Aus dem Institut für Obst-, Gemüse- und Weinbau
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Mechanisms of Frost Adaptation and Freeze Damage in Grapevine Buds

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Das Außerordentliche geschieht
nicht auf glattem,
gewöhnlichem Wege.

Johann Wolfgang von Goethe
(1749-1832)

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Contents

I Zusammenfassung

Mechanismen der Frostadaption und Gefrierschäden in Rebknospen

In der vorliegenden Arbeit wurden mit Hilfe verschiedener Methoden die Vorgänge in der Rebknospe unter Frosteinwirkung im Verlauf dreier Winter (1998-2000) untersucht. Das Material bestand aus Winterknospen der Sorte Bacchus vom 1-jährigen Holz von Rebstöcken, die 1983 gepflanzt wurden. Die untersuchten Parameter waren LTE / HTE (low temperature exotherm and high temperature exotherm), Wassergehalt, Stärke, Zucker- und Anionen-Zusammensetzung und Zelldifferenzierung.

Der Wassergehalt von Spross und Knospen wurde regelmäßig alle 2 Wochen ermittelt. Generell enthielten diese im oberen Bereich des Triebs weniger Wasser als im basalen Teil. Zwischen November und Februar war der Wassergehalt im Holz und in den Knospen am niedrigsten (ca. 40%).

Lösliche Zucker in der Knospe wurden mittels HPLC bestimmt. Saccharose war vorherrschend, Glucose, Fructose, Raffinose und Stacchyose kamen nur in geringen Konzentrationen vor. Letztere wurde erstmals in Rebknospen nachgewiesen. Die Konzentration der löslichen Zucker stieg von November/Dezember bis Anfang Januar zu einem Maximum (um die 150 mg/g Trockengewicht) an und fiel bis zum Austrieb Ende März auf ein Minimum (ca. 30 mg/g) ab. Die höchsten Zuckerkonzentrationen waren in den Knospen des basalen Triebbereichs, die niedrigsten in den apikalen vorhanden. Das vorherrschende Anion war Sulfat, während Chlorid nur in Spuren nachgewiesen werden konnte. Die höchste Konzentration lag Ende Dezember/Anfang Januar und Mitte April vor.

Zur Evaluation der Exothermen-Messmethode wurden zunächst Modellversuche mit Wassertropfen (1 µl) auf Filtrierpapier und mit kleinen

Pflanzenteilen (Blattstückchen, Blütenteile) unternommen. Sowohl die Pflanzenteile wie das destillierte Wasser auf Cellulosefasern gefrieren überwiegend zwischen –8 und –15°C (ein Einfluss des osmotischen Wertes der untersuchten Lösungen ließ sich dabei nicht feststellen). Die Gefrierpunkte, die nach zwischenzeitlichem Auftauen und neuerlichem Einfrieren erhalten wurden, waren signifikant mit den beim erstmaligen Gefrieren erhaltenen Werten korreliert. Dies zeigt, dass die Gefrierpunkte nicht einer zufälligen Streuung unterliegen, sondern durch die Eigenschaft der Probe, d.h. durch darin vorhandene Eiskeimbildner (ice nucleation sites), bestimmt werden. Diese überleben offensichtlich sogar die physikalische Zerstörung der Blattzellen während des ersten Gefriervorgangs. Weitere Modelluntersuchungen wurden zur Ausbreitung von Eiskristallen im Pflanzengewebe unternommen.

Durch Exothermen-Messung wurde ermittelt, bei welcher Temperatur Rebknospen gefrieren. Dabei erscheint eine kurze Temperaturerhöhung (Exotherme), die in der Messkurve als Gipfel sichtbar wird. Die Rebknospe liefert 2 oder mehr Exothermen: Die HTEs (high temperature exotherms) zwischn etwa – 5 °C und –10°C und eine oder mehrere LTEs (low temperature exotherms). Letztere lagen zwischen etwa –10° und –25°C, abhängig von der Kälteadaption der Knospen (Temperatur-Minimum im Januar). Die HTE deutet auf das Frieren von oberflächlichem Wasser der Knospenschuppen bzw. apoplastischem Wasser des Knospenkissens, die LTE(s) auf das Frieren von intrazellulärem Wasser in den Haupt- (und Neben)augen hin. Bei den HTEs (zwischen – 5 und – 10 °C) ließ sich ein Minimum nicht eindeutig bestimmen da sie (im Gegensatz zu den LTEs) durch Nässe in den Knospenschuppen beeinflusst wurden. Die LTEs der Knospen im unteren Bereich des Triebs lagen i.A. höher (bis -11°C) als die von Knospen im mittleren und oberen Bereich des Triebs (bis -16°C bzw. -14°C). Die LTE-Analyse zeigte zwar eindeutig die Kälteadaption der Winterknospen mit einem Maximum Ende Januar, jedoch

konnte kein eindeutiger Zusammenhang mit der Lufttemperatur gesichert werden.

Im Rahmen der histologisch-mikroskopischen Untersuchung mit z.T. eigens modifizierten Färbemethoden, sollte festgestellt werden, welche Vorgänge bei der Eisbildung wirklich ablaufen. Der Gehalt an Stärkekörnern war im Winter am höchsten. Diese wurden kurz vor dem Schwellen der Knospen aufgelöst. Eine Trennzone zwischen Knospenbasis und Knospe konnte histologisch nicht nachgewiesen werden, jedoch konnte direkt gezeigt werden, dass eine HTE keine Zellschäden in den Knospen bewirkt, während es nach dem Auftreten der LTE zu einer Desintegration der Protoplasten kommt. Dies ist ein direkter Beweis, dass die LTE tatsächlich den Tod der Augen in der komplexen Rebknospe anzeigt.

Knospen, die nach Auftreten der ersten Exotherme vor dem weiteren Abkühlen zunächst einen Tag bei dieser Temperatur gehalten wurden, zeigten danach keine LTE mehr. Diese und ähnliche Beobachtungen bei der Frostlagerung von Reb-Schnittholz ließen sich durch die (unschädliche) Eisbildung im Knospenkissen bei mäßigen Minustemperaturen erklären, die durch dessen (im Vergleich zu den nichtgefrorenen Augen) niedriges Wasserpotential bei längerer Lagerung zu einem Gefriertrocknungseffekt und dadurch zu einer weiteren Steigerung der Frostresistenz der Augen führt.

Frostadaptierte Rebtriebe aus dem Freiland waren bei 20°C nach ca. 10 Tagen deakklimatisiert. Stark von Wasser benetzte Knospen zeigten dabei häufig Exothermen bei über – 4°C. In diesen Knospen und dem Gießwasser konnten eiskeimbildende Bakterien (*Pseudomonas fluorescens*) nachgewiesen werden.

Die Analysen haben gezeigt, dass die Frostadaption der Rebknospen nicht durch einige wenige Faktoren erklärt werden kann. Entwässerung und Zuckereinlagerung liefern zweifellos einen Beitrag dazu, eine größere Rolle scheinen jedoch physikalische Effekte wie die Wasserverlagerung zwischen

nichtgefrorenem Auge und gefrorenem Knospenkissen zu spielen, die neben der Erhöhung des osmotischen Werts zu einer physikalische Isolierung oder Maskierung von Eiskeimbildnern führen könnte.

II Summary

Mechanisms of frost hardening in compound (latent) buds of the grapevine cultivar ‘Bacchus’ were tested with different methods during three winters. The investigated parameters were LTE/HTE (low temperature exotherm/high temperature exotherm), water content, starch, sugar- and anions combination and bud histology.

Water content from wood and buds was determined regularly every 2 weeks from March 1998 until Mai 2000. The lowest water content in wood and buds (about 40 %) was found between November and February. In general shoot sections and buds from the apical shoot area contained less water than in the basal area.

Sugars and anions were analyzed with HPLC. The highest concentrations of soluble sugars were found in basal buds of the shoot, the lowest concentration in buds of the apical shoot area. Sucrose was the predominant soluble sugar, it was accompanied by glucose, fructose, sucrose, raffinose, and also stacchyose which was hitherto not described for grapevine buds. The concentration of soluble sugars increased during autumn and reached its maximum (around 150 mg/g dry matter) in November/December until the beginning of January then it decreased again to around 30 mg/g at bud burst. The predominant anion was sulphate while chloride could be detected only in traces. The anions reached their maximum at the beginning of January and in mid April.

To evaluate the exotherm measuring method, model experiments were carried out with water drops (1µl) on filter paper and with small plant parts (leaf, stems, flower parts). Both the plant parts and the destilled water on the cellulose fiber freeze mainly between –8 and –15°C (an influence of the low osmotic value of the plant sap could not be found). After the first freezing the specimen were thawed and freezing repeated. The freezing points of the first and the second freezing cycle were significantly correlated. This shows that freezing does not occur at random, but is determined by ice nucleation sites characteristic for each sample. These sites even survive the physical destruction of the cells by the ice cristals. Further model experiments were carried out to get indications on possible barriers to ice cristal growth in plant tissue.

Exotherm analysis was used to determine the freezing point of grapevine buds which is accompanied by a transient temperature rise called exotherm. The grapevine buds show 2 or more exotherms, one or two HTEs (high temperature exotherms) between – 5 °C and –10°C and the LTE (low temperature exotherm, sometimes more than one) between –10°C and –25°C depending on the frost adaption of the buds. The HTEs are assumed to indicate the freezing of surface water or apoplastic water in the subtending tissue (bud pad), whereas the LTE (or LTEs) seem to be caused by freezing of the primary (and secondary) buds (shoot primordiy of the compound bud). The temperature minimum of the LTEs (down to – 25 °C) is reached in January/February and is not influenced by humidity which, however, changes the THE values occuring usually around – 10 ° and – 4 °C, which are influenced by water in the bud scales. The LTEs of the buds in the lower area of the shoot were higher as compared to the buds in the middle and upper area of the shoot. The LTE analysis clearly shows the frost adaptation of the latent buds which usually reaches a

maximum by the end of January but a clear relation to the changing air temperatures could not be established.

Histological and cytological analyses were used to test for frost damage in bud parts and for changes during the cold adaptation. A modified staining method was developed to differentiate the cells. During automn and winter the buds contained a lot of starch grains which dissolved at bud burst. A permeability barrier between bud pad and shoot primordia could not be found, however it could be directly shown, that a HTE causes no cell damage in the buds, while after the appearence of the LTE(s) a disintegration of protoplasts in primary and secondary buds could be found. This is a direct evidence that LTEs indicates the death of the eyes in the complex grapevine bud.

If after the appearance of the HTE the buds were held one day at this temperature before further cooling, no LTEs would appear. This and similar observations during the frost storage of grapevine cuttings is discussed in terms of the (harmless) ice formation in the bud base at moderate minus temperatures which would result in a freeze drying effect due to the lower water potential of the bud pad (in comparison to the non frozen eyes) and a further increase of the frost resistance of the growing points.

If frost adapted grapevine shoots from the field were kept at 20°C deacclimation occurred after about 10 days. Accidentally wetted buds showed exotherms above –4°C. In these buds and the watering water ice nucleating bacteria (*Pseudomonas fluorescens*) could be found.

The analyses have shown that frost adaptation of grapevine cannot be explained by few and/or simple factors. Although desiccation and sugar deposits may contribute to freezing resistance, a much larger role seems to

be played by physical effects such as water transfer between non-frozen eyes and the frozen bud basis which in addition to the rise of the osmotic values could lead to a physical isolation or masking of ice nucleation sites.

1 Introduction

1.1 Freezing of water

It is well known to all meteorologists – but only to few plant scientists – that the freezing point of pure water is around – 40 °C: well defined is only its melting point at 0 °C.

The freezing points of rain drops, solutions and biological fluids are usually higher and this is caused by particulate impurities present in the water or by structures on any surfaces present (e.g. cell walls) which are called ice nucleators (INs, ice nucleation sites, ice nuclei, freeze nuclei) [1].

Ice nucleation describes the process by which the ice embryo attains sufficient size to become stable and become an ice crystal (for literature see LEE et al., 1995). Ice nucleators (INs) can be very different both chemically and

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[1] This supercooling is due to the fact that under common conditions several hundred water molecules are needed before the growing ice crystal is stable: It takes energy to create a surface, and a very small crystal has a lot of surface in comparison to its volume. Usually it is wrongly assumed that freezing points below 0 °C are caused by osmotically active components. It is true that according to RAOULT'S law the melting point of a 2 M solution of a non-dissociating substance would be lowered by about 3.5 °C. However such concentrations are rarely reached in plant tissue, not even in very sweet fruits such as partly dried grapevine berries used to produce the so called "ice wine" from grapes harvested below – 7 °C. Actually osmotic effects are additive: If a drop freezes at – 7 °C due to the presence of appropriate ice nucleators, the addition of sucrose to a concentration of about 1 mol/liter would result in an overall freezing point of the solution of – 9 °C.

physically. Only few of them are well known, such as silver iodide which starts ice nucleation at – 8 °C and is used to produce ice clouds in oversaturated air, or the INs on the cell surface of *Pseudomonas* spp. and related genera which were first described by SCHNELL and VALI (1972) and which start ice nucleation already at – 2 °C. These ice nuclei are proteins encoded by ice genes (ina Z in *P. syringae*, ina W in *P. fluorescens*, ina E in *Erwinia herbicola*; for details see WARREN and COROTTO, 1989; KOZLOFF, 1991; TURNER *et al.*, 1991). In the mean time the structural genes encoding these structures have been cloned and used as reporter genes in transgenic plants (PANOPOULOS, 1995). There are INs which are acitive at still higher temperatures but they have not yet been analyzed although they are of considerable interest for commercial freezing processes (*Pseudomonas* cells are used for the production of artificial snow for skiing) and in meteorology.

Ice nucleators which are active at relatively low temperatures are rather frequent but "high temperature nucleators" (HTNS) seem rare. Therefore the probability of their presence in a small water volume is very low and small volumes of water, such as droplets in the atmosphere, small pieces of wet filter paper or of plant tissue (2-20 mm^3) generally freeze at temperatures between – 8 to – 17 °C. Freezing of larger volumes of water or of whole plant leaves occurs earlier since the growth of ice crystals, once started at one of the rare high temperature nucleation sites, spreads rapidly in free water and also in soft plant tissues if there are no mechanical barriers such as leaf veins.

1.2 Recording of freezing events

While the freezing of free water drops on a surface can be easily recognized, this is not possible in plant parts where it has to be recorded indirectly. Frost

damage becomes evident if after freezing and re-warming the tissue turns brown by the action of decompartimented hydrolases and oxidases (EIFERT, 1975). This method is used by fruit growers to determine the effect of night frosts on buds but it cannot be used on ice tolerant plant tissues, it is time consuming and gives no exact information on the freezing point. The suitability of some other viability tests such as staining with tetrazolium compounds was analyzed by STERGIOS and HOWELL (1973). Analysis of chlorophyll fluorescence to show the effects of low temperatures on grapevine buds has been used by DÜRING *et al.* (1990); the method is, however, not much faster than visual estimation of browning.

The most suitable method is therefore the measuring of exotherms as already mentioned by STERGIOS and HOWELL (1973). Freezing is an exergonic process: the energy which is dissipated is sufficient to heat an aliquot volume of water from 0 to 80 °C. If the temperature of the specimen is measured while it is slowly superchilled any formation of ice crystals will result in a transient rise of the temperature which can be recorded as a more or less sharp peak of the temperature curve (exotherm). Form and size of the exotherm depend on the structure of the sample, the location and the amount of the freezing water, the position and the mass of the thermosensor, the degree of supercooling and many other parameters so that quantitative data are difficult to obtain whereas the freezing points can be measured exactly.

The most elegant method to show freezing of plant parts is the use of infrared video thermography (WISNIEWSKI *et al.,* 1997; HAMED *et al.*, 2000) which, however, is rather expensive and is not well suited for automatic recording of a large number of repetitions. Although it has a limited resolution for fine structures it is very useful for imaging freezing events which otherwise could

only be demonstrated by temperature measurements or by still more sophisticated and expensive methods like NMR microscopy (IDE *et al.,* 1998)

1.3 Frost damage in plants

1.3.1 General aspects

In this context only freezing damage by ice crystals and plant mechanisms to prevent them will be discussed, although of course other damage types such as membrane defects are no less important.

Freeze injury is one of the main sources of losses in plant production which according to ALLEGRETTO (1984) may reach 5 – 15 %. A detailed discussion on the losses in the U.S.A may be found in RIEGER (1989). A detailed review on the formation and spread of ice in plants was compiled by ASHWORTH (1992). In the following only a short summary including more recent literature shall be given.

Unlike frost-restistant plants, frost-sensitive plants are unable to tolerate ice formation within their tissues. Freezing damage in these plant tissue results (1) from physical rupture of cells by ice crystals (2) from sudden dehydration of the symplast by rapidly growing extracellular ice crystals.

On the other hand gradual and controlled dehydration may lead to an increase of the osmotic value in the cell sap which might be sufficient to prevent nucleation. In this case an early formation of extracellular ice would be positive since at high temperatures (e.g. at – 2 °C) the ice crystals grow much slower than after strong supercooling, where they grow so fast that a controlled dehydration of the protoplast is not possible and the sudden expansion of the ice crystals may rupture the cells (for more details see ASHWORTH, 1992).

Evidently the ice resistance of many organisms relies on completely different mechanisms. Most frost-sensitive plants do not have intrinsic ice nuclei which are active at temperatures above – 5 °C (LINDOW *et al.*, 1978a). Such plants may survive frost by transient supercooling (ice avoidance: LARCHER, 1985). Very little is known on this process in frost-sensitive plants, in particular about the factors that controll the supercooling ability of plant tissues (LEVITT, 1980; LINDOW, 1983b).

The supercooling capacity of plants may be limited by the presence of INA-exhibiting bacterial strains (see above) which are widely distributed on leaves of nearly all plants in the field, usually *Pseudomonas* and *Erwinia* spp. (LINDOW, 1983a). Originally this had been described by ARNY *et al.,* (1976) in corn and has since been found in many other cultures such as citrus (YANKOVSKY *et al.*, 1981; YELENOSKY, 1983), raspberry (WARMUND and ENGLISH, 1995, 1998) strawberry (LINDEMANN and SUSLOW, 1987), *Prunus* spp. (GROSS *et al.*, 1984), *Vitis* leaves (GAIGNARD and LUISETTI, 1992, 1993; GARDEA *et al.,* 1993), peas (ELVIRA-RUENCO and VANVUURDE, 2000). In *Vitis* phytoplasmas are supposed to play a similar role (GIBB *et al.*, 1999).

On the other hand INSs may be (partly) inactivated oder hidden by proteins (MARENTES et al., 1993; HON-WAI CHING *et al.*, 1995; SALZMANN *et al.*, 1996; MCARTHUR *et al.*, 2000, FU *et al.*, 2000) or by synthetic chemical compounds which thus were used as frost protectants (a list may be found in RIEGER, 1989; for more recent research see POOL and MCINNIS, 1991; HIMELRICK *et al.*, 1991; MITTELSTÄDT, 1995). Analogous mechanisms could be active during the frost adaptation of winter hard plant parts.

Still other plants seem to control the growth of the crystals in a manner that prevents damage (in the wood of canes and in the basis of winter buds, or in leaves such as garlic and lamb's lettuce). In *Opuntia* mucilage might play such a

role (GOLDSTEIN and NOBEL, 1991, 1994). Whether the activity of urea as a freeze protectant in avocado and peach (ZILKAH *et al.,* 1996) is due to inhibition of ice nucleation is still unclear.

Both from the biological and from the technical point of view it is very important to know the reason why the actual freezing point of different plant tissues may vary between – 1 and – 38 °C depending on the season and other often unknown conditions.

1.3.2 Freezing in frost hardy woody plants

Overwintering canes of woody plants may freeze at temperatures below – 10 °C without damage. It is generally assumed that in this case ice crystals are formed in the xylem or in the apoplast but not within the cells of pith rays or meristems which freeze only at considerable lower temperatures (generally below – 30 °C) if the plants are cold adapted.

A special case are winter buds. While the base of the bud behaves like the cane tissue, the eyes of frost hardened woody plants freeze at – 20°C or below. Therefore superchilling of such buds usually produces so called high temperature exotherms (HTEs) caused by freezing of apoplastic water and one ore more low temperature exotherms (LTE) resulting from the freezing of cells. The number of LTEs depends on the complexity of the bud.

There is a large number of papers on these phenomena in different plants: *Vitis* (which will be discussed later in detail), tomato (ANDERSON and ASHWORTH, 1985), *Prunus* spp. (QUAMME, 1974), cherry (ANDREWS and PROEBSTING, 1987; CALLAN, 1990), peach (QUAMME, 1978, 1983; ANDREWS et al., 1986; ASHWORTH and DAVIS, 1984, 1987; RAJASHEKAR, 1989), apple (QUAMME, 1976), persimmon (KANG *et al.,* 1997), blackberry (WARMUND *et al.*, 1992), blueberry (BIERMANN *et al.*, 1979; FLINN and ASHWORTH, 1994a, b), strawberry

(HUMMEL and MOORE, 1997) *Forsythia* (FLINN and ASHWORTH, 1995), azalea (GRAHAM and MULLIN, 1976 a,b), *Rhododendron* (KAKU *et al.,* 1991), conifers (IDE *et al.,* 1998)

It is generally assumed that a high frost resistence depends on a gradual cold acclimatisation, the mechanisms of which are, however, far from being known. Important factors are desiccation, change of cell wall structures (in wood: WISNIEWSKY, 1993), biochemical processes such as deposition of specific proteins (MARENTES *et al.*, 1993; HON-WAICHING *et al.*, 1995 in graminees; SALZMANN *et al.*, 1996 in grapevine), proline (AIT BARKA and AUDRAN, 1997) or the conversion of polysaccharides to monosaccharides (KLIEWER, 1965). However, most of these substances have been analyzed in the canes and not in the buds which usually are very small. Now the canes will freeze in hard winters without damage even after prolonged cold adaptation. So this conversion of starch at low temperatures might be a pure coincidence as is also concluded by FLINN AND ASHWORTH (1995) who analyzed *Forsythia* canes. Other factors of frost resistance might be morphological structures such as the formation of separation tissues between eye and bud axis (JONES *et al.*, 2000) or the degeneration of vessels (e.g. VON FIRCKS, 1993). More detailed discussions can be found in QUAMME (1995) and ANDREWS (1996).

1.3.3 Freezing in grapevine

There are numerous experiments with field plants and wood cuttings of grapevine which corroborate the practical experiences of grape growers.

PROEBSTING *et al.* (1980) and STERGIOS and HOWELL (1973) submitted woody canes from the field to a slow deep freezing cycle. Whether a bud had been damaged was determined either by cutting the bud and looking for browning or by testing the shoot growth capability. By this method the

temperature was determined which led to a 50 % survival rate of 'Concord' buds. ANDREWS *et al.* (1983, 1984, 1986), WOLF and POOL (1986, 1987), and WOLF and COOK (1994) compared varieties on the basis of exotherm measurements since it had been shown by PIERQUET and STUSHNOFF (1977), PIERQUET, STUSHNOFF and BURKE (1980) and QUAMME (1986) that LTEs were reliable indicators for the death of grapevine buds. However, FLINN and ASHWORTH (1994) concluded from experiments with blue-berries and *Forsythia* that this is not necessarily true for flower buds of other plants which are anatomically different although in KADIR and PROEBSTING (1994) had used exotherm measurements to test sweet cherry cultivars for dormant bud hardiness.

The analysis of exotherms is rather time consuming because freezing cycle of several hours has to be used to approach natural conditions. Since for statistical reasons a certain amount of data is necessary, in the experiments cited above groups of buds were analyzed simultaneously with one temperature sensor so that the exotherms could not be attributed to individual buds. Therefore WOLF and POOL (1987) designed a device to minimize supercooling of the bud's subtending nodal tissue (bud pad) to avoid mixing up HTE and LTE data. The aim of the present work was to carry out histological and biochemical analyzes with buds of known "freezing history" (after occurrence of HTE only or both LTE and HTE) to get direct evidence of freezing damage and of possible relations between HTE and LTE in particular because there are contradictory statements on the nature of the different exotherms observed during freezing of the bud. Therefore an equipment was developed which allows the simultanous freezing at programmable speed of 16 buds in separate copper wells. This device allowed the analysis of over 15000 buds and other samples in three consecutive winters from 1997-2000.

2 Material and Methods

2.1 Exotherm recorder

2.1.1. Introduction

Since the analysis of slow freezing events is rather time consuming and needs much repetitions to get reliable results many authors have used microcomputers for automatic recording (ANDREWS *at al.*, 1983; WOLF and POOL, 1986; SCHUETZE, 1990, WAMPLE *et al.*, 1990). To overcome the drawbacks of these earlier devices we developed a special freezing and data acquisition system (Kryoscan) which allows programmed chilling of 16 small objects in a temperature range between + and – 40 °C. The samples are placed in small copper wells (about 1 ml, 10 mm diameter) which are soldered onto a copper block (fig. 2.1). A difference sensor (small Peltier module, 2.5 × 4 mm, composed of 6 elements) is situated on the bottom of each well (fig. 2.2 a,b). A freezing event in a specimen causes a transient rise of its temperature (exotherm) which is recorded as a temperature difference between the sample and its container. All sensors are scanned about 10 times per second so that exotherms can be recorded nearly simultaneously but separately for each specimen. The curves are displayed on a computer screen and can be saved on discs and printed on paper.

Fig. 2.1 *Left:* Kryoscan system with notebook based controller; lid of the freezing block removed. *Right*: Detail - freezing block embedded in insulating foam

To simulate natural freezing events in the field, the temperature of the sample wells can be lowered at a programmable chilling rate (between 1 and 240 °C per h) or held constant at a given temperature. Freezing events in humid samples such as plant buds, parts of fruits or leaves can be determined with a precision of +/- 0.2 °C.

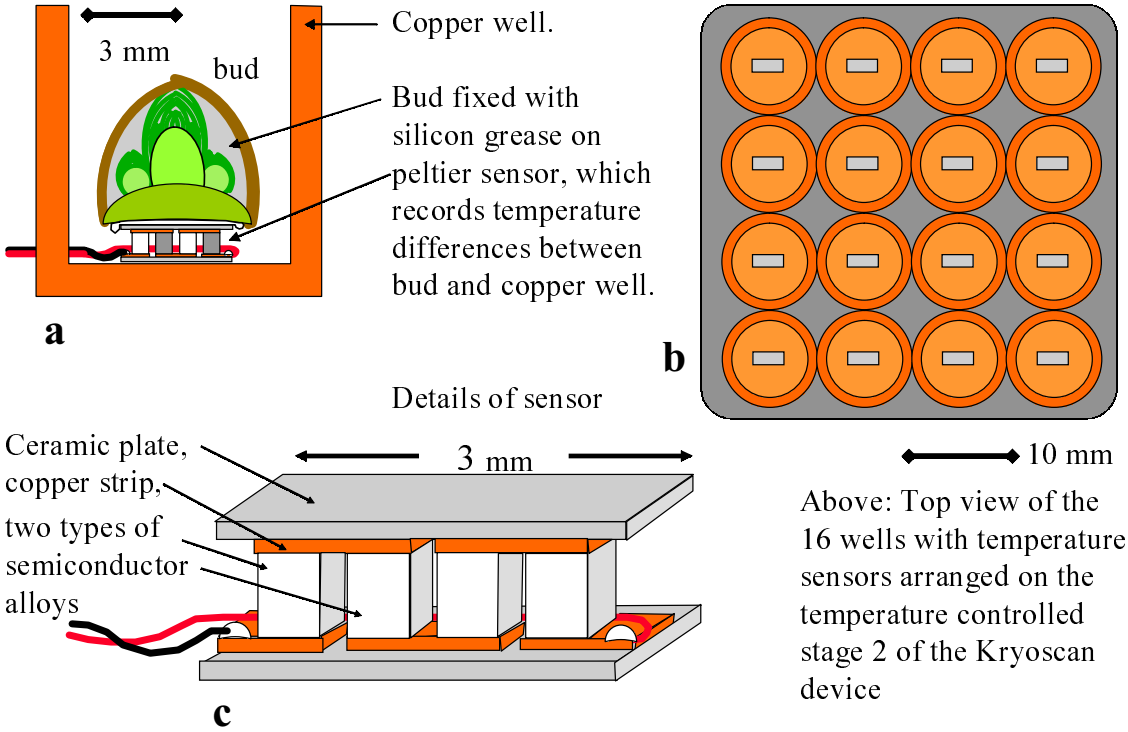

Fig. 2.2 Technical details of the Kryoscan system (after Blaich, unpublished)

2.1.2 Technical details.

The chilling block is temperated by a two stage PELTIER cascade with a constantly prechilled first stage and a proportional regulation of the second stage (fig. 2.3). The total chilling capacity of the system is around 200 W. Miniature Peltier modules instead of thermocouples are used to record the exotherms since they offer a flat surface to fix the samples with heat conducting silicon paste and yield a relatively high voltage difference which is less susceptible to electrical noise. In addition no external zero references are needed. The absolute temperature of the block is measured by a PT100 sensor and both recorded and indicated by a digital instrument which can be

calibrated. The temperature of the specimens and of the block are identical if no exotherm occurs and if the temperature changes are slow enough. If these are too fast, there may be differences because the heat transfer from the copper walls of the well and from the ceramic plate of the Peltier sensor to the specimen is relatively slow, even if heat conducting pastes are used.

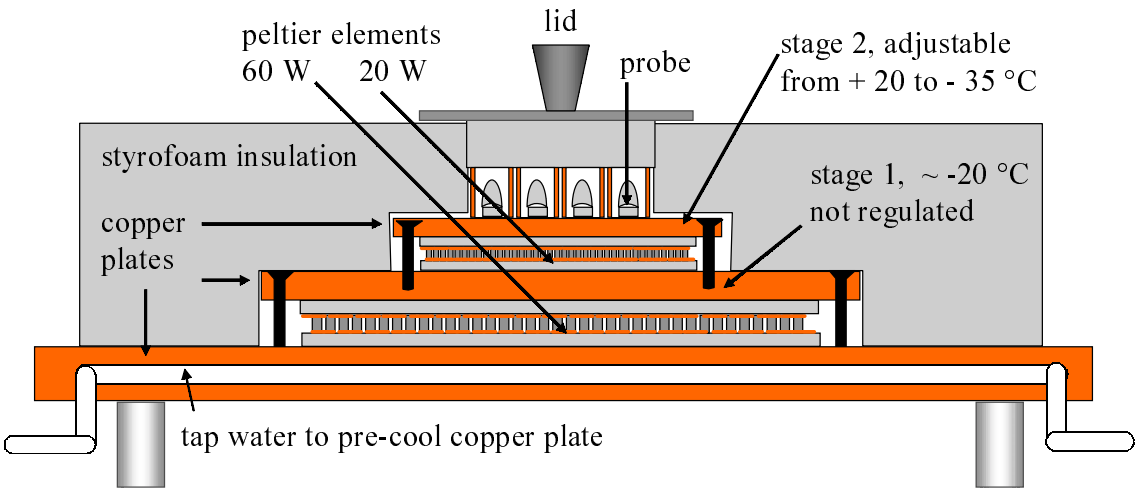

Fig. 2.3 Cross section of the peltier cascade of the Kryoscan system (after Blaich, unpublished)

The device is controlled by a PC under Windows equipped with a AD/DA-interface. Because only slow events are measured even an outdated PC was sufficient for this task. The standard software was developped in Delphi (Pascal) for a Neolab-DAQPad 1200 – interface which offers AD channels to record the exotherms, a DA channel to control the chilling temperature and

digital ports for channel selection. In addition the program allows to calibrate the device.
The exotherm recorder measures 26 × 26 × 10 cm, has a weight of 3 kg and includes an integrated direct current power supply (300 W) for the Peltier elements. It needs a water supply of 500 ml/min to precool the first stage of the Peltier system.

2.1.3 Evaluation of the exotherms.

After the freezing cycle the temperature curves were both printed out and saved on discs. After critical examination of the exotherms (problems will be discussed in chapter 3.1.2) the temperature data was registered in Excel-Sheets for statistical evaluation.

2.2 Plant material

Preliminary tests were carried out on buds of grapevines of the varieties ‘Bacchus’, ‘Schwarzriesling’ (Pinot meunier), ‘Spätburgunder’ (Pinot noir), ‘Regent’ and ‘Riesling’ of different proveniences. Since no obvious varietal differences were found, only ‘Bacchus’ was used for further tests on large numbers of buds which were carried out mainly during the three winters from 1997 – 2000. Measurements during the summer served as control experiments. These plants were grown since 18 years in the vineyard of the Institute for Fruit, Vegetables and Viticulture (370) of the University of Hohenheim. The vineyard is situated at 400 m above sea level at 48° 43’ geographical latitude

and 09° 13’ longitude, slightly sloped towards south and receives 800 mm of precipitations per year.
Well ripened canes with at least 16 well developed lateral buds were selected and collected in the morning around 8:00. The buds were cut off with a sharp knive and grouped in 3 classes: 5 buds from the basal, 6 from the intermediate region of the cane and 5 topmost buds. For more information on grapevine compound buds see schematic diagram in figures 2.4 and micrographs of sections in the following chapter. From 1997 – 2000 a total of 15680 buds were freeze tested during approximately 1200 measuring cycles.

2.4 Determination of dry weight and water content

Dry matter (dm) and water content of wood and buds were determined regularly every 2 weeks from March 1998 until May 2000. The studies were carried out during the winter and spring months of 1998-2000 on buds and stems from basal, intermediate, and apical sections of the shoots of grapevine (cv. “Bacchus”), grown at the vineyard of the Institute for Fruit-, Vegetable- and Viticulture (370) of the University of Hohenheim, Stuttgart-Germany. Buds and wood was collected weekly from 18-year-old grapevines.
Canes were collected in the vineyard, fresh weight was determined by removing buds and stem slices with a sharp scalpel, placing them in previously tared glass vials, and immediately weighing them. Tissue samples were then oven-dried for 3-8 days at 85 °C and reweighed until constant weight. Water content was calculated as percent of the total fresh weight.

2.4 Analysis of sugars and anions

Soluble sugars were quantified using high-performance anion exchange chromatography (HPAE) combined with pulsed amperometric detection (PAD) in a Dionex DX300 ion chromatograph fitted with a CarboPac PA-1 column (CHATTERTON et al. 1989). Flow rate was 1 ml*min-1 at about 1900 psi. Carbohydrate elution was effected under alkaline conditions (150 mM NaOH). The high pH (12-13) of the eluant converts hydroxyl groups of the oligosaccharides into oxyanions. The degree of oxyanion interaction with the anion exchange resin determines carbohydrate retention times. Adding a competing ion such as acetate (0-500 mM NaOAc) to the eluant reduces the retention times. Sugars in the extract were identified by comparing their HPLC-PAD retention times on various gradients with those of known sugars (i.e. glucose, fructose, sucrose, raffinose, stachyose) and by spiking the extracts with known pure sugars. Known pure sugars were used for calculation of the sugar concentration in extracts.

For anion and carbohydrate analysis, the lyopholized samples were ground. 60 mg of dried tissue was extracted twice for 15 min in 7 ml boiling H_2O dest. The two extracts were centrifuged, and the combined supernatant (14 ml) was passed through a 0.22 mm membrane filter. The detection of anions (i.e. Cl^-, SO^{4-}) was performed by HPAE-PED (electro chemical detection) using AS14 columns. Flow rate of the eluant (3.5 mM Na_2CO_3 + 1.0 mM $NaHCO_3$) was 1.2 ml×min^{-1}.

Statistical analysis was undertaken with SAS 6.12 (SAS Institute Inc., Cary, NC/USA) using PROC ANOVA/GLM procedures and Tukey's studentized range test for analysis of variance and PROC CORR procedures and Pearson's correlation coefficient for analysis of correlation.

2.5 Histological methods

2.5.1 Embedding

Buds were embedded in hard plastic according to RUDDELL (1967 a,b) modified by HERMANNS and SCHULZ (1981). Details are given in table 2.1.

2.5.2 Cutting

Cuts of 5-9 µm thickness were made from the hardened plastic blocks with a rotation microtom (Reichert-Jung Model 2040 and 2050), equipped with a hardmetal microtome blade type D. 6 µm cuts exhibited the best quality. From each block series of longitudinal and transversal cuts were made. 4 of these cuts were put on a microscope slide and stretched with a drop of distilled water. The pre-drying of the slides took place on a heat bench at 40°C over three hours; then they were dried for at least four days in a heat oven at 35°C. For further processes the cuts were stored in a slide box at room temperature.

The histological and morphological analysis comprised a total of 3600 buds. Among others 432 blocks of material from the vineyard, 288 blocks of cryoscan-material, and approximately 20000 serial cuts were made in the first experimental year, in the second year 768 blocks from material of the vineyard, 384 blocks of cryoscan-material, and around 32000 serial cuts. Only the best (good differentiated cells) were selected for further treatments and methods.

Table 2.1. Hard-plastic method embedding scheme with time and used materials

Time	Procedure and chemicals used
Day 1 •2 h •3 h •3 h •over night	Dehydration in increasing steps of ethanol concentration •Ethanol (70%) •Ethanol (90%) •Ethanol (96%) •Ethanol (96%)
Day 2 •6 h •over night	Penetration with GMA-MMA (1:1), at 4°C, shaking •Hydroxyethyl-methacrylacidester (GMA), methyl-methacrylacidester (MMA) •dto.
Day 3 •6 h •over night	Penetration with solution A, at 4°C, shaking •Solution A: GMA (60 ml) + MMA (20 ml) + ethyleneglycolmonobutylether (16 ml) as stretch medium + polyethyleneglycol 400 (2 ml) as softener + benzoylperoxid (270 mg) as catalysator •dto.
Day 4 •2-3 days	Embedding of the samples in solution B, polymerisation at 4°C •Solution B: Solution A + N,N-dimethylanilin (0.1 ml) as hardener. The polyethylen models were closed with aluminumstubs as a load, the whole formblock was covered with aluminum foil to remove the polymerisation heat

2.5.3 Staining

For general histological and morphological investigation the cuts were incubated for 20 min in aqueous haematoxylin solution (C.I. 75290 Natural, Serva after Delafield) diluted 1:10/v:v with water then rinsed for 15 min in tap water bath (GERLACH, 1984). The stained cuts were then immersed at room temperature in a series of aqueous ethanol solutions (20 vol.% , 40 vol.%, 70 vol.%, 90 vol.%, and 96 vol.%; respectively 2.5 min) then in isopropyl-alcohol (2x, 5 min.) and in

xylol (5 min), covered with with Eukitt (Kindler, Freiburg) and a cover glass and dried for approximately one week at 35°C.

A modified staining method was developed to improve cell differentiation. After variation of different stains, stain concentrations, stain durations and combinations, the method shown in table 2.2 gave the best results.

Table 2.2 Procedure of the new staining method

Step	**Time**	**Staining solution**	**Watering**
1.	6 h	Bismarck Brown Y (Sigma, 6 g / 100 ml)	Solution filtered with paper filters (Schleicher & Schüll, 240 mm)↴
			watering in tap water
2.	15 min	Lacmoid (Aldrich Chem. Co., 3 g / 100 ml water)	↴
			watering
3.	5 min	Methyl Green (Sigma, 3 g / 100 ml water)	↴
			watering

For starch staining the cuts were immersed for 1 min in Lugol's solution (IKI = potassium iodine-iodine, Merck 9261) diluted with H_2O (12:1/v:v) according to JENSEN (1962) and GERLACH (1984) then the microscope slides were submerged in a distilled water bath to remove the excess of the staining solution. After a successful staining, the microscope slides were dried, but before the microscopic analysis they had to be moistened again with distilled water, before a cover glass was put on.

2.5.4 Microscopy

The slides were investigated under a Zeiss microscope or a Zeiss fluorescence microscope equipped with a filter set of exciter filter BP 390-440, interference beam splitter FT 460 and barrier filter LP 425. Polarized light was used to differentiate primary cell walls from secondary cell walls, sclereid cells, and starch granules.

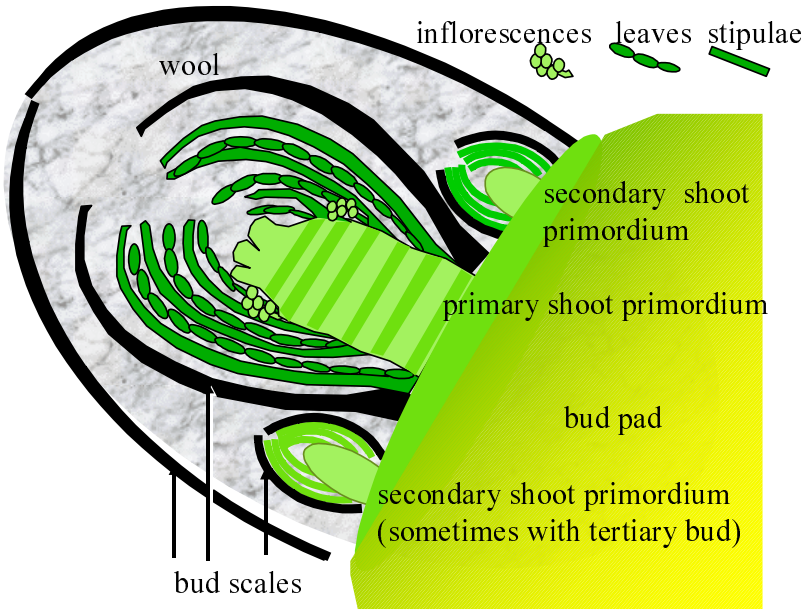

Fig. 2.4 Schematic diagram of a grapevine latent (compound) bud in the winter, the secondary primordia are usually larger (after Blaich)

3 Results and Discussion

Due to the different techniques and types of experiments it was not always appropriate to present all methods at the beginning of the thesis but rather to explain specific methods in the context of the related results. These will be discussed immediately if appropriate (e.g. methodological problems) whereas some of them will be discussed in the final concluding discussion.

3.1 Anatomy of the grapevine latent bud

3.1.1 Introduction

Understanding the bud anatomy is a prerequisite for the interpretation of the exotherm measurements. The descriptions of the bud in most recent publications usally refer to the diagrammatic figures either by KROEMER in the textbook of BABO and MACH (1923) or in the textbook of RIBEREAU-GAYON and PEYNAUD (1971) who took it from SARTORIUS (1938). Figure 2.4 is derived from these publications. Sometimes there are contradictory designations for identicals structures in different textbooks. The figures in PRATT (1959, 1974), BOTTI and SANDOVAL (1990) and KANG *et al.* (1990) show parts of shoot primordia. Microphotographs of whole bud sections of *Vitis vinifera* are rare and usually of low quality since whole buds are difficult to prepare and to cut and these sections are usually used for histochemical tests such as for amylase (AIT BARKA and AUDRAN, 1996). This is the reason why some new photos are presented in this chapter although the anatomy of grapevine buds is well known.

3.1.2 Results and Discussion

Fig. 3.1.1 shows the normal appearance of a node in the winter with a well developed latent bud. A node which was decolorized with peroxide, dried,

soaked with fluorescein solution and made translucent by immersion in xylene allowed the demostration of the vascular bundles under UV-light (fig. 3.1.2).

Fig. 3.1.1 Nodium of grapevine cane in the winter

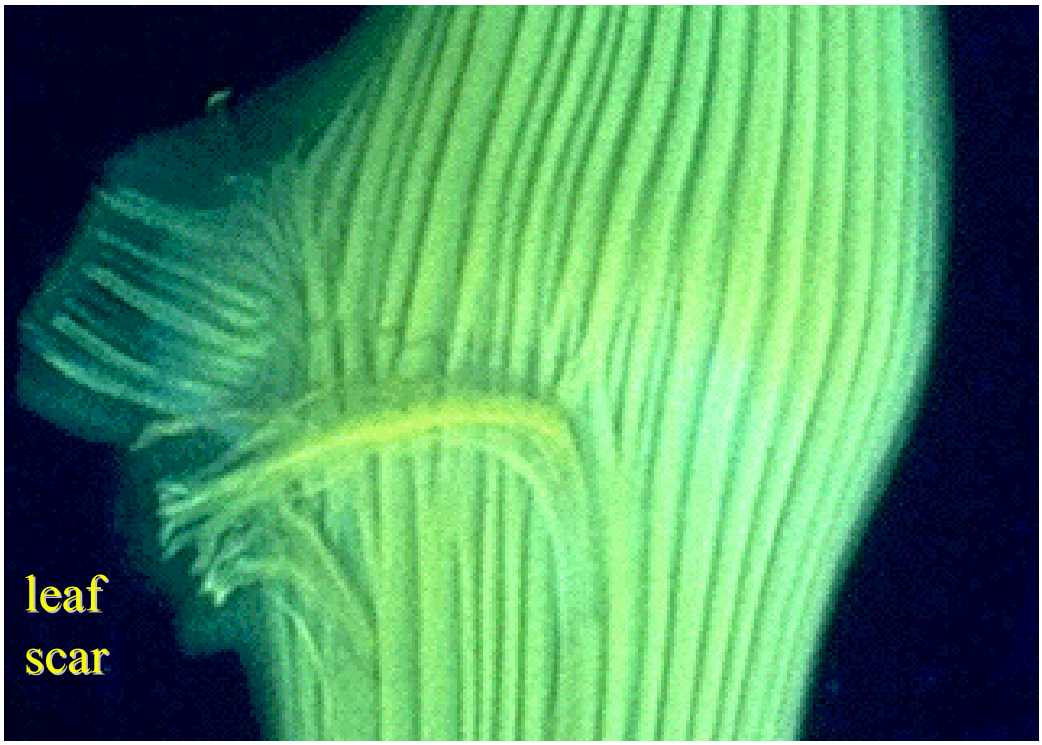

Fig. 3.1.2 Nodium of grapevine shoot treated with xylene to make it translucent; vessels are stained with fluorescent stain (photo: Wind).

Comparison of both figures shows that the xylem strands are not continued into the shoot primordia; they are restricted to the bud pad. which is also visible in figure 3.1.5. Hand cuttings of the bud (fig. 3.1.3) display the natural colors of its components. The structure of the bud is, however, more clear in the stained semithin sections in fig. 3.1.4 (cross section) and in fig. 3.1.5 (longitudinal section) of primary shoots.

It is interesting to note the different structure and stainability of leaf primordia which are wrinkled and the prophylls (bud scales, stipulae) which are smooth and contain calcium oxalate crystals.

Whereas the vessels during the winter do not reach the shoot primordia they start to differentiate by the time of bud swelling (fig. 3.1.6 and 3.1.7). This has been discussed as one of the causes for the loss of freeze resistance by that time (literature in KANG et al., 1998). Thermal imaging (HAMED et al., 2000) with deacclimated buds on canes at the time of bud burst indicated that procambial cells can resist for some minutes the ice travel across their end walls whereas differentiated xylem would allow immediate passage.

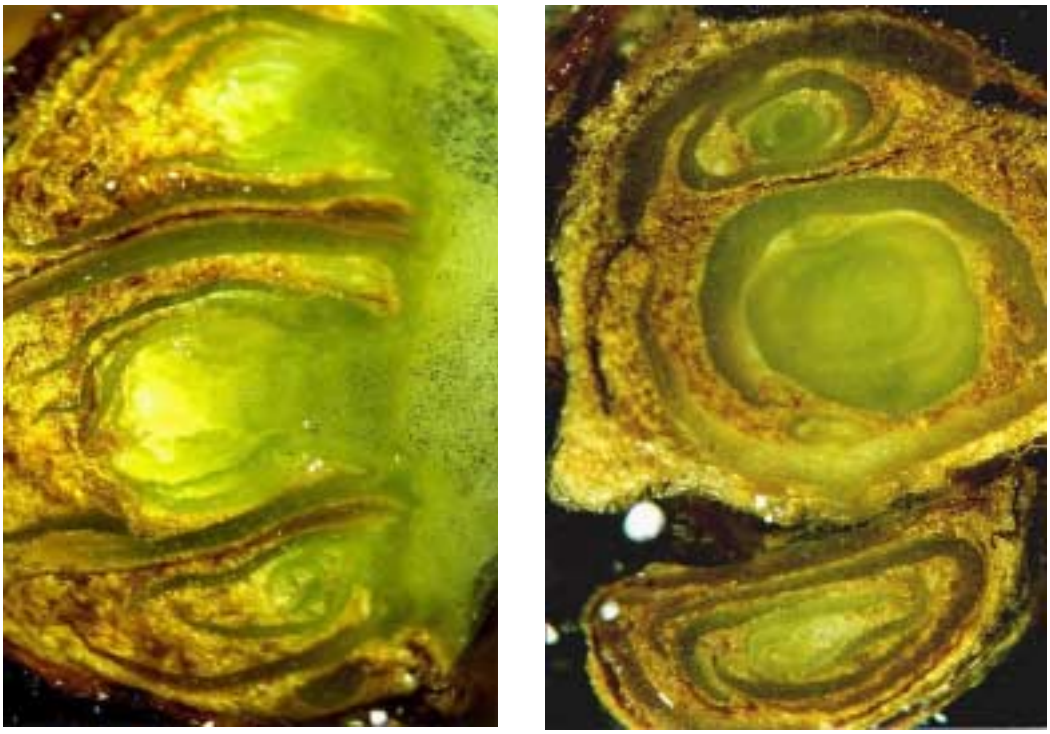

Fig. 3.1.3 Longitudinal section (left) and cross section (right) of grapevine latent buds with primary (center), secondary (bottom) and tertiary (top) shoot primordia

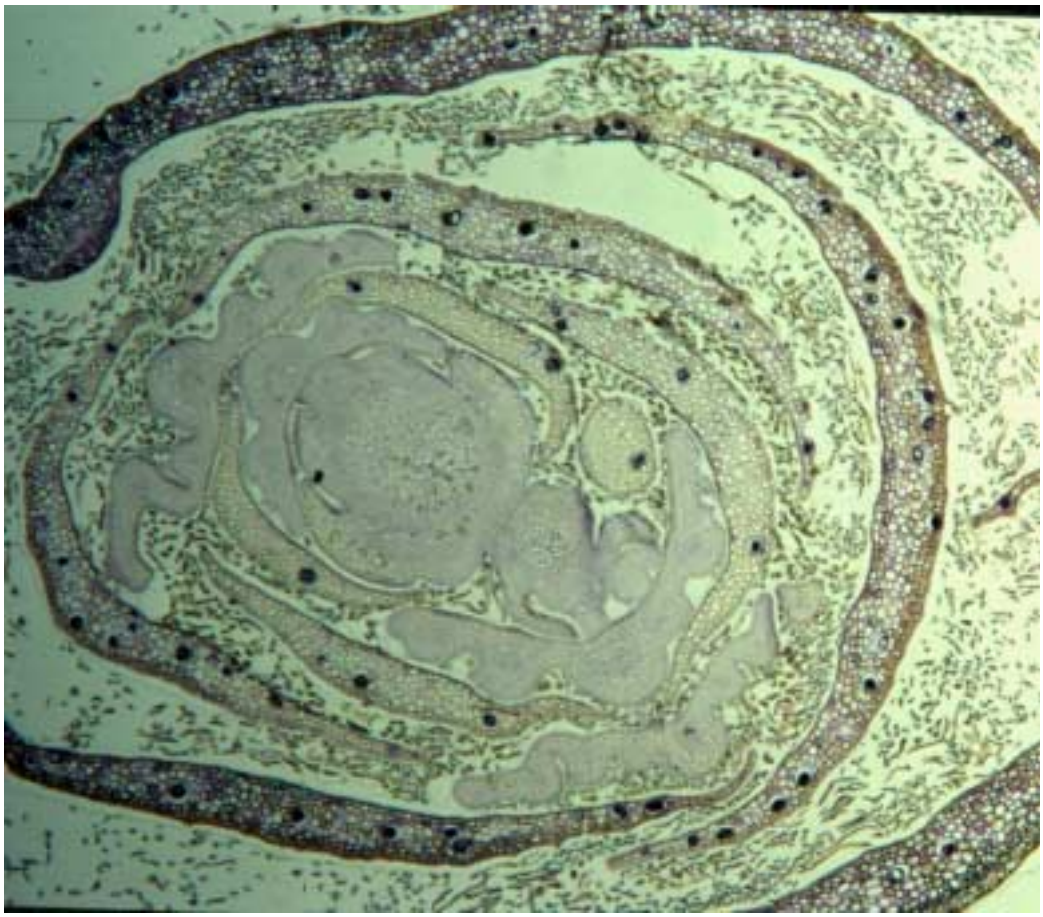

Fig. 3.1.4 Cross section through primary bud (haemato-xyline)

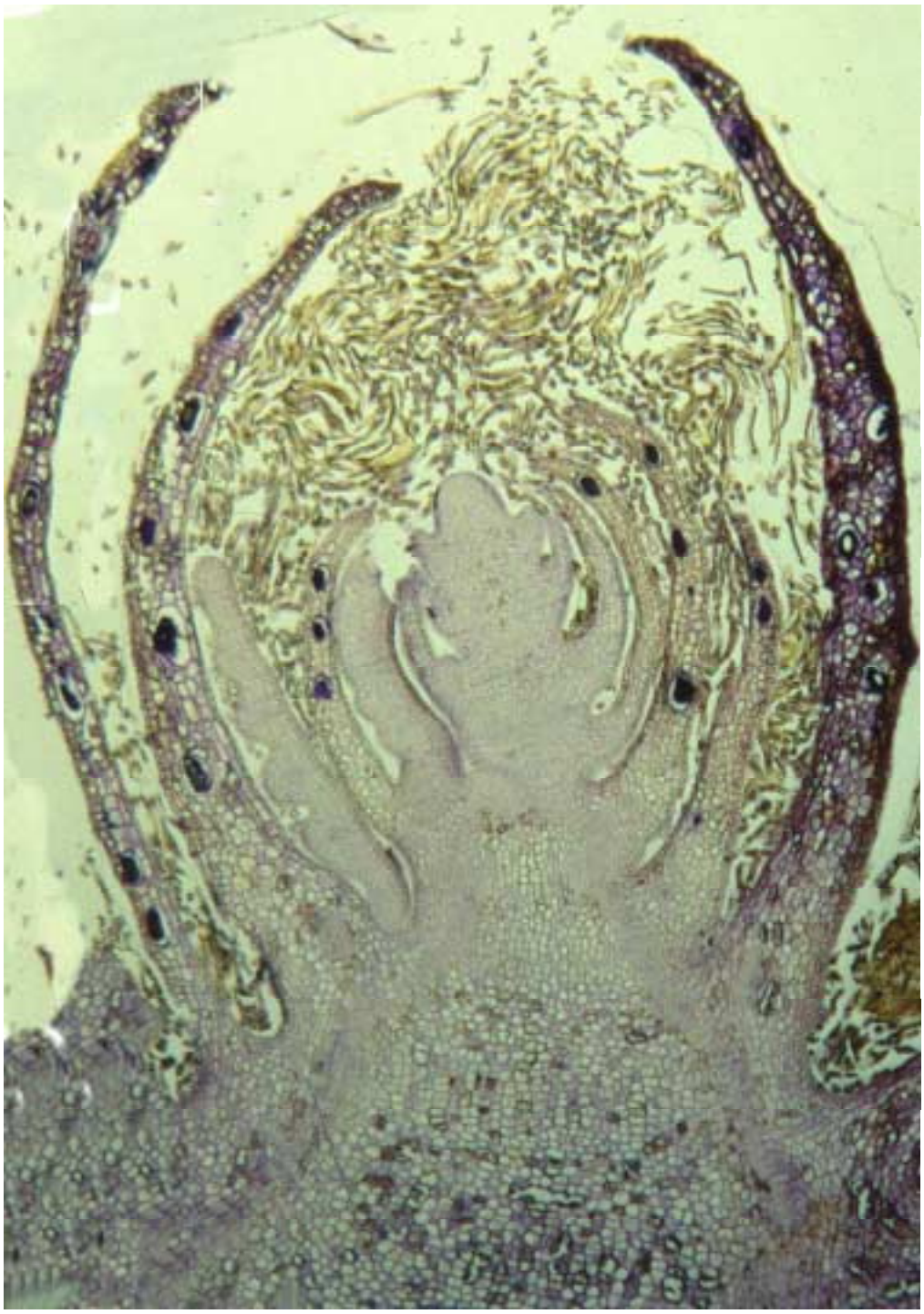

Figure 3.1.5 Longitudinal section through the primary shoot primordium of a grapevine bud (haematoxyline).

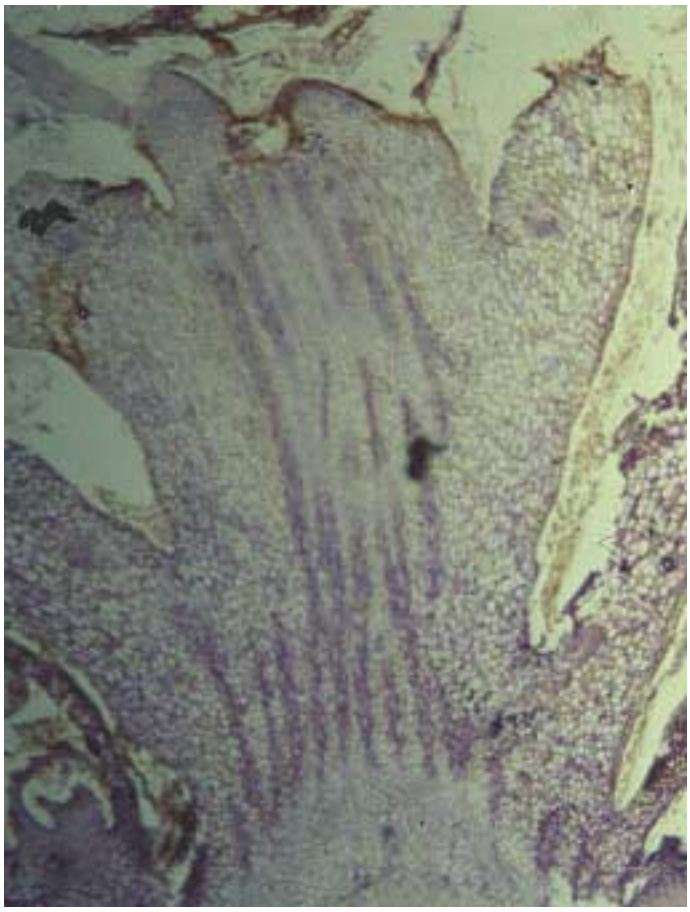

Fig. 3.1.6 Formation of vessels in the primary shoot primordium of a swelling bud (longitudinal section, haematoxyline)

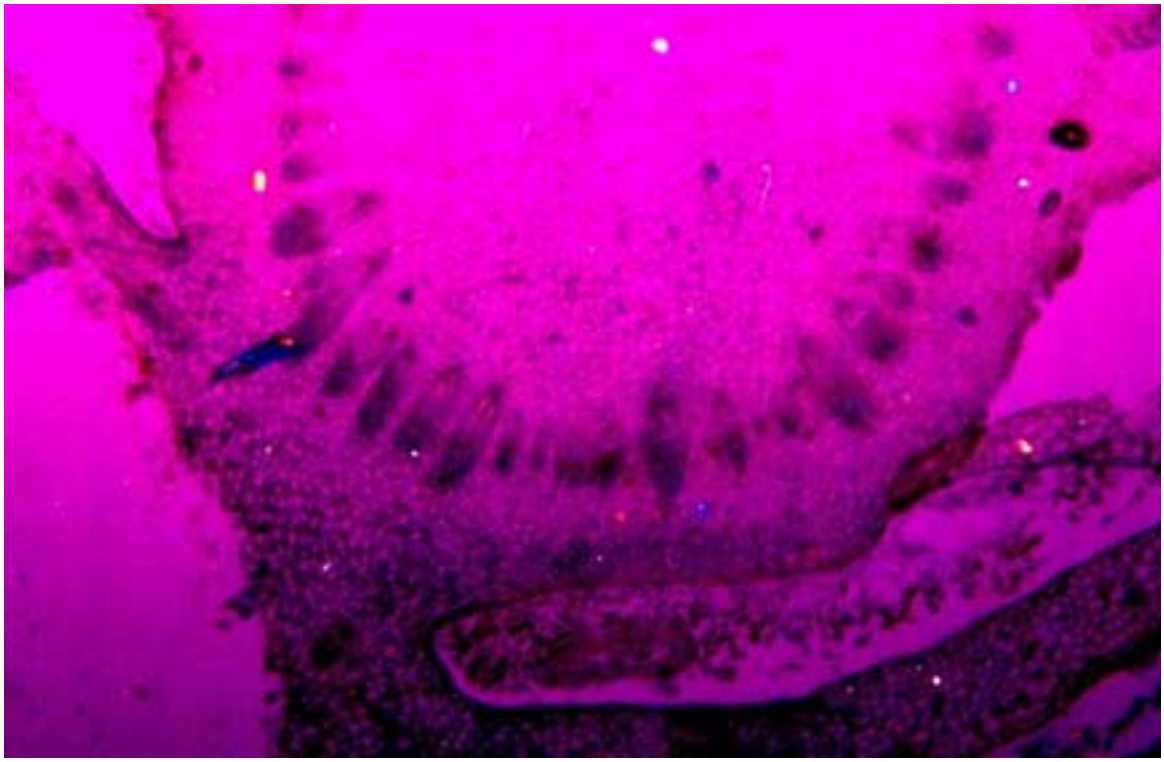

Fig. 3.1.7 Formation of vessels in the primary shoot primordium of a swelling bud (cross section, haematoxyline and magenta filter).

3.2 Evaluation of Exotherm Analysis

Although differential thermal analysis is a well established method, quite a number of basic experiments were carried out to have a better basis for the interpretation of the following measurements and the interpretation of exotherms occuring in buds.

3.2.1 Preliminary freezing experiments

Since the interpretation of exotherms generated by so complicated a structure as a compound bud, as a first step preliminary experiments on simpler structures such as leaves or petioles were carried out.

3.2.1.1 Introduction

Although the concept of ice nucleation is familiar to meteorologists and food technologists, many biologists find it hard to believe that freezing of water above – 40 °C in the tissues of organisms is also explained by this mechanism and not by osmotic or other effects. Even if the phenomenon of supercooling is recognized it is often assumed that freezing of supercooled water is just a matter of time or of a mechanical shock. However, the ice nucleating proteins and their structural genes in *Pseudomonas* are well known (TURNER *et al.*, 1991) and are even used to construct frost sensitive transgenic plants. The following experiments demonstrate, though, that there must be intrinsic INS (ice nucleation sites) in plant tissues. Nevertheless it is difficult if not impossible to find a direct proof for the presence of INS in higher plants. Therefore we tried to get indirect evidence based on the following considerations.

Small droplets of water on filter paper or pieces of plant tissue will generally supercool to around – 10 °C before freezing occurs. There is, however, a certain variability (e.g. between – 7 and – 15 °C) of the freezing points and the question arises whether freezing occurs more or less accidentally within a

certain temperatur range (which would imply freezing at higher temperatures at low cooling rates) or whether the freezing points are characteristic for each specimen if all other conditions are kept constant. This could be clarified by repeated freezing of the same specimen and by varying the cooling rate. Such experiments were already reported by VALI (1971) with water drops. He could show that the freezing point of individual drops was reproducible with few exceptions. It was, however, not clear whether freezing and thawing of plant tissue would change the conditions of the second experiment particularly in frost sensitive plants where ice crystal formation would disrupt the cellular structure and activate hydrolytic enzymes. This should not be the case for water droplets on filter paper consisting of dead and broken cotton hairs.

3.2.1.2 Material and Methods

Small droplets (0.5 – 2 µl) of distilled water or solutions on different supports and small plant parts (leaf and stem segments) were frozen, then thawed and refrozen. Both frost resistant plants (e.g. *Valerianella, Allium*) and frost sensitive plants (e.g. *Vitis, Phaseolus*) were tested.

It is frequently assumed that there exists a "freezing probability" of supercooled water. To test whether the duration of the frost period rises the average value of the freezing points we varied the chilling speed between 1 and 12 °C per hour but could find no significant difference.

To test, whether certain structures in the supercooled plant tissue might form mechanical barriers against the growth of ice crystals we tested leaf pieces including main veins and similar "barriers". Droplets of distilled water or solutions on different supports as well as small leaf and stem pieces were frozen, rewarmed to allow thawing and frozen again.

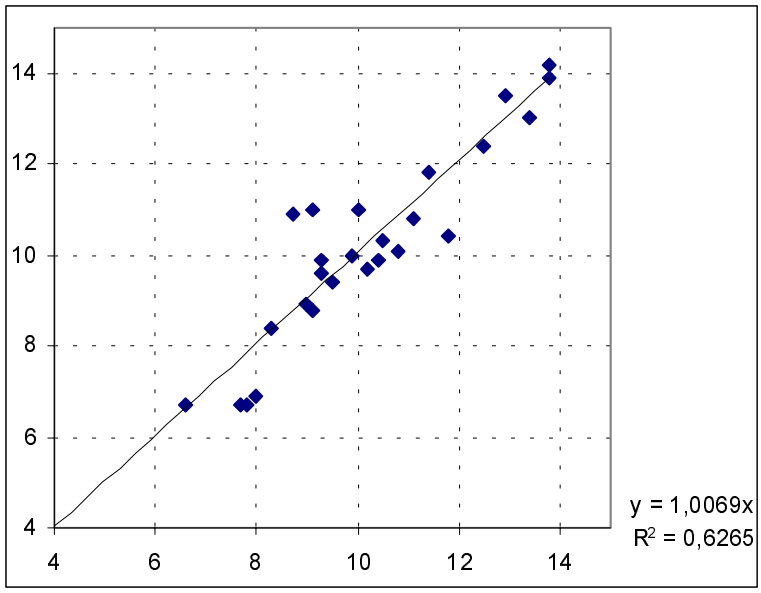

Fig. 3.2.1 Correlation between first and second freezing points of water drops (2 µl) on filter paper. Merged values of 2 paper brands.

3.2.1.3 Results and Discussion

Organic material: Repeated freezing of water droplets on ***filter paper*** indicated that the average freezing points are characteristic for the brand of the paper (fig. 3.2.1). Generally only few values out of 16 would not fit into that pattern. No correlation was found only in two cases. (1) if the variability of the results of the first freezing cycle was too small (2) if water droplets were tested on dirty cardboard instead of filter paper.

Adding of osmotically active substances lowered the freezing point according to the concentration of the substance. For example the average freezing points of droplets of a 1 M sucrose solution on a certain brand of filter paper were around – 12 °C whereas water droplets on the same supporte froze at – 10 °C. This corroborates the findings of VALI (1969) who reports that 5 % of his specimens initiated freezing at temperatures 2 °C different from those initially observed.

With ***drops on cedar wood*** it could be shown that the amount of water influences the freezing point of the drops (fig. 3.2.2). The explanation on the

basis of ice nucleation is evident: Every drop contacts a certain number of nucleation sites with different temperature specifity. Only the site with the highest nucleation temperature is relevant for the freezing point. The larger the drop the higher is the probability that it might contact a high temperature site.

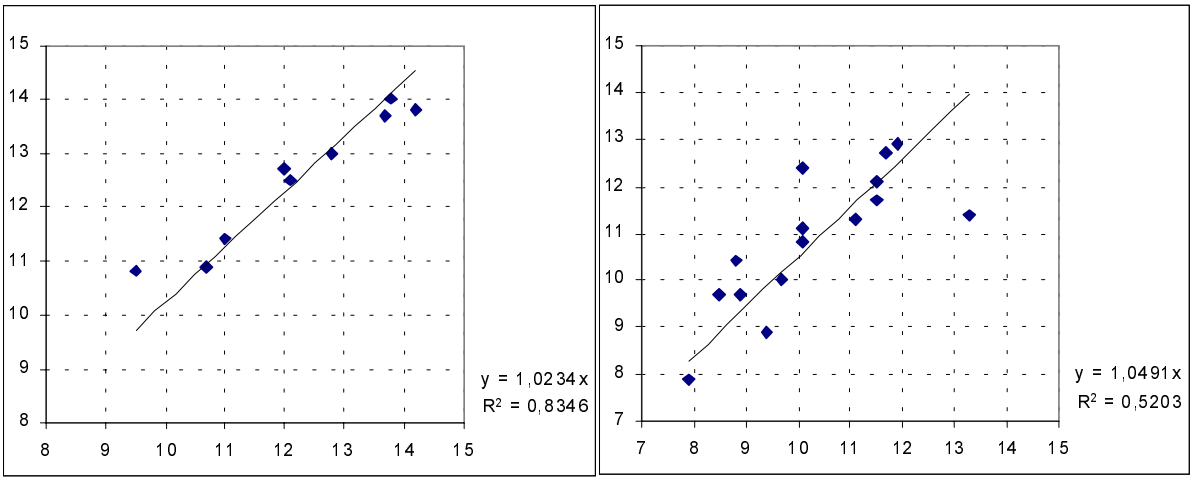

Fig. 3.2.2 Correlation between first and second freezing points of water drops on cedar wood; the average values for 1 µl drops (left diagram: -12.4 °C) and 2 µl drops (right diagram: -10.2 °C) are significantly different.

The following observation fits into that pattern: Although the values of the first and the second freezing cycle on paper or wood were significantly correlated (figs. 3.2.1 and 3.2.2), the average second freezing point was consistently between 0.3 and 0.5 °C lower than the first one. This could be explained by the evaporation of water between the two cycles which is less so in the following tests with plant material because this has a much better protection against evaporation.

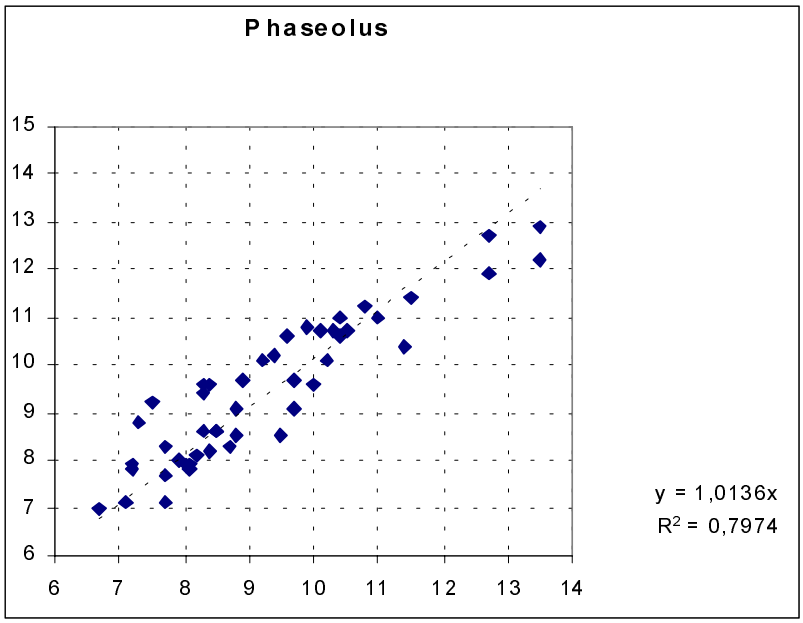

Fig. 3.2.3 Correlation between first and second freezing points of leaf pieces of *Phaseolus vulgaris.* Merged values of 3 experiments in 2 years

Plant parts: Here too, the repeating of the freezing cycle showed a clear correlation between primary and secondary freezing points (figs. 3.2.3, 3.2.4, 3.2.5 and 3.2.6). The number of inconsistent values was also around 5 – 10 % - as in our experiments and those of VALI (1969) with water drops.

However, if the specimens were left for some hours at room temperature after the first cycle the correlation got worse. This effect can be explained by the activity of hydrolytic enzymes. If thawing occurs at a low temperature and/or if the delay until the second cycle is kept short, their activity remains at a minimum. Evidently although the INS are rarely influenced by the physical damage in the cells caused by the growth of ice crystals they are changed by the activity of autolytic enzymes.

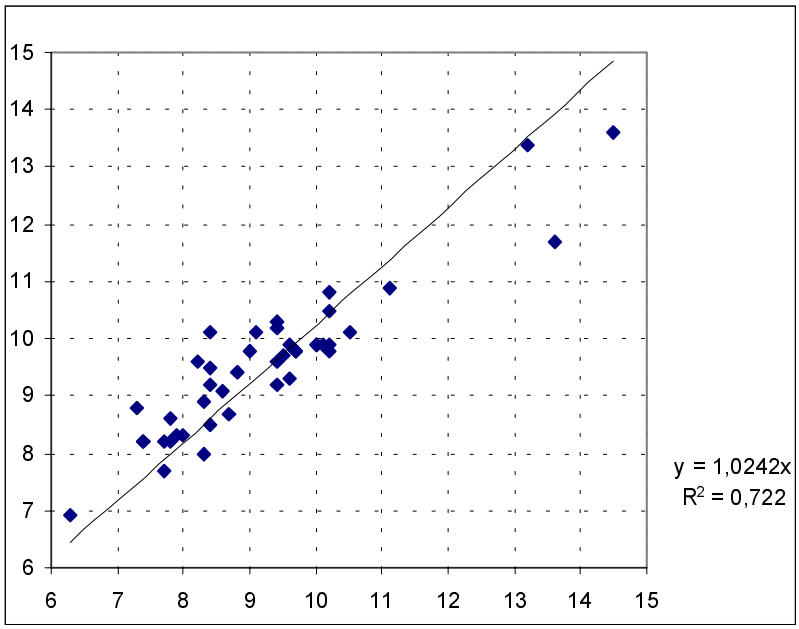

Fig. 3.2.4 Correlation between first and second freezing points of flower stems of *Phaseolus* sp. Merged values of 2 experiments from 2 years

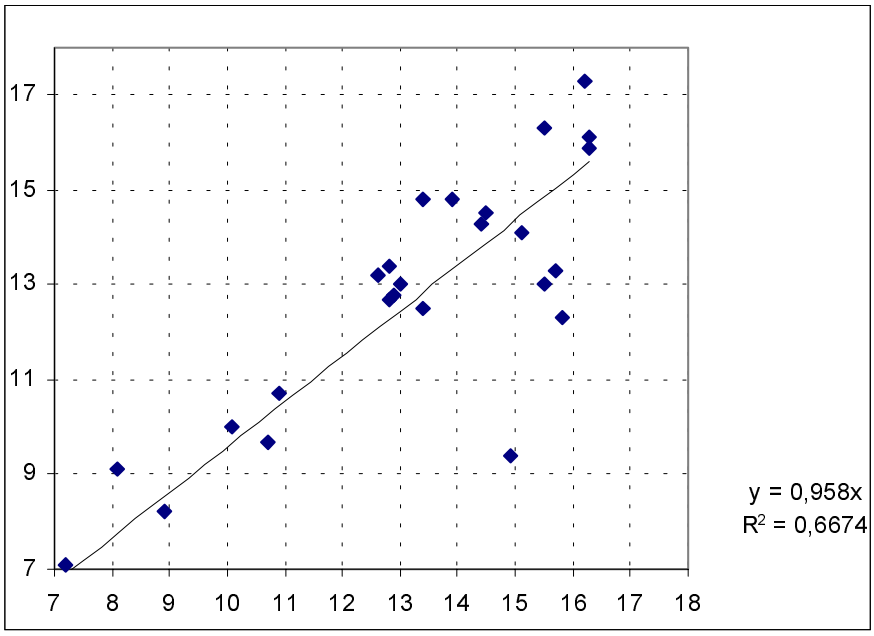

Fig. 3.2.5 Correlation between first and second freezing points of leaf pieces of *Brassica sp.* Merged values of 2 experiments in 2 years

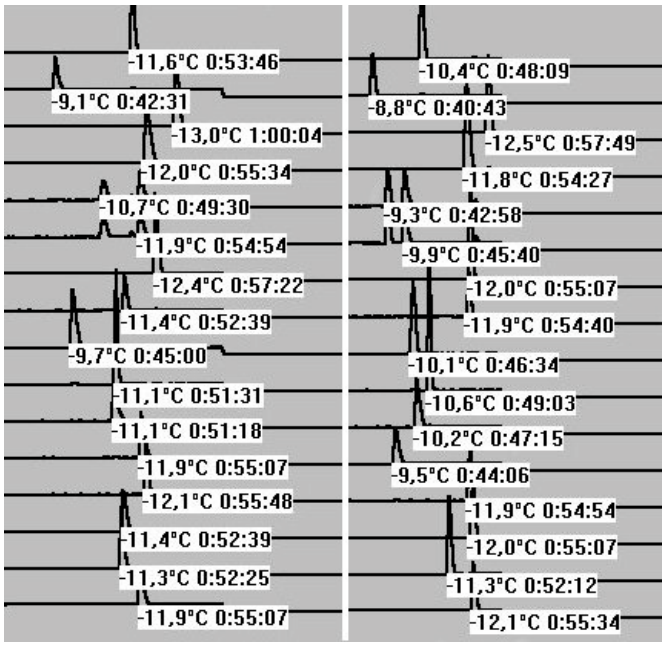

Fig. 3.2.6 a *Left:* Exotherms obtained by freezing of 1 x 1 mm pieces of a young grapevine leaf. *Right:* Exotherms after thawing and refreezing of the same samples

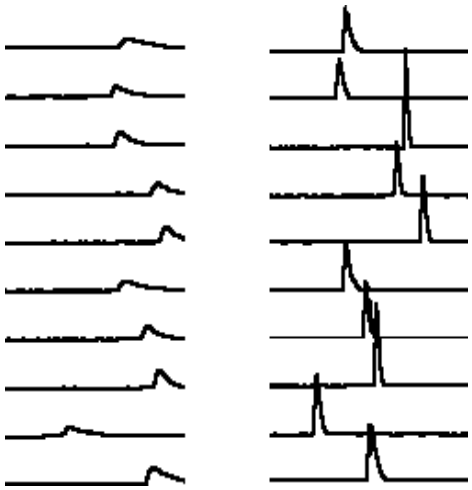

Fig. 3.2.6 b *Left:* Exotherms obtained by freezing of 1 x 1 mm pieces of an old grapevine leave. *Right:* Exotherms after thawing and refreezing of the same samples

Barriers against the growth of ice crystals*:* It is interesting to compare the different forms of the exotherm peaks of the first and the second freezing cycles in the first experiment. The second freezing event exhibits peaks which are dinstinctly sharper and higher as the peaks of the first cycle (fig. 3.2.6 b). This is characteristic only for plant parts but not for filter paper. Evidently the undamaged cells offer resistance to the growth of the ice crystals which is diminished considerably after their disruption whereas this is not the case with the hollow cotton fibers of the filter paper. In addition this effect is less pronounced in young leaves with tender cell walls (fig. 3.2.6 a).

In this context it is quite astonishing that the ice nucleation sites withstand this disruption – the absence of a correlation between the first and the second freezing point would have been no proof that freezing of plant parts occurs randomly and is not influenced by ice nucleation sites.