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Review

Spring frosts in deciduous fruit trees — morphological damage and flower hardiness

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Abstract

Freeze injury is one of the main limiting factors to crop production and distribution of horticultural crops. Despite the numerous research efforts devoted to reduce freezing injury, it still accounts for greater losses of fruits and vegetables than any other environmental or biological hazard and the ultimate causes determining cold hardiness remain uncertain. In temperate climates, important damages on deciduous fruit trees are produced in buds, flowers and developing fruits after dormancy and losses due to frosts during bloom are usually more important than those due to low winter temperatures. The information available on the influence of late spring frosts on reproductive organs of deciduous fruit trees in temperate climates is reviewed. The freezing process is examined, paying attention to how subsequent frost damage is caused at the cellular level as well as its anatomical and morphological consequences in flowers and fruits. The flower hardiness response is evaluated in terms of genotype, phenology and a number of physiological aspects as the formation of ice nucleation, moisture content and nutritive status. Finally, new perspectives are explored.

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1. Introduction

Low temperature is considered the most important factor determining the distribution of plant species on earth (Parker, 1963) and can limit both the yield and the distribution of horticultural crops (Ashworth, 1992), since many of them are either exotics or have arisen as a result of crosses of species or cultivars which

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are not indigenous to the region where they are cultivated. The importance of low temperatures in horticulture has been known since the beginning of farming. Thus, Romans considered low winter temperatures and the risk of spring frosts as important factors in selecting species to be cultivated in specific regions, and they made efforts to protect crops from freezing injury nearly 2000 years ago in the first century AD (Columela, 1965). However, in spite of the different methods of frost protection developed since then (Rieger, 1989; Perry, 1998), freezing damage to plants continues to be a problem of major economic significance, even in subtropical regions (Weiser, 1970a). This situation has led to research efforts devoted to reduce freezing injury, not only by the development of methods for frost protection (Rieger, 1989) or to delay flowering (Anderson and Seeley, 1993) but also by the study of the physiological mechanisms involved in freezing stress and injury (Weiser, 1970b; Burke et al., 1976). However, this considerable effort has resulted in only modest improvements in freezing tolerance (Thomashow, 1998) and the ultimate causes determining cold hardiness remain uncertain (Ashworth, 1986; Guy, 1990; Thomashow, 1998). Thus, freezing still accounts for greater losses of fruits and vegetables than any other environmental or biological hazard (Rieger, 1989).

Most deciduous fruit tree species require a minimum of winter chilling temperatures to break dormancy (Faust et al., 1997), and these conditions are often accompanied by periods of freezing temperatures during budbreak and blooming. In these species, freezing injury can be particularly harmful, not only because a long period of time is required to replace adult trees but because injury in reproductive organs causes dramatic crop losses, since after blooming, no new flowers are produced during that season. Consequently, cold hardiness is often a main selection criterion in breeding programs (Stushnoff, 1972; Quamme and Stushnoff, 1983; Ashworth and Wisniewski, 1991; Palonen and Buszard, 1997; Socías i Company, 1998) and much research has been focused on the mechanisms of freezing injury and acclimation to low temperatures (Quamme and Stushnoff, 1983; Ashworth and Wisniewski, 1991).

Freezing injuries to fruit trees can be associated with low temperatures prior to dormancy in the fall, in midwinter during dormancy, or during and after budbreak in the spring. In cold climates, low fall or winter temperatures affect mainly the xylem, bark, roots and buds, and can result in death of the trees (Palonen and Buszard, 1997 and references therein). While these low winter temperatures may clearly limit the area of distribution of a particular crop, low spring temperatures may seriously impair crop production in areas where a particular crop is well established. In temperate climates, losses due to frosts during bloom are more important than those due to low winter temperatures (Byrne, 1986) and consequently, the most significant damages are produced in buds, flowers, and developing fruits, although shoots can also be occasionally affected (Smeeton, 1964). In these regions, low temperatures rarely result in death of the trees, but they can be a critical factor limiting fruit production (Westwood, 1993).

The effects of low temperatures in plants have been reviewed in both herbaceous (Li, 1984) and woody plants (Parker, 1963; Weiser, 1970a), and from several points of view such as the effect on different plant tissues (Burke et al., 1976), ice formation (Ashworth, 1992), water redistribution during freezing (Olien, 1967), and cellular injuries (Mazur, 1969). Likewise, the effects of low temperatures during fall and midwinter on fruit trees have been also reviewed (Weiser, 1970b; Quamme and Stushnoff, 1983; Ashworth and Wisniewski, 1991; Palonen and Buszard, 1997). However, information concerning spring frost damage in fruit trees is dispersed and it remains to be explored to what extent the information available on the effects of low temperatures during winter applies to spring frost damage. Thus, resistance to frost of individual plants shows high variation during the year and among different tissues or parts of the plant (Weiser, 1970b) and these characteristics do not seem to be controlled by the same mechanisms before and after dormancy (Niobium, 1992; Palonen and Buszard, 1997; Wisniewski et al., 1997b). Likewise, the meteorological phenomenon that causes freezing injury can also vary among seasons and can produce different effects (Perry, 1998). In this work, the information available on the effects and responses of deciduous fruit trees to spring frosts in temperate climates is reviewed. The freezing process is examined, paying attention to how subsequent frost damage is caused at the cellular level as well as its anatomical and morphological consequences in flowers and fruits. The flower hardiness response is evaluated in terms of genotype, phenology and a number of physiological aspects such as the formation of ice nucleation, moisture content and nutritive status. Finally, new perspectives are explored.

2. Spring frost damage in reproductive organs

Frost damage is mainly caused by ice formation rather than by low temperatures per se. Following a frost, the fate of the cell depends on where the formation of ice crystals takes place (Mazur, 1969). Thus, ice formation can be either extra-cellular, which protects the cells themselves at least temporarily, or, if cooling is rapid, intra-cellular, which is fatal and causes cell death. During spring frosts, the cellular damage is usually produced by extra-cellular freezing and results in a series of anatomical effects, that can be reflected in diverse morphological manifestations.

2.1. Cell damage

Ice nucleation in most plant tissues begins on the surface of the cell-wall, in water transporting vessels or in the extra-cellular spaces (Guy, 1990). Ice crystals spread throughout the extra-cellular regions (Burke et al., 1976) causing a rapid

rise in the solute concentration of the extra-cellular solution. Thus, a water potential gradient is established, with liquid water moving out of the cell (Mazur, 1969). The cell acts as an osmotic system, with the osmotic concentration inside increasing as water diffuses out through the plasmalemma, dehydrating the cell. The protective effect of extra-cellular freezing is due to gradual dehydration which prevents intra-cellular freezing. When these ice crystals melt, most water is likely absorbed by the adjacent cells. Cell death can be caused either by a severe dehydration that produces coagulation of the protoplasm (Modlibowska, 1962a) or by disruption of cell membranes and other cellular components by ice crystals (Burke et al., 1976; Steponkus, 1984). This disruption is usually manifested as a flaccidity and/or discoloration of the affected tissue (Burke et al., 1976). Thus, ice formation is a prerequisite for injury. Although the melting point of ice is 0°C, the freezing temperature can be variable, since water and dilute solutions often remain as metastable liquids below their melting points. This lack of crystallization at temperatures below the freezing point is so-called supercooling (Modlibowska, 1962b). As a result, parenchymatic cells of supercooled tissues do not dehydrate and retain their original shape and volume during freezing (Modlibowska, 1962b; Ristic and Ashworth, 1995).

Survival of flowers or fruits following a frost depends upon the amount of damage to the vital tissues and the capacity of the remaining intact cells to continue growth and development (Modlibowska, 1962a). Thus, several anatomical and morphological manifestations of spring frost injury have been reported in reproductive organs of deciduous fruit trees.

2.2. Anatomical effects

In dormant flower buds the effect of freezing temperatures is not uniform but ice crystals are formed only in some floral tissues (Ashworth et al., 1989; Ashworth, 1990; Ashworth and Wisniewski, 1991; Warmund et al., 1992; Flinn and Ashworth, 1994). Moreover, the distribution of ice crystals in frozen buds also changes during de-acclimation. Thus, in acclimated flower buds, large ice crystals are formed within the bud scales and bud axis but there is no ice formation within the floral organs (Quamme, 1978; Ashworth et al., 1989; Ashworth, 1990), while in de-acclimated flower buds ice crystals are present within the developing flower organs as well as in bud scales and bud axis (Kader and Proebsting, 1992). These crystals are formed predominantly in the lower portions of the developing flower and peduncle, and cause a separation of the epidermal layer from adjacent cells (Ashworth et al., 1989). Although the location of ice is ultimately influenced by hardiness, several theories have been proposed to explain the selective accumulation of ice in different flower bud tissues (Flinn and Ashworth, 1994). Thus, the preservation of floral organs has been connected to the fact that there is a physical discontinuity between them and

the rest of the plant with no xylem connection (Ashworth, 1982, 1984, 1992; Ashworth and Rowse, 1982; Ashworth et al., 1989; Callan, 1990; Kader and Proebsting, 1992). Upon breaking of dormancy, xylem continuity is reestablished and ice can propagate into flower tissues. Other theories hypothesize that differences in water potentials among the tissues could cause localized ice formation (Quamme, 1978) or that ice formation is the result of intrinsic ice nucleators that are present in ice-accumulating tissues and absent in ice-free tissues (Ishikawa and Sakai, 1985).

Following a spring frost, different injuries in flowers at the balloon stage or anthesis have been reported. Those include the separation of epidermis and hypodermis from the cortex by a layer of ice (Rogers, 1952; Simons and Doll, 1976), stylar abscission (Simons et al., 1970; Olson and Steeves, 1983), abortive ovules (Modlibowska, 1957; Simons, 1969; Williams, 1970; Stösser and Anvari, 1982), and large breaks in the cortical tissue with a high number of cells destroyed (Simons, 1959a; Simons and Doll, 1976). The ovarian locule is particularly sensitive to freezing; the injury is frequently characterized by cell-wall thickening, lack of meristematic activity and destruction of the vascular tissues (Simons and Doll, 1976). Breaks can also occur around the petal and sepal bundles (Simons, 1969).

When a limited number of cells are dead, recovery can be rapid (Modlibowska, 1962a), since the damage usually occurs in a period of intense cell division (Simons, 1969). However, tissue damage before and during anthesis persists at fruit maturity. Thus, subsequent healing is accomplished by the formation of callus tissue (Modlibowska, 1962a), in a variable quantity dependent upon the severity of the injury (Simons, 1969). Callus tissue is also present in the cavities produced by ice in the inner cortex and along the locules (Modlibowska, 1962a; Simons and Doll, 1976). These injured areas show a deficient cuticle development. Furthermore, injured cortical cells and vascular tissues result in variable lesions. The regions of dead cells appear initially as light brown areas that finally form obscure masses of necrotic cells, with cracks in injured areas (Simons, 1969). The cells adjacent to the injury are usually abnormal in shape, size or orientation (Simons and Lott, 1963) and the vascular bundles affected are disrupted, producing large areas of cell disintegration, with scattered layers of suberized cells throughout the parenchymatic tissue adjacent to vascular strands (Simons, 1969). The injury generally follows the path of the major vascular bundles in the cortex and therefore, bundle cells and the adjacent cortical cells appear to be more susceptible to frost injury than other cells of the cortex (Simons and Lott, 1963).

2.3. Morphological consequences

The anatomical effects caused by spring frosts in reproductive organs usually result in internal and external morphological abnormalities that affect the normal

development of the fruit or even cause abscission. Thus, de-acclimated apple flower buds killed by spring frosts show a general browning as an immediate external symptom. Subsequently, buds desiccate and drop. However, flower buds without external symptoms can also present internal damage in the style, ovary and/or stamens and although sepals and petals may continue their development, pistil development is arrested and the fruitlet finally drops, except in some parthenocarpic cultivars of pear, where the ovary can resume growth (Modlibowska, 1957).

At full bloom, the first symptom observed after thawing in apple flowers is a brown discoloration at the base of the style (Modlibowska, 1957) and the damage may extend both to the style and to the ovary, resulting in death of the placenta, abortion of ovules (Modlibowska, 1957), or formation of large breaks in the cortical tissue (Simons et al., 1970). After severe frosts, flowers can also present other symptoms such as stylar abscission or browning of the petals (Simons, 1959a). However, the sequence of events may differ in other species. In pear, the presence of symptoms of damage may be observed in the ovary before they are observed in the style (Modlibowska, 1957), and in peach, the first tissue affected can be the pistil, the petals, or both (Oberle, 1957).

The first evidence of damage in apple fruits is the separation of several layers of cells from the cortex in the hypodermal region (Modlibowska, 1946; Simons, 1957, 1959b). This manifestation is usually accompanied by internal damages such as cracks in the cortex and core. When the epidermis remains unbroken, these internal damages may be of little significance since fruits can heal rapidly under favorable environmental conditions (Modlibowska, 1962a). In *Prunus*, the ovules seem to be the most sensitive tissue. Thus, in peach, browning of the ovule may occur with no apparent external damage. Whenever the whole ovule is affected, the embryo aborts and the fruit abscises. However, when only the integuments are affected, the fruit may continue developing in spite of the abortion of the embryo (Saunier, 1960). In European plum, external damage is apparent immediately. Thus, fruitlets may display swellings and even show browning. Internal damage is very similar to that produced in peach (Saunier, 1960). On the other hand pear fruits can develop to maturity even when the ovules have been completely destroyed in those cultivars with some parthenocarpic tendency (Modlibowska, 1962a).

Flowers and fruits affected by spring frosts can frequently recover and continue their development. Survival of a flower or fruit following a frost depends upon the amount of damage to the vital tissues, the capacity of the remaining intact cells to continue growth, and the subsequent environmental conditions, particularly the risk of desiccation of the tissue (Modlibowska, 1962a). The consequences of frost can be evident when the fruit ripens and may affect its shape, appearance and/or size. Thus, fruit malformations result from injury to the fruit cortex (Simons, 1969; Simons and Doll, 1976) and/or flattening of the fruit calyx in apple (Simons

and Lott, 1963) and neck thickening or fruit elongation in pear (Modlibowska, 1957). Occasionally, abnormal fruit-pedicel development has also been reported in apple (Simons and Doll, 1976). The external appearance of fruits can also be modified when the skin is ruptured, or so damaged that it breaks during the growth of the fruit. Thus, the healing phellogen forms cork which restricts meristematic activity and can result in blemishes and alterations in skin color (Simons, 1959a) and/or the development of cracks and cork formations (Modlibowska, 1957; Simons and Doll, 1976). These defects can develop vertically or horizontally (Modlibowska, 1957). While vertical blotches can affect only the surface or penetrate to the center of the fruit, horizontal defects tend to develop surrounding the fruit. Thus, cork formations can spread as dispersed lenticels (Simons, 1959a; Simons et al., 1970) or green blotches (Modlibowska and Glenn, 1950), "frost eyes" surrounding the calyx and "frost rings", russeted bands encircling the fruit which causes constricted growth on defined areas (Modlibowska, 1957; Simons, 1959a). Although "frost eyes" have been related with damage at an early stage of floral development and "frost rings" with damage at a later stage, the position and type of blemish seems to be a cultivar characteristic (Modlibowska and Glenn, 1950). Finally, alterations of size have been reported in pear, where simulated frost increased fruit malformation and reduced fruit weight (Strang et al., 1980a).

3. Flower hardness

While freezing temperatures can seriously damage plant tissues, the effects of spring frosts on reproductive organs of deciduous fruit trees are highly variable and depend on the characteristics of both the freezing stress and the plant material. Thus, factors influencing the extent of cold injury include the intensity and duration of the low temperatures (Mazur, 1969; Strang et al., 1980b), the rates of temperature decrease (Weiser, 1970a; Strang et al., 1980b; Friesen and Stushnoff, 1985) and of thawing (Olien, 1967; Burke et al., 1976), the temperatures of previous days and short-term temperature variations (Proebsting and Mills, 1978a; Lu and Rieger, 1993; Westwood, 1993), as well as other climatic conditions such as wind speed, relative humidity, and cloud cover (Perry, 1998). Moreover, frost damage can be produced through different climatic processes leading to different types of spring frosts: radiative, advective and evaporative, each with specific conditions (Perry, 1998). The different responses to frosts observed among plant genotypes, tissues of the same plant, and different seasons have led to the study of the mechanisms of injury with the goal of establishing both the time when injury is produced and the site of lethal injury (Guy, 1990). However, the mechanisms involved in frost damage and hardiness

have turned out to be exceedingly complex and many aspects remain uncertain (Olien, 1967; Guy, 1990; Thomashow, 1999).

Woody species usually have little cold hardiness during active growth, but during fall, dormancy induces a number of changes which enable the plant to withstand freezing temperatures (Powell, 1987). This development of cold hardiness is termed acclimation and is mainly characterized by supercooling (Modlibowska and Rogers, 1955; Modlibowska, 1962b). During late winter, physiological changes induce the breaking of dormancy (Saure, 1985; Faust et al., 1997) and flower buds gradually lose cold resistance (Weiser, 1970b, Ashworth, 1982; Sedgley, 1990; Ashworth and Wisniewski, 1991). This progressive loss of cold hardiness is termed de-acclimation (Weiser, 1970a).

Differences in hardiness between vegetative and reproductive structures have been reported for several species of trees and shrubs (Proebsting, 1970; Sakai, 1982; Cappiello and Dunham, 1994; Väinölä et al., 1997). Although the mechanisms of water supercooling in flower buds when ice is present in adjacent tissues are not well established (Ashworth and Wisniewski, 1991), cold hardiness in flower buds during dormancy seems to be the result of several factors including structural, physiological and morphological features (Weiser, 1970b; Ashworth and Wisniewski, 1991; Faust et al., 1997; Palonen and Buszard, 1997). In spite of the fact that an important lack of hardiness takes place, a frost-tolerant period occurs during de-acclimation (Andrews et al., 1983). However, cold hardiness of flower buds in spring does not seem to correspond to the ability of the plant to withstand low winter temperatures, indicating that these characteristics are not controlled by the same mechanisms (Palonen and Buszard, 1997; Wisniewski et al., 1997b). Thus, cultivars that are extremely tolerant to low temperatures in a continental climate, may be subject to severe flower bud injury following spring frosts in more variable coastal climates (Niobium, 1992; Warmund et al., 1992; Palonen and Buszard, 1997).

The fact that cold hardiness is highly variable led to the study of the biochemical and physiological changes that can alter freezing tolerance (Guy, 1990). Since a number of correlations between different tissue components and hardiness have been reported, it seems that several factors might affect hardiness and some of them can induce injury whereas others can protect against frost damage. Those relationships can be different among different plants (Mazur, 1969). While much work has been devoted to the study of these changes during acclimation, little information is available on the tolerance of reproductive organs during and after de-acclimation. Still, differences in damage have been recorded following apparently similar frost conditions even among flowers of the same tree.

A number of factors have been reported to play a part in the vulnerability or resistance of flowers to spring frosts. These are mainly related to the genotype, the stage of development, the formation of ice, the moisture content and the nutritive status of the pistil.

3.1. Genotype

Spring frost hardiness in deciduous fruit trees is influenced primarily by genotype (Westwood, 1993). Both the flower supercooling capacity (Modlibowska, 1962b) and blooming time (Anderson and Seeley, 1993), as well as other indirect characteristics influencing survival of flowers such as flower bud density and uniformity of bud development (Okie and Werner, 1996), seem to be genetically controlled. Critical temperatures causing damage to reproductive organs are different among species (Saunier, 1960; Ballard et al., 1971; Proebsting and Mills, 1978b) and among cultivars; these cultivar differences are frequently higher than those reported among species (Saunier, 1960; Strang et al., 1980a; Cain et al., 1984; Byrne, 1986; Rieger et al., 1991). However, it is not easy to separate the effects of genotype from the effects of other factors. As a consequence it has been observed that critical temperatures not only vary with the phenological stage of flower buds but also can vary among species, cultivars, orchards (Proebsting and Mills, 1978a) and even within the trees (Westwood, 1993). Although the variability of data shows that critical temperatures for species or cultivars are not biological constants, the results obtained can have an orientative value and be useful to set the values to activate frost protection systems (Proebsting and Mills, 1978b).

3.2. Phenology

Frost damage is highly dependent on the stage of development of the flower buds (Simons and Doll, 1976; Westwood, 1993). From dormancy to fruit set, the flower bud undergoes a number of developmental stages that are associated with a progressive increasing vulnerability of the pistil to low temperatures (Proebsting and Mills, 1961; Proebsting, 1963). Thus, the early developing fruit is the most vulnerable stage (Proebsting and Mills, 1978b). Andrews et al. (1983) suggest four periods during spring de-acclimation of *Prunus* flower buds: the dormant period with deep supercooling of the flower buds, a transition period when buds begin to swell and approach budbreak in which deep supercooling is progressively lost, a third frost-tolerant period before petal tip emergence and a final frost-sensitive period following petal tip emergence, in which flowers and early fruits are very sensitive to frosts.

Temperature fluctuation before and during bloom can also influence flower tolerance to low temperatures since warm conditions may increase flower vulnerability and cool temperatures may decrease it (Head, 1959; Proebsting and Mills, 1978a; Anderson and Seeley, 1993; Lu and Rieger, 1993). Likewise, temperature affects frost survival indirectly by delaying or advancing bloom (Anderson and Seeley, 1993) but it remains unclear whether intrinsic changes in flower freezing tolerance are produced.

3.3. Ice nucleation

The formation of ice on or within a plant requires a small ice crystal embryo. Since ice formation is a prerequisite for freezing injury, ice-nucleating agents can play a significant role in flower bud hardiness. One factor that induces ice nucleation in plant tissues is the presence of ice nucleation-active (INA) bacteria. Their discovery evoked considerable optimism for the development of new methods of frost protection (Lindow, 1983), since the regulation of ice nucleation would produce significant advances (Wisniewski et al., 1997b). Thus, a reduction in the size of INA bacterial populations has been related with a decrease in frost damage in reproductive organs of almond (Lindow and Connell, 1984) and pear (Lindow et al., 1996). These reports contrast with other studies under a wide range of sizes of INA bacterial populations, where no appreciably lower frost damage was observed. In those cases, ice nucleation seems to depend on the presence of intrinsic ice nucleators synthesized by the plant itself and consequently, complicates the potential ability to increase frost protection through the control of bacterial sources of ice nucleation (Gross et al., 1984; Ashworth et al., 1985; Andrews et al., 1986; Cody et al., 1987; Proebsting and Gross, 1988). These intrinsic ice nucleators have not been identified or characterized (Ashworth, 1992), but several characteristics make them distinct from INA bacteria. While the bacterial component active in ice nucleation has been identified as a protein (Lindow, 1983; Wolber et al., 1986), this does not seem to be the case in the intrinsic factor present in woody tissue (Ashworth, 1992). Recent reports have added complexity to the process of ice nucleation in fruit trees, since the sites where ice nucleation takes place can also be variable (Wisniewski et al., 1997a).

3.4. Moisture content

Moisture content has been related to hardiness of flower buds in several species (Head, 1959; Bittenbender and Howell, 1975; Hewett et al., 1978; Layne and Ward, 1978; Junntila et al., 1983; Andrews and Proebsting, 1987; Durner and Gianfagna, 1991; Lu, 1992). Although increased water content negatively affects ovary cold hardiness within a given cultivar, it cannot explain the genetic differences in ovary freezing tolerance of several *Prunus* species (Lu, 1992). Hewett et al. (1978) suggested that the water present in flower buds may increase injury in two different ways, by providing added sites for ice nucleation or by directly influencing protoplasmic resistance.

High tissue water content caused by sprinkling or rainfall seems to be also related to freezing tolerance. However, while frost damage has been reported to be higher in wet than in dry flowers and fruits in different fruit tree species (Modlibowska, 1962b; Hewett et al., 1978; Lu, 1992), it has been observed that

wetted buds had less pistil injury than dry buds before petal tip emergence in peach (Andrews et al., 1983), and reverse results were found in different years in apple buds (Hamer, 1983). These conflicting results suggest that further work is needed to determine the conditions in which sprinkling irrigation can be used as a method for frost protection.

3.5. Nutritive status

Although a causal relationship has not been established, carbohydrate content has also been associated with plant hardiness (Alden and Hermann, 1971; Flinn and Ashworth, 1995). Carbohydrates have been frequently related to cold hardiness and seem to have several functions including cryoprotection of cellular constituents (Alden and Hermann, 1971) and the ability to retard the development of ice crystals (Parker, 1963). Indeed, the accumulation of water soluble carbohydrates reduces the freezing point of the cell sap and thus the freezing temperature (Sakai and Larcher, 1987). In woody species, carbohydrate levels have been correlated with flower bud hardiness (Flinn and Ashworth, 1995). In apple, a cool treatment increased the sugar content of flower buds and their cold hardiness (Head, 1959). Likewise, cold hardiness of flower buds has been related to nutritive status in deblossomed apple trees, where the absence of fruit in the previous year increased cold hardiness of buds and their content of sorbitol, glucose, starch, nitrogen, phosphorous, potassium (Khanizadeh et al., 1989) and hydrophilic and acidic amino acids (Khanizadeh et al., 1992). These differences may explain the effect that previous crop load has on bud hardiness (Byers and Marini, 1994) and be responsible for some of the orchard to orchard differences often recorded. In peach, differences in cold hardiness among cultivars have been related to differences in the content of sugars, proteins and amino acids of flower buds (Lasheen and Chaplin, 1971). In the same way, an increase in flower bud hardiness mediated by an ethephon treatment to peach trees was associated with increased contents of sorbitol and sucrose in the pistil during de-acclimation (Durner and Gianfagna, 1991).

4. Future perspectives

Despite the formidable research effort made and the fact that spring frost risks can be considerably reduced by a proper selection of the site and the plant material, by adequate methods of freeze protection (Rieger, 1989) or by delaying blooming (Anderson and Seeley, 1993), losses caused by spring frosts continue to be a critical factor for fruit production in temperate climates. Moreover, the constantly changing fruit markets often force the expansion of cultivars to areas where spring frosts can be harmful. Likewise, the effects of climatic warming,

especially in high latitudes, could include earlier bud burst, and therefore increased risk of spring frost injury (Heide, 1993). Spring frost hardiness is a complex phenomenon depending on a number of factors that range from inherent genetic characteristics and stage of development to the impact of environmental conditions and cultural practices on the individual genotype. It is reflected in a variety of responses of buds, flowers and fruits, ranging from lethal effects causing abscission to modifications of the characteristics of the ripe fruit. Consequently, it is not possible to establish a single sequence of events or symptoms in reproductive organs. Although much research about the responses of plant tissues to low temperatures in fall and midwinter has been done, the mechanisms that control cold hardiness of reproductive organs during and after de-acclimation are not well understood and a number of aspects remain uncertain. Thus, since tree responses to freezing temperatures vary seasonally and different tissues within the same tree respond in different ways to low temperatures, further work is needed to establish the whole sequence of events throughout the year, and to know the role of cell components in cryoprotection and whether the physiological variations affecting cold hardiness in fall and midwinter are also related to spring frost hardiness.

On the other hand the different responses of buds in the same developmental stage to low temperatures among species, cultivars and orchards are also reflected inside the tree. These variations can be partially explained by orientation and height inside the tree, particularly under radiative freezing conditions (Proebsting and Mills, 1978b; Scorza et al., 1983; Friesen and Stushnoff, 1985; Lu et al., 1992). However, apparently similar flowers, in the same developmental stage and in similar position often present differences in cold resistance and while some of them are killed under a spring frost, others can continue their development. This also takes place in laboratory tests under controlled conditions (Wisniewski et al., 1997a) and appears to indicate an internal attribute that would make some flowers harder than others. Consequently, searching for differences among flowers inside the tree and their possible relationship with cold hardiness could contribute to the understanding of this behavior.

While several analytical methods are used to determine the physiological changes related to the cold hardiness of plant tissues, histological approaches can be useful in order to establish the relationship between the effects of freezing on reproductive organs and frost hardiness. However, little work has been carried out establishing a causal relationship and it has mainly involved the descriptions of symptoms of surviving flowers and/or fruits. Further work would contribute to discern the effects caused by the different types of frosts and those caused by other sources. Likewise, the use of new techniques such as infrared video thermography has allowed the observation of ice nucleation and propagation in intact herbaceous plants (Wisniewski et al., 1997a; Fuller and Wisniewski, 1998) as well as in flowering shoots of fruit trees under controlled conditions

(Wisniewski et al., 1997a), and probably will aid understanding of the process of frost injury and could open the possibility of regulating ice nucleation in fruit crops under field conditions.

Further work is needed to explore the implications that the freezing tolerance mechanisms in other species have on fruit tree hardiness and whether these mechanisms play a role determining spring frost resistance. Thus, it has been established that changes in gene expression occur during cold acclimation (Guy, 1990) and some of those genes have been isolated from a variety of different species (Hughes and Dunn, 1996; Boothe et al., 1997; Thomashow, 1998 and references therein). However, previous attempts to obtain transgenic plants showing increased levels of frost tolerance have been unsuccessful (Wisniewski et al., 1997b), probably due to the fact that frost tolerance seems to be a trait under polygenic control (Hughes and Dunn, 1996; Thomashow, 1998). Nevertheless, a growing number of cold responsive genes, which provide new tools for improving plant freezing tolerance, have been recently identified and characterized (Thomashow, 1999). Thus, the freezing tolerance of nonacclimated transgenic plants of *Arabidopsis* has been enhanced by overexpressing a transcription factor that activates a group of regulated cold responsive genes (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). Although not all plant species may have the same repertoire of cold-tolerant genes, the fact that homologs to the regulatory elements involved in the increasing of tolerance of *Arabidopsis* are known to exist in *Brassica* species (Jaglo-Ottosen et al., 1998) and that cold tolerance genes may be similarly manipulated in other species may open a way to improve the freezing tolerance of crop plants that are subject to frost during seasons in which freezing tolerance is not naturally induced (Warren, 1998). Different cold-tolerant gene candidates have been reported, including those encoding proteins with known enzymatic activities that could potentially contribute to freezing tolerance (Thomashow, 1999). Thus, the recently described plant antifreeze proteins (AFPs) accumulate at low temperature and seem to contribute to freezing tolerance in both animal (Logsdon and Doolittle, 1998) and plant species (Antikainen et al., 1996; Griffith et al., 1997). They bind to the surface of ice nuclei and inhibit their growth during freezing and thawing cycles (Griffith et al., 1997). Although transgenic lines containing fish AFPs have been generated in several plant species, the most interesting long term opportunities seem to lie with AFPs of plant origin, particularly those that are already present in the food chain (Dunwell, 1999 and references therein). Thus, an AFP from carrot that prevents ice crystal formation and subsequent destruction of the roots in frozen soil has been successfully introduced into tobacco (Worrall et al., 1998). Other hydrophilic polypeptides also accumulate during cold acclimation in different species and seem to contribute to freezing tolerance (Thomashow, 1999 and references therein). Although efforts to determine the precise mode of action of cold responsive genes as well as the polypeptides accumulated during low

temperatures, together with physiological and biochemical studies, will probably reveal higher orders of complexity of the responses of plants to low temperatures (Guy, 1990), they will also provide powerful tools to understand how plants in general, and fruit trees in particular, tolerate freezing during and after acclimation and how their freezing tolerance can be improved.

An improved understanding of freezing tolerance could aid the improvement of breeding programs searching for hardier cultivars and the development of cultural conditions leading to avoidance of frost damage, as with the development of antifreeze compounds to inhibit premature ice nucleation under field conditions (Wisniewski et al., 1997b). In practical terms, minor increases in spring frost hardness could have a major impact on fruit production.

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