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Chlorophyll Fluorescence as a Parameter for Frost Hardiness in Winter Wheat. A Comparison with other Hardiness Parameters.

By

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With 2 Figures

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Summary

CLEMENT J. M. A. M. & VAN HASSELT P. R. 1996. Chlorophyll fluorescence as a parameter for frost hardiness in winter wheat. A comparison with other hardiness parameters. – Phyton (Horn, Austria) 36 (1): 29–41, 2 figures. – English with German summary.

Frost hardiness of winter wheat leaves (*Triticum aestivum* L. cv. Urban) was measured during an eight weeks hardening period using chlorophyll fluorescence. Determination of frost induced damage after freezing, measured as the decrease of photochemical capacity of photosystem II ($F_V/F_M = (F_M-F_0)/F_M$), was compared with three conventional methods to determine the degree of frost damage: electrolyte leakage, TriphenylTetrazoliumChloridereduction, and visual assessment. There was a good correlation between frost hardiness measured by chlorophyll fluorescence and the other methods.

Winter wheat leaves reached a hardiness of –12 °C within eight weeks of frost hardening. In unhardened leaves, electrolyte leakage and TTC-reduction measurements were influenced by supercooling of the tissue, which lowered the lethal temperature about 4 °C. Supercooling could be prevented by addition of small ice crystals. After one week of hardening the effect of supercooling was no longer observed, because the plants were hardened to a lower temperature as could be achieved by supercooling. It is concluded that the determination of the ratio of variable to the maximal chlorophyll fluorescence after freezing is a reliable and rapid method to determine the frost hardiness of wheat leaves.

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Zusammenfassung

CLEMENT J. M. A. M. & VAN HASSELT P. R. 1996. Chlorophyllfluoreszenz als Parameter für Frostresistenz in Winterweizen. Ein Vergleich mit anderen Resistenzparametern. – Phyton (Horn, Austria) 36 (1): 29–41, 2 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Frosthärte der Blätter von Winterweizen (*Triticum aestivum* L. cv. Urban) wurde während einer achtwöchigen Härtungsperiode mittels Chlorophyllfluoreszenz gemessen. Als Maß für den frostinduzierten Schaden nach Kälteeinwirkung wurde die Abnahme der photochemischen Kapazität des Photosystems II ($F_V/F_M = (F_M-F_0)/F_M$) bestimmt und mit drei gebräuchlichen Parametern (elektrolyte leakage, Reduktion von TriphenylTetrazoliumChlorid, optische Schadensbemessung) verglichen. Die Ergebnisse der Fluoreszenzmessungen korrelierten gut mit denen der übrigen Methoden. Die Blätter von Winterweizen erreichten während der achtwöchigen Härtungsperiode eine Resistenz gegen Temperaturen bis –12 °C. In ungehärteten Blättern wurden die Bestimmungen von electolyte leakage und TTC-Reduktion durch Unterkühlung des Gewebes, die die letale Temperatur um rund 4 °C erniedrigte, beeinflußt. Diese Unterkühlung konnte durch Beigabe kleiner Eiskristalle vermieden werden. Nach einer Woche Frosthärtung konnten keine Unterkühlungseffekte mehr beobachtet werden, weil die Pflanzen bereits an niedrigere als die durch Unterkühlung erreichbaren Temperaturen angepaßt waren. Aus den Untersuchungen wird geschlossen, daß die Bestimmung des Verhältnisses variable/maximale Chlorophyllfluoreszenz nach Kälteeinwirkung eine schnelle und verläßliche Methode zur Bestimmung der Frosthärte von Blättern bei Weizen darstellt.

Introduction

The measurement of chlorophyll a fluorescence (further referred to as chlorophyll fluorescence) has been used for many years as a sensitive, reliable, and rapid method to determine the effect of environmental stresses, like drought, temperature, excessive light and air pollution on green plants (BOLHÀR-NORDENKAMPF & al. 1989). SMILLIE 1979, and after him many others, showed that the decrease of the maximal rate of the fast rise of fluorescence after exposure of leaves to chilling temperatures, correlated well with the visual symptoms of chilling injury in several species (MACRAE & al. 1986, SMILLIE & al. 1987, HETHERINGTON & ÖQUIST 1988). Also other fluorescence parameters (F_V, F_0, F_M, and their derived ratios) were used to evaluate chilling stress on plants (KAMPS & al. 1987, BRENNAN & JEFFERIES 1990).

Frost stress has also been investigated by this technique. Several authors studied the effect of freezing temperatures on photosynthesis by use of chlorophyll fluorescence (KLOSSON & KRAUSE 1981, STRAND & ÖQUIST 1985, STRAND & ÖQUIST 1988, BOLHÀR-NORDENKAMPF & LECHNER 1988). Others used chlorophyll fluorescence as a parameter in order to rank species and varieties for frost tolerance (SUNDBOM & al. 1982, BARNES & WILSON 1984, LINDGREN & HÄLLGREN 1993, ÖQUIST & al. 1993). In most of these studies the change in variable fluorescence upon freezing was

measured. This parameter however is dependent on species, chlorophyll content, leaf thickness and leaf side (BJÖRKMAN & DEMMIG 1987).

The ratio of variable to maximal fluorescence (F_V/F_M which is equal to $(F_M-F_0)/F_M$) appeared to be a reliable and quantitative indicator of the photochemical capacity of photosystem II (BJÖRKMAN & DEMMIG 1987). In contrast to the variable fluorescence (F_V), this ratio was found to be highly constant in unstressed leaves of diverse species. The value for 37 investigated C_3 species was 0.832 $\pm$ 0.004 (BJÖRKMAN & DEMMIG 1987). Several forms of stress seem to reduce the photochemical capacity of photosystem II and consequently the ratio F_V/F_M (POWLES 1984, LICHTENTHALER 1988, BOLHÀR-NORDENKAMPF & al. 1994). Therefore, it is suggested that the F_V/F_M ratio is a suitable parameter for the assessment of stress damage in plants (BJÖRKMAN & DEMMIG 1987, BOLHÀR-NORDENKAMPF & al. 1994).

In this study we tested the possibility to measure the development of frost hardiness in winter wheat by use of the fluorescence parameter F_V/F_M. To get insight into the reliability of this method, chlorophyll fluorescence measurements were compared with three conventional methods to determine the degree of frost damage: electrical conductivity, TTC-reduction, and visual assessment of damage after freezing.

Materials and Methods

Abbreviations

F_0, minimal fluorescence (dark); F_M, maximal fluorescence (dark); F_V, variable fluorescence (dark); LST, Lowest Survival Temperature; TTC, TriphenylTetrazolium-Chloride.

Plant material

Seeds of winter wheat (*Triticum aestivum* L. cv. Urban) were germinated on moist filter paper for 2 days at 23 °C in the dark. After germination the seedlings were placed on containers with 20 l of 12.5% strength Hoagland type nutrient solution (SMAKMAN & HOFSTRA 1982) and grown in a climate chamber (20/16 °C day/night temperature, 10 h photoperiod of fluorescent light (Osram L 58W/21 and 31; 250 µmol m^{-2} s^{-1} PAR), R. H. 70/60 %). After one week the young plants were placed on containers with 30 l of 25% strength of the Hoagland solution (80 plants per container).

Low temperature hardening of plants

Sixteen days old plants were transfered to a climate chamber and hardened under low temperature and short day conditions (4/3 °C day/night, 10 h of fluorescent light (Philips TLD 58W 83/84; 60 µmol m^{-2} s^{-1} PAR), R. H. 80–90%) for 1 to 8 weeks. The low light intensity was used to prevent photoinhibition. The nutrient solution was changed once a week.

Freezing treatment

For assessment of frost hardiness by chlorophyll fluorescence 1 cm leaf strips from the middle part of the second and third leaf were placed on moist filter paper on a cuvette (3 rows of 16 strips). The cuvette consisted of a massive aluminium block (61*12*4 cm) that could be cooled at both ends by heat exchange units which were coupled to 2 ethanol cooling baths (Julabo F40, Julabo Labortechnik, GmbH, Seelbach, Germany). The cuvette was covered with a PVC lid which had holes with a transparent perspex window above the leaf strip. The holes were closed with rubber stoppers for darkness. The cuvette was kept at 4 °C at one side and cooled at the other side at a rate of 4 °C h^{-1} until a temperature gradient from +4 °C to –20 °C was established. Leaf temperatures were registered with copper/copper-nickel thermocouples (Comark Ltd, Herts, U. K.) which were tightly positioned to the abaxial side of the leaf. The temperatures for each row of leaf strips were similar. After reaching a temperature of –20 °C, the cuvette was warmed up to 4 °C at a rate of 30 °C h^{-1}.

In some experiments 1 cm leaf strips were frozen in test tubes as described for the electrical conductivity and TTC-reduction measurements. After thawing at 4 °C overnight, the strips were placed on the cuvette at a temperature of 4 °C for measurement of the chlorophyll fluorescence.

For determination of the frost hardiness by electrical conductivity or TTC-reduction measurements, leaf parts of 3 mm were placed in test tubes (6–10 parts per tube). The tubes were closed with rubber stoppers on which 1 cm^2 of moist filterpaper was connected to maintain high humidity in the tubes. The samples were placed in an ethanol freezing bath (Julabo F40, Julabo Labortechnik, GmbH, Seelbach, Germany), equilibrated at 4 °C for 30 min, and then frozen at a rate of 4 °C h^{-1}. In some experiments little ice crystals, created by means of a pipe cleaner dipped in liquid nitrogen, were added at a temperature of –1.5 °C. Leaf sample temperatures were measured with a copper/copper-nickel thermocouple which was tightly positioned to the abaxial side of the leaf. Samples were taken from the bath at different temperatures (5 (electrical conductivity) or 3 (TTC-reduction and chlorophyll fluorescence) tubes per temperature) and stored overnight at 4 °C in the dark. Control samples were stored at 4 °C throughout the whole freezing procedure.

Measurement of chlorophyll fluorescence

Chlorophyll fluorescence was measured with a fiberoptic light guide of a PAM fluorometer (PAM 101 and 103, H. Walz GmbH, Effeltrich, Germany), positioned into the holes in the PVC lid of the cuvette, just above the leaves. Minimal (F_0) and maximal chlorophyll fluorescence (F_M) of the adaxial leaf side were measured at 4 °C after a 30 minutes dark period before and after (at different times) the freezing treatment. F_0 was measured with a modulated light source (PAM 101 ED; peak wavelength 650 nm; light intensity < 1 µmol m^{-2} s^{-1}). F_M was measured at a saturating light intensity of 3000 µmol m^{-2} s^{-1} (light pulse 1 s, Schott KL 1500).

As the ratio F_V/F_M did not change between 6 and 16 h thawing at 4 °C (Fig. 1), and for comparison with the electrical conductivity and TTC-tests, the ratio F_V/F_M was measured after a thawing period of 16 hours. The degree of frost hardiness was expressed as the lowest survival temperature (LST), the lowest temperature where the ratio F_V/F_M was not significantly different from that of the leaves kept at 4 °C. These leaf strips remained fully turgescent and showed less than 50% yellowing after 3 days.

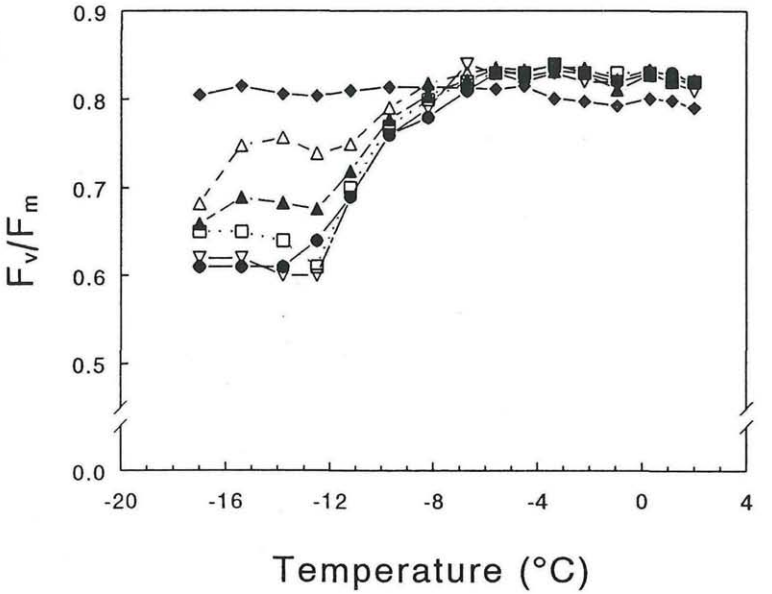

Fig. 1. Photochemical capacity (F_V/F_M) time course of 2 weeks hardened winter wheat leaf strips which were exposed to different levels of freezing and then thawed at 4 °C for different time intervals. Chlorophyll was measured at 4 °C before (◆) the freezing treatment and after thawing for 0.75 (△), 2 (▲), 4 (□), 6 (▽) and 16 (●) hours.

Measurement of electrical conductivity

After thawing for 16 h at 4 °C in the dark, 5 ml demineralized water was added to the tubes and electrical conductivity of the solution was measured according to STUIVER & al. 1992. The degree of frost hardiness, expressed as LT_{50} (the temperature at which 50% of the electrolytes had leaked out of the leaves), was calculated from a four parameter logistic model according to JANÁČEK & PRÁSIL 1991.

Measurement of TTC-reduction

After thawing at 4 °C in the dark for 16 h, the TTC-reduction capacity of the leaves was determined according to VAN HASSELT 1973. The amount of formazan in the extract formed after TTC-reduction was expressed as percentage of the formazan content of control leaf samples, which were stored at 4 °C during the freezing treatment. The degree of frost hardiness was expressed as the temperature at which the amount of formazan formed was 50% of that formed in control leaves. This temperature and the standard deviations were calculated from a four parameter logistic model in the same way as described for the electrical conductivity measurements.

Visual assessment of frost damage

After freezing on the temperature cuvette or in test tubes, leaf samples were placed in petri dishes on demineralized water (abaxial surface down) in a climate chamber (20/16 °C, 10 h fluorescent light (Osram L 58W/21 and 31; 100 µmol m^{-2} s^{-1} PAR).

After 72 hours the leaves were evaluated for injury based on the percentage of yellowing of the leaf parts. Leaf yellowing of more than 50% resulted in complete bleaching after 7 days, so these leaf parts were considered to be irreversibly damaged. The degree of hardiness was expressed as the lowest temperature where 50% or less yellowing occurred.

Results

Chlorophyll fluorescence of leaf strips was measured before and at different time intervals after freezing. In the leaves that were not exposed to temperatures causing damage, the ratio F_V/F_M remained at a level of 0.82 during 16 hours (Fig. 1). In damaged leaf strips the ratio F_V/F_M decreased below this point. The decrease was gradually in time, but a minimal level was reached after 6 hours thawing at 4 °C (Fig. 1). These damaged leaf parts had lost turgor and they showed more than 50% yellowing after 3 days and complete bleaching after 7 days floating on demineralized water in a climate chamber.

Frost hardiness of winter wheat leaves, measured as lowest survival temperatures with chlorophyll fluorescence, showed an increase from –4.3 °C to -8.2 °C during the first 8 days of hardening (Tab. 1). Results obtained from visual assessment of damage were similar (Tab. 1).

Table 1

Frost hardiness of winter wheat leaves frozen on moist filter paper expressed as lowest survival temperature (LST) during a hardening period of up to 8 days, measured with chlorophyll fluorescence measurements and by visual evaluation of frost damage. Means of 3 leaf strips per temperature ± SD. N.D. not determined.

hardening period (days)	Lowest Survival Temperature (°C)	
	Chlorophyll fluorescence (F_V/F_M)	Visually assessed
0	–4.3 ± 0.0	–4.9 ± 1.2
1	–5.4 ± 0.0	N. D.
2	–5.4 ± 0.0	–6.1 ± 0.6
6	–6.9 ± 0.7	–7.3 ± 0.0
8	–8.2 ± 0.9	–9.9 ± 1.5

After another 7 weeks hardiness increased to a temperature of –12 °C (Tab. 2). Except for the LT_{50} after 6 weeks hardening as determined by the TTC-test, LST's measured with chlorophyll fluorescence were similar with the LT_{50}'s from the two other methods during hardening (Tab. 2). However, in unhardened leaves there was a striking difference between the LST determined by the chlorophyll fluorescence method and the LT_{50} of both the electrical conductivity and the TTC-reduction test (Tab. 2).

Table 2

Frost hardiness of winter wheat (°C) during an eight weeks hardening period determined as LST with chlorophyll fluorescence and as LT_{50} with the electrolyte leakage test and the TTC-reduction test. Means of 3 (chlorophyll fluorescence and TTC-reduction) or 5 (electrolyte leakage) measurements ± SD. Data with the same letter in the same row were significantly different at $p < 0.05$ (Bonferroni's t-test).

weeks of hardening	chlorophyll fluorescence (F_V/F_M)	electrical conductivity	TTC-reduction
0	− 4.2 ± 1.7^{ab}	− 8.6 ± 0.3^{a}	− 8.2 ± 0.2^{b}
2	− 9.7 ± 1.5	− 9.6 ± 0.3	− 9.3 ± 0.3
4	−10.4 ± 1.0	−10.8 ± 0.5	−10.5 ± 0.3
6	−11.5 ± 0.0^{a}	−11.2 ± 0.3^{b}	−14.1 ± 0.5^{ab}
8	−12.0 ± 2.0	−11.7 ± 0.3	−12.3 ± 0.5

This discrepancy of the degree of hardiness of unhardened plants measured between the three methods could be due to the fact that the leaf parts used in the chlorophyll fluorescence method were frozen on moist filter paper in contrast to dry freezing in the electrical conductivity and TTC-tests. Possibly, supercooling of the extracellular water in the two last mentioned methods, might have been responsible for the observed difference (–4 °C versus –8 °C) in frost hardiness. To check this hypothesis, unhardened leaf parts were frozen in dry test tubes with or without addition of small ice crystals at –1.5 °C and the degree of hardiness was measured with the electrical conductivity test. Leaf parts that were inoculated with ice crystals showed an LT_{50} of –4.6 °C (Fig. 2A), which was comparable to the LST of the chlorophyll fluorescence method (Tabs 1 and 2). In leaf parts without ice nucleation the LT_{50} was about 5.5 °C lower, –10.1 °C (Fig. 2A). Standard deviations around the LT_{50} point were much higher in the experiment without ice inoculation (Fig. 2A).

The same pattern could be observed when chlorophyll fluorescence was measured of unhardened leaf strips which were not frozen on moist filter paper but in dry test tubes with or without ice inoculation. Leaf parts nucleated with ice crystals were already killed at temperatures below –4.3 °C, whereas those frozen without addition of ice crystals showed an LST of –8.3 °C (Fig. 2B).

Discussion

Winter wheat hardened to a temperature of –12 °C in 8 weeks at 4°C, which was comparable to the moderate hardening observed in winter wheat by GUSTA & al. 1982 and TRUNOVA 1982.

The LST, measured as a decrease of the ratio F_V/F_M, correlated well with the visual observable damage, and with the LT_{50} of both the conductivity and the TTC-reduction tests. BARNES & WILSON 1984 already

proved that frost sensitivity of *Trifolium* species could be determined with differences in variable fluorescence (F_V) after exposure to frost. Also in conifer needles, visually estimated freezing damage correlated well with a decrease of the ratio F_V/F_0 (STRAND & ÖQUIST 1988) or F_V/F_M (ADAMS & PERKINS 1993, LINDGREN & HÄLLGREN 1993). Chlorophyll fluorescence, and especially the determination of photochemical capacity after freezing, seems to be a suitable method to determine the frost hardiness of green plants.

The decrease of the ratio F_V/F_M after freezing indicates a change of photosystem II reaction centers from functional to down-regulated or nonfunctional ones (POWLES 1984, KRAUSE 1988). It represents a disturbance of photosynthetic performance, since photochemical capacity (F_V/F_M) was empirically correlated with quantum yield of photosystem II (BJÖRKMAN & DEMMIG 1987, ADAMS & al. 1990). However, the decrease of photochemical capacity does not necessarily imply a direct rupture of the thylakoid membrane after freezing. As was shown before, the thylakoid membrane is not the primary site of freezing injury (SUNDBOM & al. 1982, BARNES & WILSON 1984, ADAMS & PERKINS 1993), but its inactivation is due to an inhibition of photosynthetic CO_2 assimilation after freezing (KRAUSE & al. 1988, SOMERSALO & KRAUSE 1990). The inhibition of CO_2 assimilation seems to be due to a diminished light-activation of Calvin cycle enzymes, which may be caused by a change of the properties of the chloroplast envelope upon freezing (KRAUSE & al. 1988). STEFFEN & al. 1989 showed that following a freeze-thaw stress primary damage occurred to the plasmalemma. The subsequent leakage of ions or toxic compounds from the cell will result in an altered cellular environment, which in turn may disturb functioning of other cell organelles, as chloroplasts and mitochondria (SENSER & BECK 1979, KRAUSE & al. 1988, STEFFEN & al. 1989).

This secondary effect of frost on chloroplast functioning may also explain why WULFF & al. 1994 did not find chlorophyll fluorescence to be as sensitive as the electrolyte leakage rate in detecting effects of acid mist on frost hardening. In that study, frost damage, measured as the decrease of F_V/F_M, was evaluated rather quickly after freezing, leading to small and not significant differences between treatments and clones. If longer periods would have been used, the effect of freezing on F_V/F_M might have been more pronounced, as was shown for winter wheat leaves in our study or spruce needles (ADAMS & PERKINS 1993).

From this study it also became clear that the way leaves are frozen during laboratory freezing tests may have implications for the degree of frost tolerance measured. Supercooling of the extracellular water could lower the lethal temperature by 4 to 6 °C. Addition of small ice crystals could prevent supercooling and resulted in a more uniform freezing process, indicated by the much reduced standard deviations.

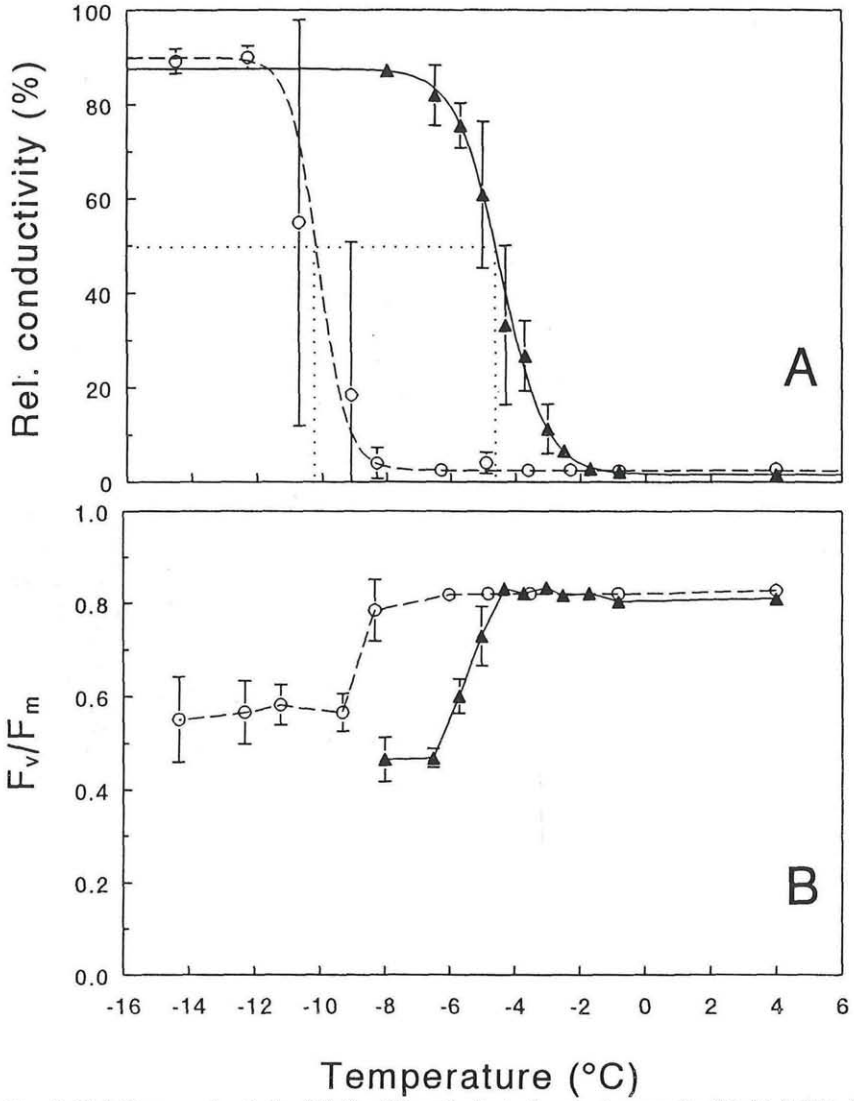

Fig. 2. Relative conductivity (A) (in %) and photochemical capacity (F_V/F_M) (B) of unhardened winter wheat leaf parts frozen in test tubes at different temperatures with (▲) and without (o) ice nucleation at –1.5 °C. Means of 5 (relative conductivity) or 3 (F_V/F_M) measurements ± SD.

MARCELLOS & SINGLE 1976 found that ice nucleation in wheat plants was necessary for frost damage at temperatures above –10 °C. Similar supercooling was observed in dry Solanum leaves (RAJASHEKAR & al. 1983). Under natural conditions, one would seldom expect supercooling to occur,

because factors like a fast cooling rate, a long exposure time to frost, a low plant temperature, the presence of nucleants, strong wind, and the presence of dew would all induce ice formation and prevent supercooling (LINDOW & al. 1982, ASHWORTH & al. 1985). As LEVITT 1980 already noted, it is necessary to inoculate leaf samples for studying frost hardiness under artificial freezing conditions in order to correlate these tests with actual field survival. Otherwise, the degree of hardening will be overestimated.

After hardening for 8 days, plants had reached the same degree of hardiness, as could be achieved in experiments with dry leaves by supercooling of the extracellular water. Also, in Solanum leaves hardened to –6.5 to –8 °C, ice inoculation did not affect the lethal temperature, in contradiction to non-hardened plants (RAJASHEKAR & al. 1983). Therefore, supercooling seems to influence the measurements only in the first week of hardening. In later stages, when the plant has reached a hardiness of –8 °C (or lower) it apparently tolerates extracellular ice formation. Possibly this can be caused by the formation of ice nucleating and antifreeze proteins which can control extracellular freezing, as was shown in hardened winter rye (GRIFFITH & al. 1993).

In conclusion, chlorophyll fluorescence is a useful tool to determine the degree of frost hardiness in winter wheat. The measurement of the ratio F_V/F_M is reliable and can be rapidly done. For assessing frost tolerance in laboratory freezing tests, one has to be aware that supercooling has to be prevented, because it can affect the freezing temperatures significantly.

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