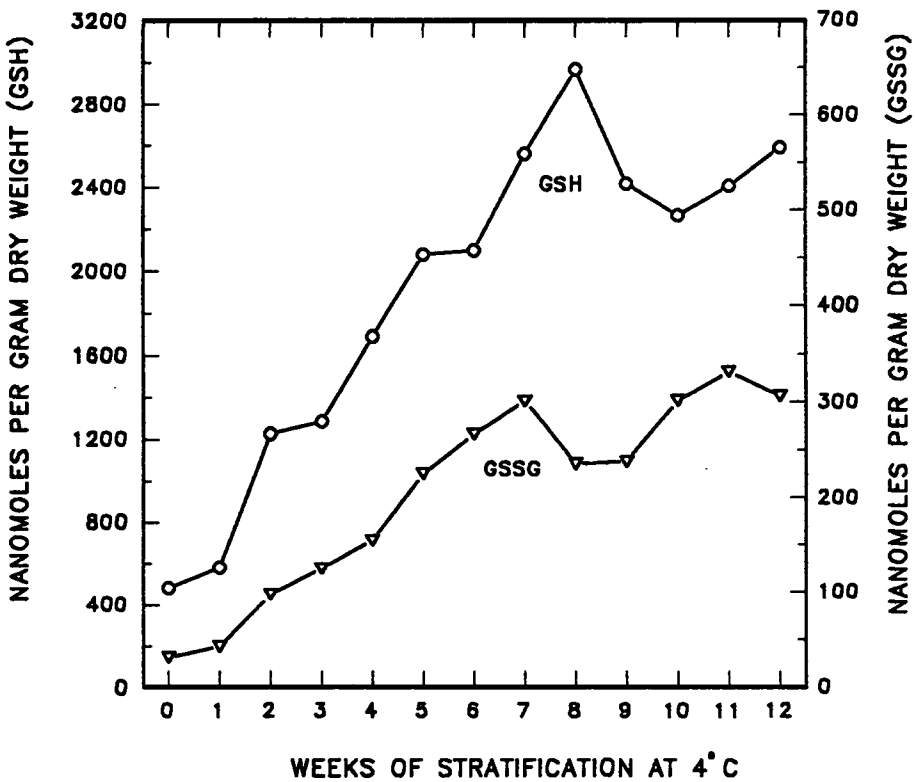
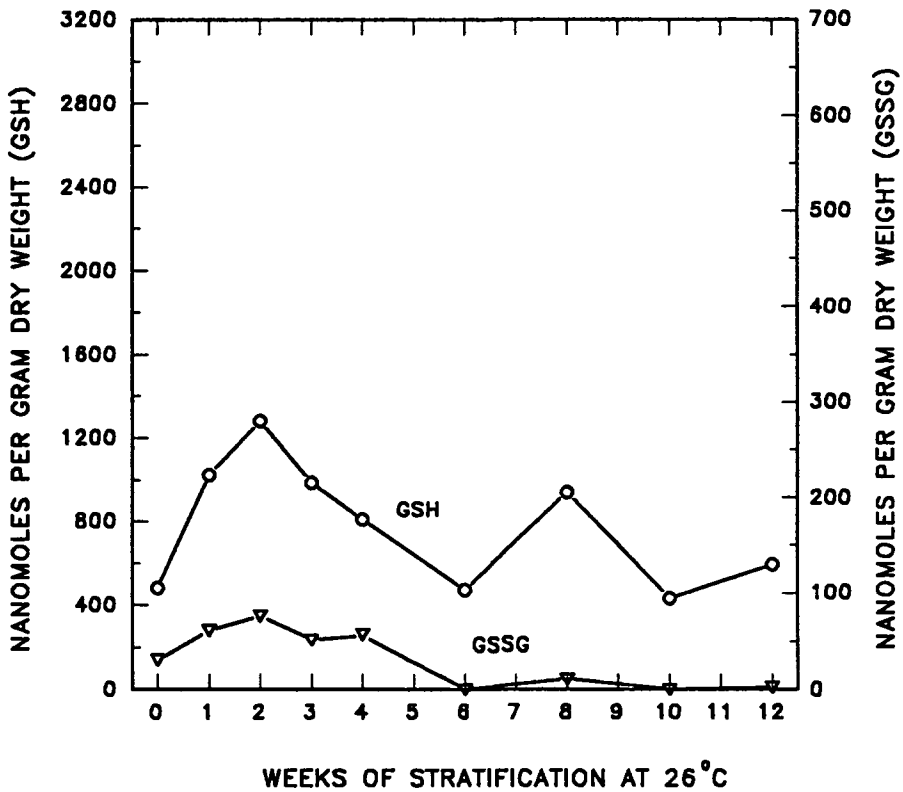
APPENDICES

Appendix A.1. Changes in GSH and GSSG content of cherry seeds induced by low temperature stratification.



Appendix A.2. Changes in GSH and GSSG content of cherry seeds induced by high temperature stratification.



Appendix A.3. Maximum and minimum temperatures and daily and accummulated chilling units from October 1990.

DATE	MAXIMUM	MINIMUM	AVERAGE	DAILY CU	CUMM CU
OCT 1	83	72	77.50	0	0 **
OCT 2	85	47	66.00	0	0
OCT 3	93	50	71.50	0	0
OCT 4	71	49	60.00	0	0
OCT 5	86	46	66.00	3.5	3.5
OCT 6	83	42	62.50	11.0	14.5
OCT 7	86	37	61.50	10.0	24.5
OCT 8	89	40	64.50	9.0	33.5
OCT 9	85	42	63.50	1.0	34.5
OCT 10	68	49	58.50	4.5	39.0
OCT 11	67	36	51.50	12.0	51.0
OCT 12	65	49	57.00	5.0	56.0
OCT 13	62	49	55.50	8.5	64.5
OCT 14	53	43	48.00	17.5	82.0
OCT 15	62	52	57.00	5.0	87.0
OCT 16	69	44	56.50	9.5	96.5
OCT 17	61	40	50.50	12.0	108.5
OCT 18	56	38	47.00	13.5	122.0
OCT 19	59	39	49.00	20.5	142.5
OCT 20	56	38	47.00	18.5	161.0
OCT 21	52	45	48.50	13.0	174.0
OCT 22	62	43	52.50	12.0	186.0
OCT 23	66	41	53.50	13.5	199.5
OCT 24	73	44	58.50	11.5	211.0
OCT 25	60	45	52.50	12.0	223.0
OCT 26	62	48	55.00	12.5	235.5
OCT 27	66	46	56.00	5.5	241.0
OCT 28	59	43	51.00	12.5	253.5
OCT 29	61	42	51.50	14.5	268.0
OCT 30	52	49	50.50	12.0	280.0
OCT 31	54	46	50.00	17.5	297.5

Appendix A.4. Maximum and minimum temperatures and daily and accummulated chilling units from November 1990.

DATE	MAXIMUM	MINIMUM	AVERAGE	DAILY CU	CUMM CU
NOV 1	53	40	46.50	22.5	320.0
NOV 2	51	37	44.00	22.5	342.5
NOV 3	54	45	49.50	17.5	360.0
NOV 4	59	48	53.50	9.0	369.0
NOV 5	57	39	48.00	21.0	390.0
NOV 6	50	36	43.00	23.0	413.0
NOV 7	51	43	47.00	18.0	431.0
NOV 8	56	48	52.00	8.5	438.5
NOV 9	59	51	55.00	5.0	443.5
NOV 10	64	50	57.00	7.0	450.5
NOV 11	63	42	52.50	14.5	465.0
NOV 12	57	44	50.50	16.0	481.0
NOV 13	53	44	48.50	17.0	498.0
NOV 14	53	40	46.50	20.0	518.0
NOV 15	59	41	50.00	18.0	536.0
NOV 16	57	35	46.00	20.5	556.5
NOV 17	48	42	45.00	24.0	580.5
NOV 18	51	38	44.50	16.5	597.0
NOV 19	46	35	40.50	20.0	617.0
NOV 20	47	39	43.00	24.0	641.0
NOV 21	49	43	46.00	21.5	662.5
NOV 22	52	47	49.50	17.0	679.5
NOV 23	52	47	49.50	14.5	694.0
NOV 24	54	50	52.00	12.0	706.0
NOV 25	52	39	45.50	22.0	728.0
NOV 26	49	36	42.50	22.0	750.0
NOV 27	50	42	46.00	22.5	772.5
NOV 28	48	33	40.50	19.5	792.0
NOV 29	47	40	43.50	24.0	816.0
NOV 30	49	34	41.50	21.0	837.0

Appendix A.5. Maximum and minimum temperatures and daily and accummulated chilling units from December 1990.

DATE	MAXIMUM	MINIMUM	AVERAGE	DAILYCU	CUMM CU
DEC 1	45	40	42.50	24.0	861.0 **
DEC 2	46	38	42.00	24.0	885.0
DEC 3	50	40	45.0	22.5	907.5
DEC 4	52	36	44.00	21.0	928.5
DEC 5	50	35	42.50	18.0	946.5
DEC 6	45	35	40.00	21.0	967.5
DEC 7	43	38	40.50	24.0	991.5
DEC 8	58	39	48.50	15.5	1007.0
DEC 9	49	48	48.50	12.0	1019.0
DEC 10	50	38	44.00	16.0	1035.0
DEC 11	48	37	42.50	20.5	1055.5
DEC 12	39	33	36.00	15.5	1071.0
DEC 13	44	34	39.00	16.0	1087.0
DEC 14	46	37	41.50	22.0	1109.0
DEC 15	51	33	42.00	18.0	1127.0
DEC 16	44	34	39.00	19.0	1146.0 **
DEC 17	52	43	47.50	15.0	1161.0
DEC 18	47	32	39.50	18.5	1179.5
DEC 19	32	17	24.50	0.0	1179.5
DEC 20	23	9	16.00	0.0	1179.5
DEC 21	23	5	14.00	0.0	1179.5
DEC 22	23	7	15.00	0.0	1179.5
DEC 23	28	15	21.50	0.0	1179.5
DEC 24	33	15	24.00	0.0	1179.5
DEC 25	36	29	32.50	2.0	1181.5
DEC 26	44	35	39.50	23.0	1204.5
DEC 27	45	37	41.00	24.0	1228.5
DEC 28	40	22	31.00	6.0	1234.5
DEC 29	29	16	22.50	0.0	1234.5
DEC 30	33	13	23.00	0.0	1234.5
DEC 31	38	29	33.50	0.0	1234.5