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ORIGINAL ARTICLE

Direct and indirect measurements of freezing tolerance: advantages and limitations

Marcin Rapacz[1] · Monika Sasal[1] · Magdalena Wójcik-Jagła[1]

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Abstract The freezing tolerance of 69 accessions of field-grown, common wheat (*Triticum aestivum*) was assessed in three consecutive winters. To measure freezing tolerance directly, field-grown plants were subjected to a range of freezing temperatures in a controlled environment and plant regrowth was subsequently assessed. Indirect assessments of freezing tolerance, as measured by chlorophyll fluorescence transient measurements followed by a JIP-test (an in vivo measurement of the adaptive behavior of the photosynthetic apparatus), were performed on detached leaves frozen at the same time as whole plants. Both direct and indirect tests were also used on plants cold acclimated in the laboratory. These results were compared with results of a field survival study performed at seven experimental sites. An analysis of the data indicated that only some of the JIP-test parameters were suitable for the prediction of freezing tolerance and winter survival. Estimates of cold hardiness were very similar, regardless of the experimental year, but were dependent on the method of cold acclimation and time of sampling. Indirect measurements of cold hardiness were more in line with the field survival data for field-cold-acclimated plants sampled in mid-winter than for plants that were either sampled earlier or cold acclimated in the laboratory. Indirect measurements taken on leaves that had not frozen failed to provide accurate estimates of cold hardiness. Our observations, together with previously reported findings, indicate that cold acclimation under natural field conditions activates a greater array of freezing tolerance mechanisms than cold acclimation performed in under controlled environmental conditions in a laboratory.

Keywords Chlorophyll fluorescence transient · Wheat · Freezing tolerance · JIP-test · Winter survival

Abbreviations

ABS/RC and ABS/CS	Absorption flux (of antenna Chls) per CS and RC, respectively
GUS	The Central Statistical Office of Poland
CS	Leaf cross section
Chl	Chlorophyll
DI_0/CS and DI_0/RC	Dissipated energy flux per RC per CS and RC, respectively
ET_0/CS and ET_0/RC	Electron transport flux (further than Q_A) per RC, respectively
F_v/F_m	Maximum quantum yield of PSII photochemistry (of energy trapping)
φE_0	Quantum yield of electron transport
H	The coefficients of heritability in a broad sense
JIP-test	A method for the analysis of chlorophyll fluorescence induction curve
O	Phase of Chl fluorescence induction curve when Q_A pool is fully oxidized
P	Phase of Chl fluorescence induction curve when Q_A pool is fully reduced
PSII	Photosystem II
ψ_0	Quantum yield of overall photochemical reactions

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✉ Marcin Rapacz
rrrapacz@cyf-kr.edu.pl

[1] Department of Plant Physiology, University of Agriculture in Kraków, Podłużna 3, 30-239 Kraków, Poland

PI_{ABS}, PI_{CSm} and PI_{CS0}	Performance indexes of PSII normalized for equal absorption, equal number of active reaction centers at *P* and *O* phase of chlorophyll fluorescence induction curve, respectively
Q_A	Quinone A
RC	Reaction center
RC/CS_0 and RC/CS_m	The number of active PSII reaction centers per CS at *O* and *P* phase of Chl fluorescence induction curve, respectively
TR_0/CS and TR_0/RC	Trapping flux (leading to Q_A reduction) per CS and RC, respectively

Introduction

Advances in our understanding of plant cold hardiness has increased greatly during the last decade and was recently summarized by Gusta and Wisniewski (2013). They suggest that the complexity of the process of cold acclimation is underappreciated in the design of many experiments, resulting in data that may not reflect actual mechanisms of cold hardiness in the field. Therefore, a thorough analysis of methods utilized to assess the freezing tolerance of plants cold acclimated under controlled environmental conditions in comparison to various field conditions, together with explanations for the basis of any observed differences, is crucial for predicting the effects of climate change on plant biodiversity. Such an assessment is also necessary for successful breeding of plants that are more winter hardy given predicted changes in climate (Rapacz et al. 2014).

Insufficient cold hardiness of winter wheat is a problem of huge economic importance in Central and Eastern Europe. For example, 32.4 % of the acreage sown to winter wheat was lost to winter injury during a severe winter in 2011/2012 according to the Central Statistical Office of Poland (GUS), representing a loss of approximately 6 billion Euros (GUS 2012). Chlorophyll fluorescence-based techniques have been developed as reliable, non-invasive and easy-to-use tools for the estimation of freezing tolerance (Rizza et al. 2001; Rapacz 2007; Rapacz and Woźniczka 2009; Rapacz et al. 2011). Chlorophyll fluorescence induction transient analysis (JIP-test) provides a useful approach for researchers to obtain indirect information regarding the structure and function of the photosynthetic apparatus. It is based on a theory of energy flow in thylakoid membranes (Force et al. 2003) and is based on the relationships between PSII activity and fluorescence signals (Bussotti et al. 2007; Kalaji et al. 2011a, b). By using a JIP-test, it is possible to characterize the equilibrium between the inflow and outflow of the entire energy flux within PSII and estimate the possible fate of the absorbed light-energy.

The potential of employing chlorophyll fluorescence measurements for estimating freezing tolerance is based on the premise that the acclimation of the photosynthetic apparatus to low temperatures reflects increases in the freezing tolerance of cold-acclimated, whole plants (Rapacz et al. 2008; Crosatti et al. 2013; Hüner et al. 2013). Crosatti et al. (2013) has even suggested that the ability of chloroplasts to cold acclimate could be the rate limiting factor in whole plant adaptation to low temperature (Crosatti et al. 2013). Winter survival of plants in the field, however, is dependent on the interaction of many other factors than just temperature alone (Gusta and Wisniewski 2013). For instance, a prolonged period of a freezing stress or ice encasement may require different adaptive mechanisms than short freezing events, even though the temperature of the prolonged freezing event may be milder than the short freezing event (Waalen et al. 2011). This premise was previously established using chlorophyll fluorescence-based tests of freezing tolerance. A very high correlation between chlorophyll fluorescence parameters, affected by thylakoid membrane damage, was observed in winter wheat and triticale when measurements were taken shortly after freezing and subsequent thawing (Rapacz 2007; Rapacz et al. 2011). On the other hand, significant correlations between freezing tolerance and F_v/F_m, considered as a secondary, photoinhibition-related indicator of freeze damage, was observed when F_v/F_m was measured after plants were given a period of time to recover from exposure to freezing temperatures (Rapacz 2007; Rizza et al. 2001, 2011). A high correlation between chlorophyll fluorescence parameters and laboratory-measured freezing tolerance was observed in common wheat cultivars when the leaves used for chlorophyll fluorescence analysis were collected from field-grown plants during the winter, while correlations with winter field survival were low and often not significant (Rapacz and Woźniczka 2009).

The objective of the present study was to compare the results of the protocol used previously in triticale (Rapacz et al. 2011) with the results obtained on plant survival after controlled freezing of winter wheat, as well as with results obtained from multiple field studies. This was done in order to assess the reliability of chlorophyll fluorescence-based testing of freezing tolerance in wheat and to determine the conditions in which indirect methods, such as the JIP-test, most accurately reflect actual levels of cold hardiness based on regrowth assays and\or field survival.

Materials and methods

Plant material

Sixty-six candivars (advanced breeding lines, ready for official registration tests), and three cultivars (KWS Ozon, Tonacja, and Finezja), of common wheat (*Triticum aestivum*) were used in the study. The wheat candivars were developed by five breeding companies: Danko Plant Breeding (Choryń, Poland), Małopolska Plant Breeding (Krakow, Poland), Poznań Plant Breeders (Tulce, Poland), Smolice Plant Breeding (Smolice, Poland), and Strzelce Plant Breeding (Strzelce, Poland).

Winter hardiness

In the autumn 2010/2011, plants were sown in three, 10 m^2 replicate blocks at a density of 400 seeds per 1 m^2, with full randomization inside each block. The plantings were fertilized prior to winter at the following rate: *P*, 30–45 kg ha^{-1}; *K*, 15–25 kg ha^{-1}, depending on the soil mineral content in each specific experimental field. The plantings were established in seven experimental sites in Poland: Dębina (N 54.130323, E 19.032393), Kobierzyce (N 50.972848, E 16.947629), Nagradowice (N 52.317566, E 17.145104), Polanowice (N 50.202783, E 20.084715), Smolice (N 51.698700, E 17.184260), Strzelce (N 52.317495, E 19.404706), and Szelejewo (N 51.858932, E 17.159140). An assessment of freezing injury within each planting, based on plant appearance, was recorded after the winter using a score ranging from 1 to 9. In this scale system, 1 denoted a totally winter-killed field and nine indicated a field where no visual symptoms of damage were observed.

Direct measurements of freezing tolerance of plants cold acclimated in the field and assessed in the laboratory (field-laboratory method)

Freezing tolerance of plants was evaluated during the winters of 2010/2011, 2011/2012, and 2012/2013 at 2 or 3 different times throughout the winter. During 2010/2011, the tests were performed on plants from three experimental sites (Kraków: N 50.069014, E 19.845528, Smolice and Antoniny: N 51.855306, E 16.604474), whereas during the following winters, only plants in Kraków were evaluated. The plants were sown in the beginning of October in plastic boxes (30 cm × 38 cm × 9 cm) filled with a mixture of sand:clay soil:peat (1:1:1, v:v:v). Eight to twelve replicates of each accession were randomly distributed in separate boxes, where a replicate consisted of a row of twelve seeds. The boxes were then placed in each of the experimental fields—they were dug in the ground, so that the soil level in the box and the soil level outside the box were even. Weather conditions in the experimental fields were monitored with electronic weather stations (Fig. 1), the boxes experienced the same weather conditions (e.g., snow cover) as the plants sown directly in the ground. At the date indicated in Fig. 1, boxes containing four replicates of each accession were transferred to a freezing chamber that was set at 0 °C and equipped with an air-flow system to avoid a temperature gradient (the air flow of 175 m^3 h^{-1}). The temperature in the chamber was monitored using thermocouples and a multichannel data logging system AR205 (APAR, Warszawa, Poland). The temperature of the chamber was then lowered at a rate of 1.5 °C/h to −15 °C. This temperature was maintained for 14 h and then raised by 1.5 °C/h to 2 °C. The boxes were subsequently transferred to an unheated glasshouse and plants were cut 1.5 cm above the soil level. After 3 weeks of growth at approximately 10–15 °C and 40–60 % relative humidity, the number of plants exhibiting regrowth was recorded. Freezing tolerance was recorded as the percentage of plants exhibiting regrowth out of the total number of plants (approx. 48) exposed to –15 °C.

Direct measurements of freezing tolerance of plants cold acclimated in the laboratory (laboratory method)

Plants were sown as described for the field-laboratory method. The experiment was conducted in 2010 and 2011. In the middle of November, when the temperature in the field dropped below 10 °C, the boxes containing the plants were put into an environmental chamber and subjected to a 24-day-long cold-acclimation protocol consisting of an 8-h light period at 4 ± 0.7 °C and a 16-h dark period at 2 ± 0.4 °C. Relative humidity was set at 60 ± 5 %. Both temperature and air humidity in the chamber was maintained with a control system (Test-Therm, Kraków, Poland) and monitored independently with a data monitoring system (Panex, Wrocław, Poland) equipped with five temperature (Pt 100) and one air humidity sensor. Horizontal and vertical temperature gradients were not observed during the acclimation protocol. Light was provided by HPS 'Agro' lamps, (Philips, Brussels, Belgium). PAR at the canopy level was approximately 200 (±10) μmol m^{-2} s^{-1} during both experiments and was monitored at the beginning and end of the experiment using a QSPAR sensor (Hansatech, Kings Lynn, UK).

Plants were subjected to the same freeze–thaw protocol and regrowth conditions as described for the field-laboratory method.

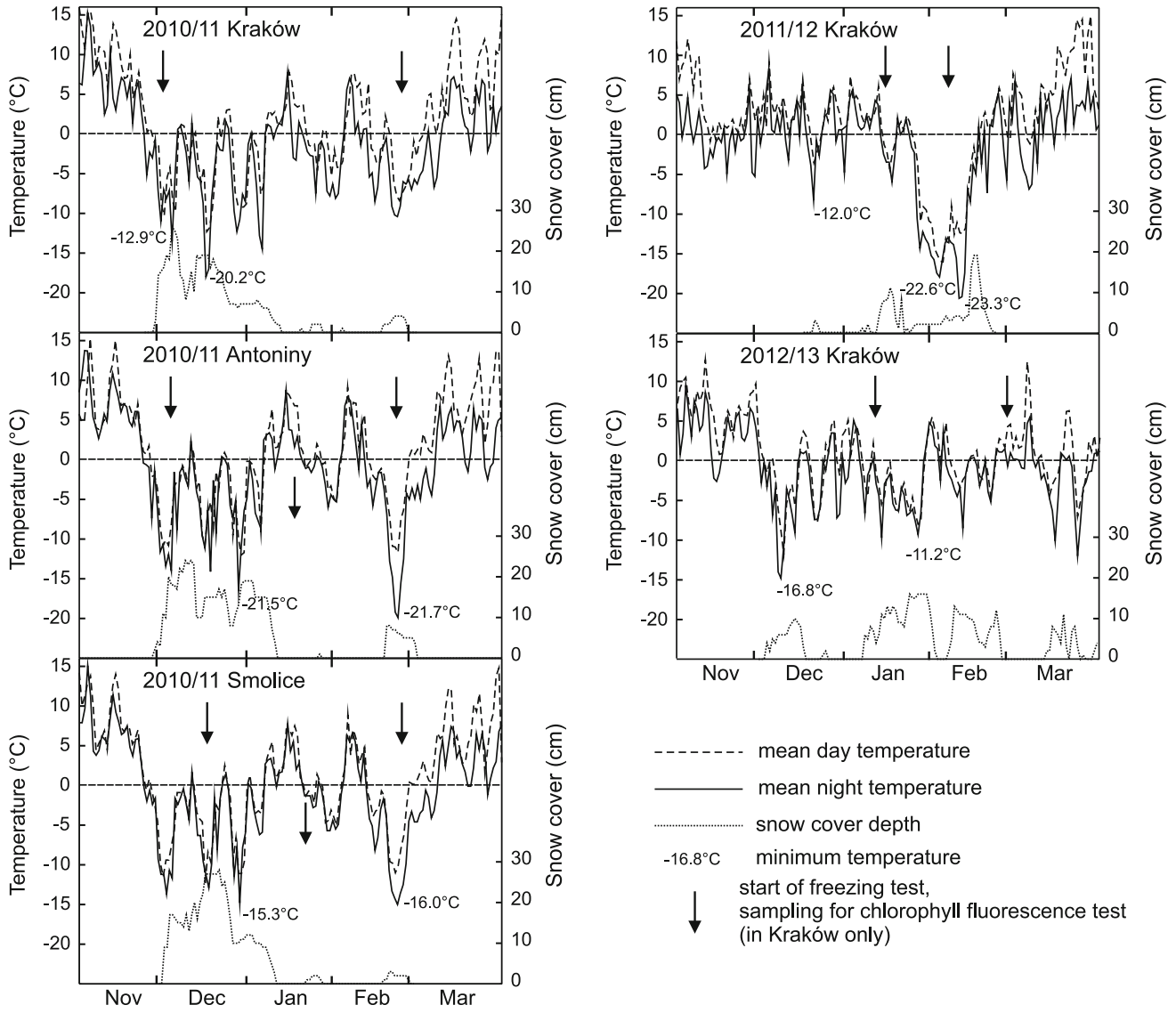

Fig. 1 Meteorological conditions of the field-laboratory experiments. Mean day and night temperatures were calculated on hourly basis

Indirect assessment of freezing tolerance by means of chlorophyll fluorescence measurements

After the plants were removed from the field (field-laboratory studies of freezing tolerance), ten leaves with no visual symptoms of injury were collected from each accession (2–3 from each box) and placed into polyethylene bags with string closures. Snow was added to the bags to ensure uniform initiation of sample freezing when the freeze test was conducted. The bags containing the leaves were then placed into a programmable freezer. The freeze–thaw cycle which was utilized was very similar to the method described for the boxes with the exception that the minimum test temperature was −18 °C and the duration of exposure to the lowest temperature was 4 h. After thawing, leaves were kept in the dark at +2 °C for no longer than 5 h, until the chlorophyll fluorescence measurements were conducted. Measurements commenced after an initial 20 min of dark adaptation in a leaf clip (Hansatech, Kings Lynn, UK) at room temperature. Polyphasic chlorophyll *a* fluorescence transients were measured using a Handy PEA fluorometer (Hansatech) with the following settings: a light pulse intensity of 3000 μmol m^{-2} s^{-1}, pulse duration of 0.3 s, fixed gain (1×). The fluorescence measurements were taken in 10 replicates (separate leaves).

The method for calculating the chlorophyll fluorescence parameters (JIP-test) used in the present study has been previously described in detail (Strasser et al. 1995, 2000; Rapacz 2007). The measured parameters included energy

fluxes per (active) PSII reaction center (RC) and phenomenological fluxes per leaf cross section (CS), calculated for the energy absorbed (ABS), trapped in PSII reaction centers (TR_0), used for electron transport (ET_0) and dissipated from PSII reaction centers (DI_0). The quantum yields of energy trapping (F_v/F_m), electron transport (φ_{E0}) and overall photochemical reactions (ψ_0) were also calculated. The third group of parameters included performance indexes of PSII normalized for equal absorption (PI_{ABS}), equal number of active reaction centers at *P* (PI_{CSm}) and *O* (PI_{CS0}) phase of the chlorophyll fluorescence induction curve, respectively. The number of active PSII reaction centers per leaf cross section at *O* (RC/CS_0) and *P* (RC/CS_m) phase of chlorophyll fluorescence induction curve was also estimated.

Statistical analysis

A completely randomized block model was used as the experimental design for the statistical analysis of both field and field-laboratory data. All data analyses utilized Statistica 10.0 PL software (Statsoft Inc., Tulsa OK, USA). Normal distribution of the data was checked by visual analysis of histograms and a Shapiro–Wilk *W* test. Arcsine transformation was used for the normalization of the plant survival data after freezing tests prior to any further data processing. Chlorophyll fluorescence and field survival data were characterized using a normal distribution. Results were analyzed using GLM, with accession and environment (year, location, and sampling date) as factors. Significant differences between the experiments were analyzed using the Tukey's HSD test. Linear correlation coefficients (Pearson's) were calculated for winter hardiness, plant survival and chlorophyll fluorescence data using means for single accessions. The coefficients of heritability in a broad sense (H) were calculated using mean squares for accessions (m_1) and accession × environmental interaction (m_2): $H = (m_1 - m_2)/m_1$ (Baker et al. 1968).

Results

Winter hardiness of wheat plants was assessed during the winter of 2010/2011 in plants obtained from multiple field locations. Results indicated that field survival differed between locations. The correlation coefficients between the results at different sites varied from 0.81 (between Nagradowice and Smolice) to statistically insignificant values (Table 1). Results obtained in Szelejewo and Kobierzyce were clearly distinct from the other locations where the survival score varied from 7.7 to 9.0.

Estimates of mean field survival for wheat plants in the Kraków planting were positively correlated (0.69) with the mean estimates of freezing tolerance measured using the field-laboratory method in all three consecutive winters. A similar correlation (0.66) was observed between the data obtained for these parameters in all three locations during the winter of 2010/2011. However, the correlation coefficients between field winter survival and field-lab freezing tolerance assessment within a single year and a single location were lower; and in one case, even negative (Table 1). Despite the fact that very different estimates of freezing injury levels were observed at the different experimental sites and series (locations and dates) in the freezing tolerance studies conducted in 2010/2011, the values of the correlation coefficients between winter survival and the results of single laboratory assessment of freezing tolerance were very similar (Table 1). On the other hand, at the Kraków location, the strength of the correlation was clearly affected by the year in which the laboratory freezing tolerance was conducted. The effect of year on the correlation was most likely associated with differences in environmental conditions. During 2010/2011 the course of the winter was very similar at all three experimental sites where freezing tolerance was assessed, but only in the first part of winter. On the other hand, the experimental sites differed in the minimum temperatures (Fig. 1). The autumn was rather warm with a sudden temperature drop at the end of November followed by strong temperature fluctuations throughout the winter months. In Antoniny and Smolice two periods of extreme low temperatures occurred: in December and in the end of February, while in Krakow extreme low temperatures were observed only in December. In Smolice and Antoniny the lowest temperatures were recorded in February together with thin snow cover (below 5 cm). On the contrary, December frost was accompanied by approximately 20-cm-thick snow cover. The winter was milder in Smolice than in Antoniny, where both minimum and mean night temperatures were approximately 5 °C lower.

During following winters freezing tolerance tests were performed in Kraków only. Winter of 2011/2012 was extremely harsh. Both autumn and early winter were characterized with optimum temperatures for cold acclimation with the mean day temperature oscillating between 0 and 5 °C (Fig. 1). Then a sudden decrease in temperature was observed. The mean night temperature dropped to below −20 °C and minimum temperature reached −23.3 °C. At that time snow cover in the field was thinner than 5 cm. Results of the field-laboratory freezing tolerance analysis recorded that year had the lowest correlation with the field survival data from the winter of 2010/2011 (Table 1). Regarding the experimental sites with the highest winter survival (Nagradowice, Smolice, Szelejewo), no significant or a negative (Szelejewo) correlation with the field-laboratory assessment of freezing tolerance

Table 1 Freezing tolerance of wheat, as measured by plant survival for wheat plants cold acclimated in the field and subjected to controlled freezing in the laboratory (field-laboratory method), cold acclimated in the laboratory and subjected to controlled freezing (laboratory method), and by assessing survival in the field after the winter (winter hardiness)

Trait/winter/location	Linear correlation coefficients (Pearson's)										
	Parameter value			Field survival in							
	Mean	Min.	Max.	Dębina	Kobierzyce	Nagradowice	Polanowice	Smolice	Strzelce	Szelejewo	Mean
Freezing tolerance—field-laboratory method, plant survival (%)											
2010/2011											
Kraków (1)	75.0a	10.3	100.0	0.62	0.33	0.43	0.34	0.43	0.35	–	0.58
Kraków (2)	9.5ef	0.0	60.1	0.49	0.28	0.31	0.36	0.34	–	–	0.47
Kraków ($\bar{x}$)	*a* 44.4	5.1	76.7	0.66	0.34	0.35	0.40	0.36	0.32	–	0.57
Smolice (1)	16.4e	0.0	77.1	0.59	0.28	0.32	0.50	0.31	0.31	–	0.54
Smolice (2)	11.5e	0.0	56.5	0.59	0.26	0.31	0.49	0.31	0.30	–	0.54
Smolice (3)	9.4ef	0.0	60.4	0.56	0.25	0.25	0.45	0.26	0.27	–	0.49
Smolice ($\bar{x}$)	*bc* 12.4	0.0	62.1	0.59	0.27	0.29	0.50	0.30	0.30	–	0.54
Antoniny (1)	15.4e	0.0	68.2	0.62	0.38	0.28	0.44	0.26	–	–	0.50
Antoniny (2)	14.0e	0.0	52.8	0.61	0.41	0.33	0.42	0.30	0.34	–	0.56
Antoniny (3)	40.5c	0.0	89.7	0.51	0.35	0.29	0.27	0.36	0.24	–	0.48
Antoniny ($\bar{x}$)	*b* 23.3	0.0	67.4	0.61	0.40	0.34	0.38	0.35	0.28	–	0.55
$\bar{x}$ 2010/2011	21.5	0.3	55.9	0.74	0.38	0.40	0.54	0.41	0.36	–	0.66
2011/2012											
Kraków (1)	10.5ef	0.0	54.5	0.45	0.31	–	0.42	–	–	−0.28	0.36
Kraków (2)	4.3f	0.0	23.9	–	–	–	0.40	–	0.38	–	0.38
Kraków ($\bar{x}$)	*c* 7.4	0.0	30.8	0.44	0.33	–	0.47	–	0.27	–	0.42
2012/2013											
Kraków (1)	50.3bc	0.0	97.2	0.73	0.44	0.42	0.64	0.43	0.33	–	0.68
Kraków (2)	27.3d	0.0	86.1	0.71	0.49	0.37	0.56	0.34	–	–	0.59
Kraków ($\bar{x}$)	*a* 38.8	0.0	86.4	0.76	0.49	0.40	0.64	0.41	0.29	–	0.68
3-year mean											
Kraków	29.5	3.0	59.7	0.76	0.48	0.43	0.60	0.44	0.34	–	0.69
All exp.	22.1	0.9	57.5	0.75	0.39	0.40	0.58	0.42	0.35	–	0.68
Freezing tolerance—laboratory method, plant survival (%)											
2010/2011	55.1b	0.0	100.0	0.46	0.45	0.26	0.36	0.28	–	–	0.45
2011/2012	57.7b	0.0	96.7	0.45	0.45	–	0.37	0.26	–	–	0.45
Mean	56.3b	0.0	98.3	0.46	0.43	0.26	0.36	0.26	–	–	0.43
Winter hardiness—field survival (1–9)											
Dębina	6.4	3.0	8.3								0.78
Kobierzyce	8.7	7.7	9.0	0.44							0.39
Nagradowice	8.5	5.7	9.0	0.41	–						0.82
Polanowice	6.6	5.7	7.3	0.58	0.38	0.44					0.67
Smolice	8.3	5.5	9.0	0.40	–	0.81	0.43				0.80
Strzelce	6.6	3.0	9.0	0.35	–	0.67	0.35	0.67			0.78
Szelejewo	8.9	7.7	9.0	–	–	–	–	–	–		–
Mean	7.7	5.9	8.5	0.78	0.39	0.82	0.67	0.82	0.78	–	

The values of mean % survival labeled with the same letter did not differ at $P = 0.05$ according to Tukey's HSD test. Values for means from different years (italicized letters) and separate testing dates were tested independently. All the correlation coefficient values shown in the table are significant for $P = 0.05$

performed in 2011/2012 was observed. The results of the field-laboratory assessment of freezing tolerance performed during winter 2012/2013 had a similar or higher correlation to field-survival data from 2010/2011 than the data from the laboratory freezing tests that were conducted during the winter of 2010/2011 (Table 1). The temperature profile

during the autumn and winter 2012/2013 was similar to the corresponding period in 2010/2011. The difference was that in 2010/2011 lower minimum and mean temperatures were observed together with thicker snow cover (−20.2 °C/19 cm and −16.8 °C/7–8 cm, respectively; Fig. 1). The level of correlation between field winter survival and the field-laboratory assessment of freezing tolerance in 2012/2013 was similar to what was observed using the field-laboratory method in 2011/2012 (Table 1).

The correspondence between the field survival and laboratory estimates of winter hardiness appeared to be dependent on the field temperature and snow cover depth prior to the collection and transfer of plants to the environmental (freezing) chamber (Table 2; Fig. 1). An examination of the results from the winter 2010/2011 indicate that the lowest correlation coefficients with other experimental locations and dates were observed for the first testing date (late November) in Kraków. Further examination of the temperature profile during that period suggests that conditions were such that the plants were insufficiently cold acclimated. This premise may be additionally confirmed by a very low correlation between these results and the results of the laboratory assessment of freezing tolerance (Table 2).

The collective results of the field-laboratory assessment of freezing tolerance of plants located in Kraków during three successive winters exhibited a similar level of correlation as observed for the single winter of 2010/2011 (Table 2). In this case, the lowest correlation was observed for the second lab assessment of freezing tolerance conducted in 2011/2012, when plants were heavily injured by frost prior to conducting the test (Tables 1, 2; Fig. 1).

Both sets of freezing tolerance data obtained from plants that had been acclimated under controlled laboratory conditions of freezing tolerance provided similar marginally significant correlation (approx. 0.5) to results obtained using the field-laboratory method (Table 2).

A major objective of the present study was to compare the estimates of freezing tolerance obtained by measuring chlorophyll fluorescence parameters on detached leaves with estimates obtained using whole, field-acclimated plants subjected to controlled freezing in the laboratory and qualitative measurements of winter hardiness observed in wheat plants planted at several locations. The detached leaves used for the chlorophyll fluorescence measurements were obtained from the same plants used in the whole plant studies (field-laboratory method), and subjected to the same freezing protocol. Chlorophyll fluorescence measurements were made after the leaves had been subjected to freezing. Both the detached leaf and whole plant tests were conducted at the same time. As described above, the results obtained with the field-laboratory assessment of freezing tolerance and the field assessment of winter survival were both affected by the prevailing environmental conditions at the time the assessment was made. This was confirmed by the analysis of heritability coefficients (Table 3). The results indicated that field survival was much more affected by the environment than the survival of plants subjected to laboratory freezing tests. However, a direct comparison of H values for these two traits may be problematic due to existence of different variables, such as year and location.

In contrast, the results of chlorophyll fluorescence studies may be directly compared with the results for plant survival obtained in the freezing tests performed in the same conditions. The various chlorophyll fluorescence parameters exhibited different levels of genotype × environment interaction. Only in the case of ET_0/CS, however, were the levels of genotype × environment interactions comparable to those observed for results obtained for the freezing tolerance of plants evaluated using the field/lab method. A relatively low level of environmental effect (high H values) was observed for TR_0/CS, φE_0, RC/CS_0 and RC/CS_m.

Results of the chlorophyll fluorescence analyses conducted over three consecutive winters were compared directly with mean winter survival of field plants, percentage plant survival obtained using the field-laboratory method, and plant survival obtained using controlled cold acclimation and laboratory assessment of freezing tolerance. The highest correlations were obtained when the chlorophyll fluorescence measurements were made on the second date (mid-winter) (Table 4). The strength of the correlation between the JIP-test parameters and both field winter hardiness and freezing tolerance of whole plants depended on the chlorophyll fluorescence parameter (Table 4). The highest levels of correlation coefficients between plant freezing tolerance measured using the field-laboratory method and JIP-test parameters were observed for phenomenological energy fluxes, electron transport and trapping (ET_0/CS, TR_0/CS, respectively), quantum efficiencies of these processes (F_v/F_m, φE_0), and the number of reactive PSII reaction centers per leaf cross section (RC/CS_0 and RC/CS_m). The same parameters also exhibited the highest correlations with field winter survival. Importantly, most of these parameters were ones that were least impacted by an environmental effect (Table 3). In contrast, parameters connected with energy absorption in PSII (ABS/…), as well as with electron transport within individual active PSII reaction centers (…/RC), were not correlated with plant survival or the correlation was negative; contrary to what was expected. The correlations for PSII performance indexes (PIs) were also either low or insignificant. The relationship between highly correlated parameters and plant survival in the field and field-laboratory tests are presented in Figs. 2 and 3, respectively. The relationships were clearly linear with a slightly higher

Table 2 Linear correlation coefficients, significant at $P = 0.05$, between locations, test dates, and methods of freezing tolerance assessment

Method/year/location (test date)	2010/2011									2011/2012		2012/2013		$\bar{x}$ (Kraków)	$\bar{x}$	2010
	Kraków (1)	Kraków (2)	Smolice (1)	Smolice (2)	Smolice (3)	Antoniny (1)	Antoniny (2)	Antoniny (3)	Mean	Kraków (1)	Kraków (2)	Kraków (1)	Kraków (2)			
Field-laboratory method (%survival)																Lab.
2010/2011																
Kraków (2)	0.48															
Smolice (1)	0.35	0.44														
Smolice (2)	0.36	0.48	0.92													
Smolice (3)	0.35	0.44	0.89	0.95												
Antoniny (1)	0.30	0.41	0.72	0.71	0.73											
Antoniny (2)	0.39	0.54	0.68	0.69	0.69	0.82										
Antoniny (3)	0.30	0.40	0.60	0.57	0.58	0.75	0.75									
$\bar{x}$	0.59	0.64	0.87	0.87	0.87	0.82	0.84	0.76								
2011/2012																
Kraków (1)	0.38	0.41	0.47	0.47	0.42	0.56	0.45	0.46	0.58							
Kraków (2)	–	0.27	–	–	–	0.26	0.32	0.27	0.30	0.43						
2012/2013																
Kraków (1)	0.55	0.58	0.54	0.52	0.47	0.58	0.62	0.62	0.70	0.59	0.45					
Kraków (2)	0.58	0.66	0.63	0.61	0.62	0.70	0.65	0.71	0.80	0.56	0.33	0.79				
$\bar{x}$ (Krakow)	0.74	0.74	0.60	0.60	0.56	0.64	0.67	0.56	0.82	0.70	0.48	0.91	0.90			
$\bar{x}$	0.54	0.60	0.88	0.88	0.87	0.86	0.84	0.79	0.98	0.62	0.34	0.74	0.82	0.83		
Laboratory method (Lab.)																
2010	0.33	0.37	0.43	0.36	0.32	0.46	0.47	0.51	0.53	0.52	0.46	0.55	0.48	0.49	0.47	
2011	0.35	0.39	0.42	0.38	0.31	0.45	0.48	0.48	0.51	0.54	0.45	0.51	0.50	0.50	0.53	0.92
$\bar{x}$	0.37	0.40	0.41	0.35	0.33	0.47	0.48	0.52	0.55	0.50	0.42	0.54	0.45	0.53	0.51	0.97

Table 3 Genotype × environment interactions for wheat winter hardiness, freezing tolerance (measured as % survival), and chlorophyll fluorescence measurements after freezing of detached leaves calculated as a heritability coefficient in a broad sense (*H*) (Baker et al. 1968)

Trait	Heritability (*H*)
Winter hardiness	0.411
Freezing tolerance (% survival in the field-laboratory method)	0.741
Chlorophyll fluorescence parameters after freezing of detached leaves	
ABS/CS	0.303
TR_0/CS	0.625
ET_0/CS	0.697
DI_0/CS	0.092
ABS/RC	0.051
TR_0/RC	0.202
ET_0/RC	0.317
DI_0/RC	0.096
F_v/F_m	0.590
ψ_0	0.543
φE_0	0.643
PI_{CS0}	0.527
PI_{CSm}	0.421
PI_{ABS}	0.455
RC/CS_0	0.651
RC/CS_m	0.612

Calculation for winter survival was based on an experiment performed at seven locations during the winter of 2010/2011. Calculations for freezing tolerance and chlorophyll fluorescence parameters were based on experiments performed in Kraków during the winters of 2010/2011, 2011/2012, and 2012/2013

convergence in the case of accessions with low freezing tolerance. With respect to the correlation between RC/CS_0 and winter survival, the coefficient was still high (0.603) after excluding the minimally freeze tolerant candivar 53 from the analysis (data not shown).

When comparing levels of freezing tolerance obtained using chlorophyll fluorescence measurements with winter field survival results at different locations (Table 5), the highest correlations were observed in locations where the correlations between freezing tolerance and winter survival were also the greatest; i.e., the places where freezing tolerance was a predominant factor in determining winter survival—Smolice, Dębina, Nagradowice (Table 1). In contrast, field survival in Szelejewo was not correlated with the levels of freezing tolerance that were obtained by measuring chlorophyll fluorescence parameters.

The freezing tolerance of plants that were cold acclimated under controlled conditions was less correlated with the results obtained using chlorophyll fluorescence measurements when compared to the correlations between chlorophyll fluorescence parameters and either plant survival in the field or in the field-laboratory method (Table 4). Parameters that were dependent on energy absorption and energy fluxes for individual reaction centers were not useful for predicting freezing tolerance, while φE_0 and F_v/F_m were best correlated with the level of plant survival obtained in the laboratory tests.

The results obtained for freezing tolerance using chlorophyll fluorescence parameters in a single test were also compared with mean plant survival obtained using: the field-laboratory protocol, the controlled cold-acclimation protocol, and results obtained from field evaluations of winter survival (Tables 6, 7). Remarkably, the level of the correlation was dependent on the year of the experiment. The highest correlations were observed when the chlorophyll fluorescence measurements were taken during the winter of 2012/2013, which was mild and optimal for cold acclimation (Tables 6, 7). The lowest correlations with both 3-year means but also for each year of the field-laboratory analysis of plant survival after freezing, were observed for chlorophyll fluorescence measurements which were taken during the winter of 2011/2012. During that winter, plants in the field were seriously damaged prior to the date on which they were sampled. Irrespective of the year of the measurements chlorophyll fluorescence parameters measured after controlled freezing of leaves detached from field-cold-acclimated plants were better correlated with field survival than with the results of controlled freezing of the plants cold acclimated in the laboratory (Table 7).

The averaged plant survival after controlled freezing and mean survival in the field were correlated with the results obtained using chlorophyll fluorescence parameters in the winters of 2010/2011 and 2012/2013 at a similar or greater level as with the results for plant survival in a single year or a single location (Tables 1, 2, 6, 7). For example, RC/CS_0 measured in 2012/2013 was highly correlated ($r = 0.684$) with mean freezing tolerance estimated using the field-laboratory method and F_v/F_m measured in 2012/2013 was highly correlated ($r = 0.603$) with mean field survival. The highest r value (0.678) obtained for a single freezing tolerance measurement and mean winter field survival was from the extremely harsh winter of 2011/2012, while the r value was 0.581 for other winters (Table 1).

Discussion

Direct tests of freezing tolerance and winter survival potential

In the present study, the freezing tolerance of 69 candivars and three cultivars of common wheat (*T. aestivum*) was

Table 4 Linear correlation coefficients (Pearson's), statistically significant at $P = 0.05$, between chlorophyll fluorescence (JIP-test) parameters measured on the first or the second date that measurements were taken (means for 3 years), freezing tolerance, and winter hardiness of plants

JIP-test parameter	Date of the measurements					
	Freezing tolerance—laboratory method (%survival)		Freezing tolerance—field-laboratory method (%survival)		Winter hardiness (field survival 0–9)	
	1	2	1	2	1	2
ABS/CS	–	–	–	–	–	−0.444
TR_0/CS	0.300	0.429	0.285	0.743	–	0.739
ET_0/CS	–	0.469	–	0.767	–	0.722
DI_0/CS	–	−0.313	–	−0.431	–	−0.628
ABS/RC	–	–	–	–	–	−0.318
TR_0/RC	0.374	0.269	0.428	0.436	0.315	0.315
ET_0/RC	–	−0.421	–	−0.610	–	−0.700
DI_0/RC	–	–	–	−0.300	–	−0.498
F_v/F_m	0.406	0.495	0.289	0.739	–	0.746
ψ_0	–	–	–	0.308	–	0.447
φE_0	–	0.524	–	0.749	–	0.713
PI_{CS0}	0.438	0.370	0.514	0.631	0.436	0.640
PI_{CSm}	0.455	0.461	0.526	0.649	0.422	0.603
PI_{ABS}	–	–	–	–	–	–
RC/CS_0	0.398	0.468	0.264	0.747	–	0.754
RC/CS_m	0.456	0.460	0.392	0.717	–	0.717

Freezing tolerance of wheat, as measured by plant survival for plants cold acclimated in the field and subjected to controlled freezing in the laboratory (field-laboratory method, mean of 12 independent tests), cold acclimated in the laboratory and subjected to controlled freezing (laboratory method, mean of two independent tests), and by assessing survival in the field after the winter (winter hardiness, measured in one winter)

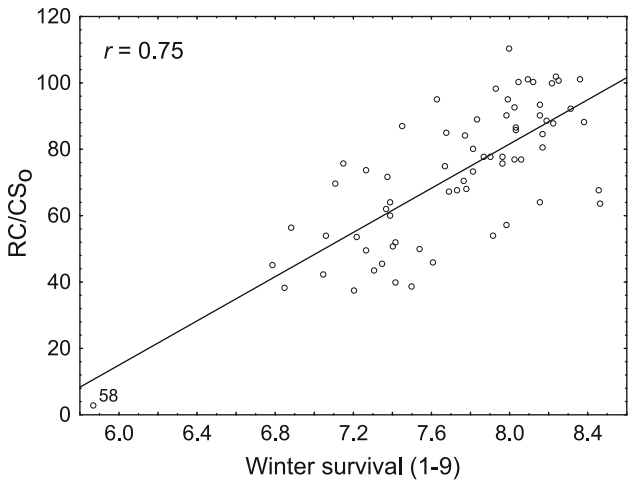

Fig. 2 The relationship between winter field survival of 69 candivars and three cultivars of winter wheat observed during the winter of 2010/2011 (mean of 7 locations) and the number of PSII reaction centers active at the *O* stage of the chlorophyll fluorescence induction curve (RC/CS_0). Measurements were taken on detached leaves after they had been frozen and thawed. Leaves were collected in the second half of February from plants growing in the field in Kraków, Poland (mean for 2011, 2012 and 2013). The linear correlation coefficient (*r* value) is presented in the figure

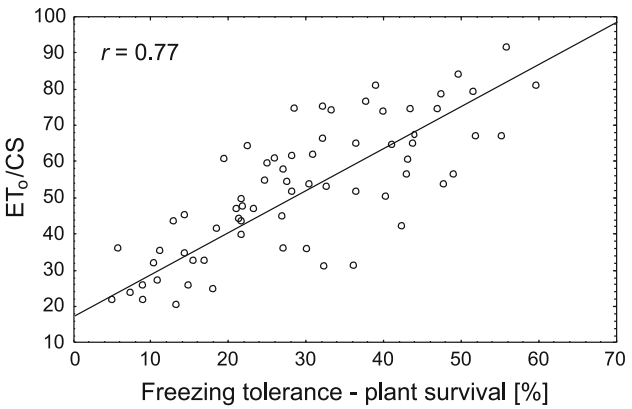

Fig. 3 The relationship between the freezing tolerance of 69 candivars and three cultivars of winter wheat measured in field-laboratory tests performed in Kraków, Poland (3-year mean), and the phenomenological (calculated for leaf cross section) energy flux for electron transport ET_0/CS measured after freezing of detached leaves. Both plant survival and chlorophyll fluorescence measurements were performed simultaneously on the same plants and used the same freezing–thawing cycle. The linear correlation coefficient (*r* value) is presented in the figure

Table 5 Linear correlation coefficients (Pearson's), statistically significant at $P = 0.05$, between chlorophyll fluorescence (JIP-test) parameters measured on the second date that measurements were taken (3-year mean) and winter hardiness (field survival) of plants registered in seven locations during winter 2010/2011

JIP-test parameter	Field survival (1–9) in						
	Smolice	Strzelce	Polanowice	Dębina	Szelejewo	Kobierzyce	Nagradowice
ABS/CS	−0.568	−0.409		−0.263			−0.515
TR_0/CS	0.672	0.409	0.554	0.658		0.300	0.645
ET_0/CS	0.624	0.412	0.561	0.644		0.339	0.601
DI_0/CS	−0.707	−0.488	−0.267	−0.448			−0.712
ABS/RC	−0.270			−0.323			−0.233
TR_0/RC	0.265			0.314			0.212
ET_0/RC	−0.567	−0.403	−0.564	−0.651		−0.317	−0.523
DI_0/RC	−0.619	−0.393	−0.166	−0.338			−0.623
F_v/F_m	0.667	0.413	0.564	0.664		0.344	0.665
ψ_0	0.393	0.353	0.155	0.347			0.391
φE_0	0.613	0.391	0.565	0.641		0.371	0.621
PI_{CS0}	0.535	0.380	0.430	0.561		0.287	0.542
PI_{CSm}	0.505	0.328	0.478	0.538		0.294	0.497
PI_{ABS}					0.358		
RC/CS_0	0.685	0.410	0.563	0.668		0.331	0.673
RC/CS_m	0.627	0.386	0.536	0.649		0.316	0.613

evaluated in three successive winters at several locations. Freezing tolerance was measured as a survival rate determined after controlled freezing of plants cold acclimated in the field or in an environmental chamber. In addition, freezing tolerance was also estimated by measuring various chlorophyll fluorescence parameters on detached leaves after they had been subjected to the same level of freezing as in the other assessment methods. The obtained results were compared with an estimate of plant field survival at seven experimental sites at the end of winter. The environmental impact (location and year) on plant survival obtained using the field-laboratory method ($H = 0.74$) was lower in our evaluation of wheat than what was previously reported for winter barley (Gut et al. 2004: $H = 0.35$) or for winter triticale (Rapacz et al. 2011: $H = 0.18$). The interactions calculated for some chlorophyll fluorescence parameters, however, were similar to those observed for plant survival and to what was previously calculated for triticale (Rapacz et al. 2011).

The selection of an appropriate method for evaluating freezing tolerance and cold acclimation of the plants, is crucial in obtaining an accurate estimate of freezing tolerance (Gusta and Wisniewski 2013). In the present study, the lowest correlations between estimates of freezing tolerance and actual winter survival in the field were observed for plants that were cold acclimated in an environmental chamber using a specific acclimation protocol and for plants growing in the field during the winter of 2011/2012. That winter was characterized by initial temperatures that were optimal for cold acclimation followed by very low minimum winter temperatures without snow cover. Such conditions are similar to those typically simulated in a laboratory protocol of cold acclimation and freezing evaluation. Indeed, during the winter of 2011/2012, the results obtained for plant survival using the field-laboratory method were better correlated with the level of plant survival obtained for plants that were cold acclimated in the laboratory than during the other two winters. These observations suggest that freezing tolerance may represent just one component of winter hardiness (Olien 1967). Different plant parameters (adaptive responses) may contribute to the overall winter hardiness observed in field plantings. The parameter(s) measured in controlled freezing tests, may represent one of the components, albeit a major one, that contributes to the overall potential for winter survival (Waalen et al. 2011; Gusta and Wisniewski 2013). For example, a controlled freezing test measures the ability of plants to survive an "acute" or short-term freezing stress; however, the ability of a plant to maintain freezing tolerance when exposed to warm temperatures, or reacclimate if warm temperatures are followed by freezing temperatures, may be just as important to overall winter survival as the ability of a plant to withstand a short-term freezing stress. It is also possible that the use of winter hardiness experiments performed over several years and the calculation of field-survival indexes (FSI: Fowler and Gusta 1979) which potentially reduces the experimental error associated with field trials, may result in higher correlation between field-laboratory measurements of freezing tolerance based on survival rate and winter hardiness. In the study of Fowler and Gusta (1979) the LT50 (freezing temperature, which kills 50 % of the plants) estimated on

Table 6 Linear correlation coefficients (Pearson's), statistically significant at $P = 0.05$, between chlorophyll fluorescence (JIP-test) parameters taken on the second date when the measurements were taken on detached leaves taken from field-acclimated plants during three winters and the results of field-laboratory analyses (plants acclimated in the field and subjected to controlled freezing tests in the laboratory) performed at the same time in Krakow (means of two experiments)

JIP-test parameter	Chlorophyll fluorescence measurements taken during the winter											
	2010/2011				2011/2012				2012/2013			
	Field-laboratory estimation of freezing tolerance during winter											
	2010/2011	2011/2012	2012/2013	$\bar{x}$	2010/2011	2011/2012	2012/2013	$\bar{x}$	2010/2011	2011/2012	2012/2013	$\bar{x}$
ABS/CS	−0.286	–	–	−0.259	–	0.277	0.261	0.264	0.258	–	–	0.240
TR_0/CS	0.478	0.343	0.531	0.585	0.387	0.349	0.503	0.514	0.534	0.317	0.668	0.670
ET_0/CS	0.489	0.423	0.551	0.618	0.395	0.354	0.504	0.517	0.515	0.359	0.624	0.636
DI_0/CS	−0.352	–	−0.316	−0.348	–	–	–	–	−0.256	−0.272	−0.409	−0.390
ABS/RC	–	–	–	–	–	–	–	–	−0.546	−0.336	−0.586	−0.621
TR_0/RC	0.567	0.391	0.427	0.551	0.241	–	–	–	0.286	–	0.307	0.309
ET_0/RC	0.484	–	0.326	0.396	–	–	–	–	−0.546	−0.335	−0.589	−0.623
DI_0/RC	−0.328	–	−0.263	−0.291	–	–	–	–	–	–	–	–
F_v/F_m	0.362	0.281	0.446	0.490	0.378	0.320	0.498	0.502	0.535	0.345	0.672	0.671
ψ_0	0.493	0.371	0.401	0.506	–	–	–	–	0.455	0.248	0.480	0.498
φE_0	0.401	0.362	0.475	0.534	0.345	0.335	0.460	0.468	0.525	0.374	0.623	0.635
PI_{CS0}	0.362	0.354	0.457	0.509	–	–	–	–	0.472	0.360	0.524	0.558
PI_{CSm}	0.315	0.328	0.418	0.464	0.345	0.395	0.428	0.459	0.444	0.350	0.469	0.513
PI_{ABS}	0.310	0.319	0.403	0.452	–	–	–	–	0.485	0.373	0.522	0.558
RC/CS_0	0.445	0.336	0.524	0.571	0.347	0.328	0.500	0.497	0.551	0.325	0.677	0.684
RC/CS_m	0.376	0.314	0.477	0.520	0.337	0.298	0.415	0.429	0.509	0.328	0.618	0.630

the basis of field-laboratory freezing tests similar to those performed in the present study, did not reveal differences in winter hardiness for cultivars that were similar in this trait. This also might be observed in our experiment, where the group of wheat accessions of rather low variation in their winter hardiness (Polish plant breeding programs only) was studied. In the paper by Gusta et al. (2001) the correlation coefficient between LT50 for field-cold-acclimated plants and winter survival (FSI) for the group of winter-hardy genotypes was 0.51 (0.15 for semi-hardy accessions), thus it was almost the same as in the case of the accessions studied in our experiment (0.43). In the same study freezing tolerance of plants cold acclimated in the field were better correlated with FSI than the freezing tolerance of the plants cold acclimated in the laboratory (0.73 vs. 0.56). In our experiment, the respective correlations were similar (0.68 and 0.43). In both Gusta et al. (2001) and our study the correlation between winter hardiness and freezing tolerance of plants cold acclimated in the laboratory were lower than for field cold-acclimated plants. In the paper of Gusta et al. (2001), the results clearly pointed out the possibility of overestimation of winter hardiness when the plants are cold acclimated under controlled environment. In the case of our study no clear direction of the differences was observed (data not shown). This may result either from the lower light intensity during controlled cold acclimation used in our study, or from different conditions of winters in Poland and western Canada. For example, prolonged freezing tests may be a good alternative in estimation of winter hardiness under the conditions of western Canada (Gusta et al. 1997; Waalen et al. 2011). These kinds of tests seem not to be reliable for prediction of winter hardiness in Poland (Rapacz et al. under analysis).

JIP-test as an indirect alternative for field winter survival estimation

Chlorophyll fluorescence-based method used in the current study may be a valuable, indirect tool for estimating freezing tolerance, but only when the two conditions listed below, and subsequently discussed, are met.

1. The appropriate chlorophyll fluorescence parameter is used.
2. Plant leaves are collected from the field in the middle of winter and directly frozen to predetermined

Table 7 Linear correlation coefficients (Pearson's), statistically significant at $P = 0.05$, between chlorophyll fluorescence (JIP-test) parameters from the second date that measurements were taken during three winters and the results of winter field survival at seven locations after the winter of 2010/2011, as well as freezing tolerance measured on laboratory cold-acclimated plants subjected to controlled freezing in the laboratory (mean for twice-repeated study)

JIP-test parameter	Chlorophyll fluorescence measurements taken during winter:					
	2010/2011		2011/2012		2012/2013	
	Field survival	Freezing tolerance	Field survival	Freezing tolerance	Field survival	Freezing tolerance
ABS/CS	−0.439				0.251	0.299
TR_0/CS	0.577	0.477	0.271	0.327	0.534	0.428
ET_0/CS	0.547	0.486			0.458	0.422
DI_0/CS	−0.536				−0.282	
ABS/RC					−0.703	−0.432
TR_0/RC	0.545	0.287			−0.704	−0.432
ET_0/RC	0.496	0.391		0.276	0.307	
DI_0/RC	−0.476				−0.246	
F_v/F_m	0.484	0.399		0.290	0.603	0.475
ψ_0	0.440	0.304			0.473	0.367
φE_0	0.435	0.375			0.493	0.467
PI_{CS0}	0.353	0.267	0.481	0.412		0.338
PI_{CSm}	0.297		0.416	0.417		0.285
PI_{ABS}	0.258					0.367
RC/CS_0	0.590	0.460		0.254	0.560	0.459
RC/CS_m	0.490	0.393	0.293	0.345	0.347	0.394

temperatures, and then thawed prior to taking the fluorescence measurements.

1. Rapacz et al. (2011) reported that RC_{CSm} and PI_{CSm} were the best indicators of triticale freezing tolerance. While both parameters in the present study were also good, they were not the best parameters of chlorophyll fluorescence to use for estimating the freezing tolerance of wheat. Another distinct difference between the wheat and triticale studies was that while F_v/F_m, a parameter indicating the energy trapping efficiency in PSII antennas, was one of the parameters that was best correlated with plant survival using controlled freezing tests and with estimates of winter hardiness of wheat in field plantings, this was not the case in triticale plants. Measurements of F_v/F_m are most often used as an indicator of freezing injury in leaf tissues (Strand and Öquist 1988; Clement and van Hasselt 1996; Herzog and Olszewski 1998; Rizza et al. 2001, 2011). This parameter was previously reported to be related to secondary, photo-inhibitory damage of the photosynthetic apparatus during the recovery of wheat after freezing (Rapacz 2007). It is unclear whether the difference observed between triticale and wheat is mainly due to variation in the environmental conditions (more severe winters) in the years that the evaluation of wheat occurred, differences in the level of freezing tolerance in wheat vs. triticale (winter triticale is in general more freezing tolerant than wheat, data not shown), or is due to other undefined genetic factors. Regardless of which factors are responsible, the high correlation between F_v/F_m and survival observed in the present study is most likely due to a higher degree of photo-inhibitory damage observed in the field before the analysis of wheat freezing tolerance. Concerns have been raised about F_v/F_m as an indicator of wheat freezing tolerance when measurements are made directly after freezing but not during the period of recovery (Rapacz 2007; Rapacz and Woźniczka 2009). In both of these previous studies, however, the experimental protocol excluded the consideration of photo-inhibitory injury before the freezing of the leaves was conducted. Apart from F_v/F_m, the chlorophyll fluorescence parameters dependent on electron flow upstream of Q_A were the best for estimating freezing tolerance and winter survival in the present study. This finding is consistent with the results of previous studies in wheat (Rapacz 2007; Rapacz and Woźniczka 2009). In contrast, PI_{ABS}, which has been recommended for estimating wheat freezing tolerance by Rapacz and Woźniczka (2009), cannot be recommended for wheat using the protocols in the current study. It should be noted that the protocol used by Rapacz and Woźniczka (2009) included a procedure of freezing the field-collected

leaves approximately 1 day after their collection, which may have had an impact on the obtained results.

There was a lack of any correlation or even the presence of a negative correlation in the present study between freezing tolerance/winter hardiness and energy fluxes calculated for a single PSII reaction center (…/RC). This was probably the result of the detrimental effect of freezing on the number of active PSII reaction centers (RC/CS), which may raise energy flow in the remaining (active) centers. Similar effects of increasing energy flows in single active reaction centers with increasing stress level have been previously noted (Soja et al. 1998).

2. The experimental protocol utilizing detached leaves in the current study had two basic elements: (a) the plants were grown under field conditions until the middle of winter and, (b) the leaves were frozen directly before taking the chlorophyll fluorescence measurements.

Estimates of freezing tolerance obtained using chlorophyll fluorescence were not correlated with estimates of field survival after the winter in locations where winter field survival were not correlated with the estimates obtained by controlled freezing of whole plants in the laboratory. More simply stated, estimates of freezing tolerance obtained by any of the controlled freezing tests used in the present study, were not always correlated with the estimates of winter hardiness obtained by evaluating survival in field plantings at the end of the winter. This indicates that the leaves of field-grown plants were subjected to other winter-related environmental stresses in addition to freezing stress. Therefore, what was measured in the controlled freezing studies (using whole plants or detached leaves) was freezing tolerance and not winter hardiness in a broad sense.

A correlation between plant survival based on the controlled freezing of whole plants and estimates of survival based on chlorophyll fluorescence measurements taken on detached leaves from the same plants was not surprising, since the plants may have already been damaged prior to the initiation of either protocol. The relationship between the two protocols, however, is far more complicated. No significant correlation between the chlorophyll fluorescence parameters and plant survival based upon the controlled freezing of whole plants was observed when the detached leaves were not frozen prior to taking fluorescence measurements (data not shown). Additionally, the correlation coefficients of the chlorophyll fluorescence parameters with plant survival were lower when the leaves were frozen at a higher temperature (−15 vs. −20 °C, data not shown). The higher reliability that chlorophyll fluorescence measurements taken in the middle/late, but not early winter, will be significantly correlated with plant survival, may be explained as previously suggested by Rapacz et al. (2011). Namely, cellular membranes, including those of thylakoids, can be more resistant to freezing injury in late winter due to the adaptation resulting from repeated freezing events over the winter (Steponkus 1984). Thus, the differences in the freezing tolerance of photosynthetic apparatus in freezing-tolerant versus freezing-susceptible plants may be more apparent in late winter. This hypothesis was supported by the observation that the effect of sampling date on the correlation with field survival was observed only in chlorophyll fluorescence-based measurements but not in the case of plant survival after controlled freezing.

Rapacz et al. (2011) reported that chlorophyll fluorescence measurements taken in plants cold acclimated under laboratory conditions were highly correlated with estimates of freezing tolerance of the same plants obtained by electrolyte leakage (EL), less correlated with the survival of plants cold acclimated in the laboratory, and barely correlated with the freezing tolerance of plants cold acclimated in the field. In the present study, the results obtained with the JIP-test within the field-laboratory protocol were poorly correlated with estimates of freezing tolerance obtained in the controlled freezing test using laboratory-acclimated plants. As previously stated, freezing tolerance may consist of two different, but equally important components. The first one is connected with damage to the plasma membranes, including those of thylakoids (Krause et al. 1988; Sror et al. 2003). Plasma membrane damage can be estimated in the EL analysis and damage to the thylakoid membranes can be estimated by means of the JIP-test taken on detached leaves after they have been subjected to freezing (Rapacz 2007; Rapacz and Woźniczka 2009; Rapacz et al. 2011). The membrane-related type of freezing tolerance is induced by the cold-acclimation protocols used in the laboratory and seems to be associated with the CBF-dependent pathway of cold acclimation, which is strongly connected with cold acclimation of the photosynthetic apparatus (Kurepin et al. 2013). The second type of freezing tolerance is expressed only in the field as a result of complex environmental factors (Gusta and Wisniewski 2013). In this case, winter survival may be the result of root or crown injury, or a result of plants response to a prolonged period of freezing stress (Waallen et al. 2011). Field survival may require the activation of adaptive mechanisms that are very different from each other and not solely reflected in the freezing tolerance of leaves to an acute stress (Gusta and Wisniewski 2013).

Conclusions

- The level of winter hardiness predicted from laboratory freezing studies was not well correlated with winter survival observed in the field.

- The variation in freezing tolerance observed after controlled freezing tests of plants field-cold acclimated in different locations and years, as well as cold acclimated in the laboratory is similar to the variation in plant winter hardiness between locations.
- Chlorophyll fluorescence (JIP-test) measurements performed on field-collected and laboratory-frozen leaves may be used as indirect method for estimating winter survival and freezing tolerance in common wheat resulting in similar or even lower errors than direct tests, under certain conditions:
  - Only some of the analyzed JIP-test parameters are reliably correlated with freezing tolerance and plant survival;
  - Sampling for the chlorophyll fluorescence measurements should be performed during late winter in plants which had not been seriously damaged before.

Author contribution statement MR and MS were responsible for conception and design of the study. All of the authors contributed to analysis and interpretation of the data. MR and MS prepared the draft of the article. MR and MWJ provided a critical revision of the article for important intellectual content. All of the authors approved of the final version of the article to be submitted. MR is responsible for the integrity of the work as a whole, from inception to finished article.

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