

Response to chilling of *Zea mays*, *Tripsacum dactyloides* and their hybrid

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Abstract

Maize (*Zea mays* ssp. *mays*) and eastern gamagrass (*Tripsacum dactyloides*) are known for their susceptibility to chilling injuries. Their hybrid (*Z. mays* × *T. dactyloides*) showed higher tolerance to low temperatures (-2 °C) in the field than its parents. Exposure to 5 °C for 2 or 3 d reduced the variable to maximal chlorophyll fluorescence ratio (F_V/F_M), an indicator of the maximum photochemical efficiency of the photosystem 2, and the variable to minimal fluorescence ratio (F_V/F_0) more in maize and eastern gamagrass than in hybrid plants. Chlorophyll contents for rewarming plants (25 °C for 3 d) were lower than before chilling in both parents while values for hybrid plants were similar. Electrolyte leakage was higher in chilled than control plants but it did not show significant differences among genotypes. Our data suggest that hybrid plants have higher capacity to recover from chilling injury in controlled conditions than their parents.

Additional key words: maize, eastern gamagrass, chilling tolerance, chlorophyll fluorescence, intergeneric hybrid.

Introduction

Maize (*Zea mays* ssp. *mays*) is known for its great susceptibility to chilling injuries (Hodges *et al.* 1995). Eastern gamagrass (*Tripsacum dactyloides*) is a warm-season, perennial grass with high palatability and productivity. After cold winters, plants survive by regrowth from its rhizomes. Eastern gamagrass has been employed to investigate the possibility of introgressing disease tolerance (Bergquist 1981, Gay 1984), constitutive aerenchyma (Ray *et al.* 1999) and gametophytic apomixis (Leblanc *et al.* 1995, Kindiger *et al.* 1996a,b) into maize. With regard to vegetative morphology, maize × *Tripsacum* F₁ hybrids resemble *Tripsacum* more than maize: they are perennial and tillering plants. However, a maize influence can be observed in their floral morphology (Mangelsdorf 1947, James 1979).

A hybrid of *Zea mays* × *Tripsacum dactyloides* (García *et al.* 2000) was more tolerant to chilling temperatures in the field than its parents. For instance,

minimum temperatures of -2 °C during 2 d resulted in irreversible injuries in both parents without visible damages in the hybrid (unpublished results). Injuries can be observed as wilting, followed by discolouring and necrosis of leaves.

In chilling sensitive plant species, chilling induces a phase transition of the membrane lipids which results in increased leakage of electrolytes from leaves (Markowski and Skrudlik 1995, Szalai *et al.* 1996). In addition, simultaneous exposure of the plants to low temperature and high irradiance can cause photoinhibition (Long *et al.* 1983, Bolhár-Nordenkamp and Öquist 1993, Koscielniak and Biesaga-Koscielniak 1999, Maxwell and Johnson 2000). It takes place in maize during bright mornings when the temperature drops below 12 °C (Farage and Long 1987).

Photosystem 2 (PS2) is the most vulnerable part of the photosynthetic apparatus, with damage to PS2 often being the first result of stress (Greer 1990). Chlorophyll

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; F_0 - minimum chlorophyll fluorescence yield; F_M - maximum chlorophyll fluorescence yield; F_V - variable fluorescence; PAR - photosynthetically active radiation; PS2 - photosystem 2.

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fluorescence is used as a sensitive probe of photosynthetic function.

After illumination of dark-adapted photosynthetic tissues with a weak light, there is an immediate rise in fluorescence to a so-called minimal level (F_0) which is attained when radiation has been absorbed by the chlorophyll antenna, but before the density of excitons is high enough to produce a continuous charge separation in the reaction centres of PS2. It depends also on the chlorophyll content (Csapó *et al.* 1991). Fluorescence measuring systems obtain F_0 level with a modulated exciting radiation being low enough to prevent a detectable photochemistry from a sample (Bolhár-Nordenkamp and Öquist 1993, Maxwell and Johnson 2000). Upon irradiation with a sufficiently strong radiation, fluorescence increases from F_0 to a maximum fluorescence (F_M). Variable fluorescence (F_V) equals to the $F_M - F_0$. The ratio F_V/F_M estimates the efficiency of the excitation energy captured by open PS2 reaction centres (Genty *et al.* 1989). It has a typical range from 0.75 to 0.85 and as can be shown this ratio is proportional to the maximum quantum yield of photochemistry (Bolhár-Nordenkamp and Öquist 1993). The F_V/F_0 ratio reflects changes in the maximum ratio of quantum yields of photochemical (F_V/F_M) and concurrent non-photochemical processes (F_0/F_M) in PS2 related to the dark-adapted state (Roháček 2002).

The light-dependent effect of the decrease of photosynthetic rate is attributable to the chilling-induced damage of PS2. At the photochemical level, this causes a decrease of the F_V/F_M ratio (Butler and Kitajima 1975, Baker *et al.* 1983, Havaux and Lannoye 1984). The F_M

declines also (Greer 1990) and, as $F_0/F_M + F_V/F_M = 1$ (Butler and Kitajima 1975), the F_0/F_M ratio markedly increases if plants are stressed or damaged (Roháček 2002).

Maize seedlings are more damaged by constant than by fluctuating low temperatures (Stamp 1987). The effect of varying temperatures prior to chilling stress was investigated to reveal chilling adaptation in maize seedlings (Nie *et al.* 1992, Koscielniak and Biesaga-Koscielniak 1999). Many plants become more resistant to freezing temperatures when first exposed to low, non-freezing temperatures (cold acclimation) (Travert *et al.* 1997). Cold acclimation in maize seedlings is obtained by exposure to 14 °C for 1 d (Prasad 1997). At approximately the three to four leaf stage, the seed reserves of maize are exhausted (Cooper and MacDonald 1970) and the plantlet has to rely on its photosynthetic activity. Autotrophic growth proceeds very slowly when the temperature is lower than 15 °C (McWilliam and Naylor 1967). Under limiting temperatures, the rate of assimilate production is often inadequate to sustain autotrophic growth so that the plant starves and eventually dies (Hardacre and Eagles 1980).

The objective of this research was to evaluate the parents and the hybrid of *Zea mays* × *Tripsacum dactyloides* for cold tolerance, expressed during autotrophic growth under temperature-limiting conditions determined in controlled environments. The aim of this comparative study was to determine chlorophyll (Chl) content, Chl fluorescence parameters (F_V/F_M and F_V/F_0), and the electrolyte leakage in the leaves during growth at 5 °C.

Materials and methods

Plants, growth and acclimation: *Zea mays* L. ssp. *mays* (2n = 40; N107B) was supplied by Maize Genetic Stock Center, Urbana, Illinois. Four replicates of 25 maize seeds each were germinated on cotton and filter paper moistened with distilled water in Petri dishes at 25 °C for 5 d in the dark, at which time coleoptile lengths were higher than 1 cm. Maize seedlings were placed in plastic pots (one seedling per pot) filled with 300 cm³ soil issued from horizon A of a Typic Argiudol. Twenty pots were arranged in a growth chamber (16-h photoperiod) at 25 °C with artificial light (Philips HPLR, 400 W) which gave irradiance of 400 µmol m⁻² s⁻¹ PAR at leaf level. Relative humidity was > 60 %. Plants were grown for 6 d in these conditions, reaching the four-leaf stage of development. Of the 20 plants, 10 plants at random were placed in controlled environment conditions in a growth cabinet (model CPM 40/480, Ibertest, Madrid, Spain) at 25 °C (16-h photoperiod, 400 µmol m⁻² s⁻¹ PAR, 80 % RH) during 48 h, followed by two periods of 24 h at 15 and 10 °C, respectively, and finally at 5 °C for 72 h. The

other 10 plants (control) rested these 7 d at 25 °C. All the experiments were realized at the atmospheric CO₂ concentration. After chlorophyll fluorescence and conductivity measurements, all chilled plants were cultivated at 25 °C in the same place as the control plants.

Tripsacum dactyloides L. (2n = 72), from Mexican origin, was introduced in the Instituto Fitotécnico de Santa Catalina (I.F.S.C.) and cultivated in the field from 1970 to the present. Due to the high dormancy of eastern gamagrass seeds (Tian *et al.* 2002) plantlets were obtained by placing one rhizome, containing an sprouted tiller, in each of 20 plastic pots filled with 300 cm³ soil from horizon A. Pots were arranged in the same growth place as described above for maize seedlings (16-h photoperiod) at 25 °C. Adequate regrowth plants (3 - 4 leaves per tiller) were obtained after 25 d. Control (25 °C) and chilling treatments were achieved at the same time and in the same conditions as described for maize.

Z. mays × *T. dactyloides* hybrid plants were obtained by hand pollination of *Z. mays* inbred N107B (2n=40)

with *T. dactyloides* and subsequent embryo rescue. Ears were harvested 12 d after pollination. Developed caryopsis were cut off and disinfected with 2.5 % sodium hypochlorite solution for 20 min. Embryos were isolated from the caryopsis, plated on the basic medium by García *et al.* (1992) supplemented with 4 μ M 2,4-dichlorophenoxyacetic acid (2,4-D) and incubated at 28-30 °C with 16-h photoperiod. Shoots were regenerated by organogenesis and rooted on the basic medium free of plant growth regulators. Regenerated plants were rusticated for 10 d at 25 °C in 300 cm³ pots with horizon A soil and a plastic cover to ensure adequate light transmission. Afterwards, they underwent the same treatments and conditions as the eastern gamagrass clones and maize seedlings.

Measurements of chlorophyll fluorescence: Chlorophyll fluorescence was recorded with a *Fluorescence Modulated System* (model FMS 2, Hansatech, Kings Lynn, UK) and parameters (F_0 , F_M , F_V/F_M , F_V/F_0) of the fluorescence induction kinetics were measured. Measurements were made 6 h after completing the first dark period at 5 °C, on the fourth leaf stage of seedlings (maize) or regrowth plants (*Tripsacum* and F_1 plants). Leaves were dark-adapted for 15 min before light excitation (Koscielniak and Biesaga-Koscielniak 1999) using leaf clips. Measurements were taken 2 to 3 s after the fiber probe was inserted in the leaf clip. Measurements were repeated 24 and 48 h thereafter on the fourth leaf of the same plants. Temperature of the growth place was maintained all time at 5 °C. In control plants, grown at 25 °C, measurements were made similarly as in stressed plants. For each genotype and treatment, values of the different fluorescence parameters were the average of seven leaves from different plants. All data were collected from the upper side of fully expanded primary leaves attached to the plant.

Results

Exposure to low temperature significantly reduced maximum photochemical efficiency of PS2 measured in dark-adapted state (F_V/F_M ratio). All the F_V/F_M ratios in control plants were higher than in chilled plants (Fig. 1). After applying 5 °C during 2 or 3 d, maize and eastern gamagrass showed lower values than F_1 plants. For instance, after 3 d at 5 °C, means F_V/F_M ratios decreased 81.2, 76.2 and 50.3 % in maize, *Tripsacum* and the hybrid, respectively, in relation to control plants. Ratios between the F_V/F_M parameter for stressed plants [obtained for each genotype as $\Sigma(F_V/F_M \text{ data})/21$] vs. F_V/F_M for control plants, were also lower in the parents than in their hybrid. All regression coefficients for the F_V/F_M ratio vs. days of growth at 5 °C were negative and the slope of the

Leaf chlorophyll content: After cold acclimation, temperature for chilled plants was maintained all time at 5 °C for 4 d. Then, chilled plants were returned to the original growth conditions (25 °C) for 3 d. Chlorophyll content was measured according to Inskeep and Bloom (1985). Two discs from fully-developed leaf (1 cm² each) were placed into test tube and chlorophyll was extracted with 5 cm³ of N,N-dimethylformamide (DMF). Tubes were stored for 3 d at 4 °C in the dark and shaken for 5 min once a day. Absorbance values were measured at 647 and 664.5 nm in a spectrophotometer (model UV 160A, Shimadzu, Columbia, USA). Each treatment contained four replications for each genotype.

Measurements of electrolyte leakage: After completing chlorophyll fluorescence measurements, electrolyte leakage was measured as a change in conductivity in the following way. The third leaf of each seedling or regrowth plant was separated, weighed (approximately 327 mg fresh mass), cut in slices across the leaf of, approximately, 1 cm long, washed in distilled water and put into 10 cm³ distilled water at 20 °C during 3 h 25 min. Conductivity of the solution was measured for each genotype and treatment with an electric conductivity meter (*Digicond IV*, Luftman, Argentina) and repeated six-fold. Stress/control ratios were computed for all traits investigated to overcome possible biases due to scale effects (Hodges *et al.* 1995, Brandolini *et al.* 2000).

Statistical analysis: Data were analyzed by analysis of variance (*ANOVA*). When *ANOVA* indicated the presence of differences among means, a least significant difference (LSD) test was used to make comparisons among individual means. A simple regression analysis was performed for the F_V/F_M ratios versus days of growth at 5 °C and for the correlation analysis between F_V/F_0 versus F_V/F_M (Steel and Torrie 1980).

regression for the hybrid was significantly higher (-0.123; $P \leq 0.01$) than those for maize (-0.219) or eastern gamagrass (-0.191) (Fig. 1). Conversely, all the F_0/F_M ratios ($F_0/F_M = 1 - F_V/F_M$) in control plants were lower than the mean ratios in chilled plants. For instance, after 3 d at 5 °C, F_0/F_M increased 416 % in maize, 489 % in eastern gamagrass but only 262 % in the hybrid, related to the respective controls (data not shown).

All the F_V/F_0 ratios in control plants were higher than mean ratios in chilled plants ($P \leq 0.01$) (Table 1). Experiments resulted in higher F_V/F_0 mean data at 25 °C (3.307) than in chilled plants (1.314, 0.654 and 0.339 for 1, 2 and 3 d at 5 °C, respectively). After applying 5 °C for 2 or 3 d all genotypes were affected, but maize and

eastern gamagrass showed lower values than F_1 plants. For each genotype, F_V/F_0 means had the same tendencies described above: control data were higher than means for stressed plants while plants after 1 d at 5 °C showed higher F_V/F_0 means than plants after 2 or 3 d at 5 °C. For instance, means after 2 d at 5 °C, approximately reduced an 89 % in maize, 87 % in *Tripsacum* and 65 % in F_1 plants, related to control plants. Fluorescence comparisons clearly indicate that the progenitors and the hybrid differed in their responses to chilling. Correlation coefficient for F_V/F_0 vs. F_V/F_M ($r = 0.925$; $n = 84$) was highly significant ($P < 0.001$) (Fig. 2).

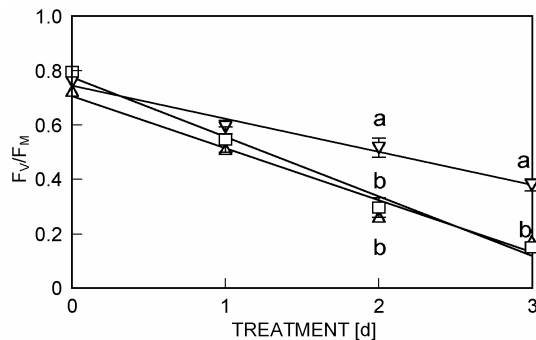


Fig. 1. Regressions of F_V/F_M ratio vs. days at 5 °C. Regression equations are: $F_V/F_M = 0.776 - 0.219x$ (b) (x - day of treatment) (for maize, squares); $F_V/F_M = 0.706 - 0.191x$ (b) (for *Tripsacum*, triangles); $F_V/F_M = 0.748 - 0.123x$ (a) (for the hybrid, reversed triangles). Slopes of regression equations followed by the same letter are not significantly different ($P \leq 0.01$).

Table 1. F_V/F_0 ratios in leaves of control (25 °C) and chilled (5 °C) plants. Values are the means of seven measurements (\pm SE) on different samples. Means followed by the same small letter are not significantly different ($P \leq 0.05$) according to the LSD test.

Chilling	Maize	<i>Tripsacum</i>	Hybrid
Controls	3.956 \pm 0.184a	2.685 \pm 0.125c	3.281 \pm 0.164b
1 d	1.342 \pm 0.221de	1.074 \pm 0.075f	1.528 \pm 0.191d
2 d	0.447 \pm 0.079gh	0.362 \pm 0.030hi	1.154 \pm 0.187ef
3 d	0.177 \pm 0.009i	0.212 \pm 0.022i	0.628 \pm 0.065g

Electrical conductivity data of washed leaves from plants cultured at 25 °C do not differ significantly among genotypes (Table 2). For each genotype, conductivities from control plants were lower than from leaves after 3 d at 5 °C. Accordingly, mean value for chilled plants was higher than the corresponding mean for control plants. F_1 chilled plants were less affected than its parents but the difference in relation to maize was not significant ($P > 0.05$) (Table 2). Mean values were 71.3, 80.6 and 93.7 % higher for F_1 , eastern gamagrass and maize,

respectively, than those for the controls. Stress/control ratios were all higher than 1.7 with no significant differences among genotypes.

The behaviour of chilled plants placed back in an environment with 25 °C was also different. After one week, maize seedlings died and leaves from eastern gamagrass plants too, while stressed hybrid plants remained alive as did in the field.

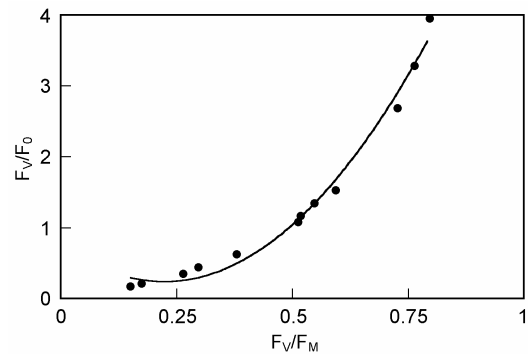


Fig. 2. Graphical relationship between the chlorophyll fluorescence parameters F_0/F_M and F_V/F_M . Each value is the mean of seven repetitions. The correlation coefficient between parameters is: $r = 0.925$ ($n = 84$, $P < 0.001$).

Table 2. Electric conductivity ($\mu\text{S cm}^{-1} \text{ g}^{-1}(\text{f.m.}) \text{ h}^{-1}$) of leaf slices washed in distilled water from three genotypes. Means \pm SE of six repetitions. Data for chilled and control plants or stress/control ratios followed by the same letter are not significantly different ($P \leq 0.05$).

Genotypes	Chilled plants	Controls	Chilled/controls
Maize	71.1 \pm 9.8cd	36.7 \pm 3.5a	1.92 \pm 0.13a
<i>Tripsacum</i>	84.9 \pm 4.3d	47.0 \pm 3.0ab	1.80 \pm 0.11a
Hybrid	61.5 \pm 7.9c	35.9 \pm 3.2a	1.70 \pm 0.07a

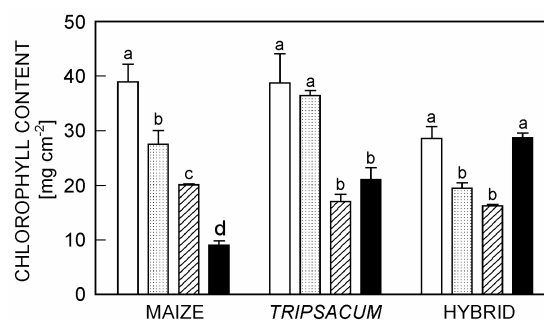


Fig. 3. Chlorophyll content at different temperatures. Control and rewarming temperature, 25 °C; chilling temperature, 5 °C. Empty columns - control, checked columns - 3 d at 5 °C, stripped columns - 4 d at 5 °C and 1 d at 25 °C, full columns - 4 d at 5 °C and 3 d at 25 °C. For each genotype and treatment values are the mean (\pm SE) of four repetitions. Means followed by the same letter are not significantly different ($P \leq 0.05$).

After applying 5 °C for 3 d (Fig. 3) or 4 d (data not shown) a reduction of chlorophyll content was observed for maize and F₁ plants. Returned back for 1 d at original growth conditions, the chlorophyll content for maize and

the hybrid was stabilized but eastern gamagrass showed lower values than previously. After 3 d of post-chilling rewarming only F₁ plants showed normal chlorophyll content (Fig. 3).

Discussion

It is a well documented fact that the function of a photosynthetic apparatus is sensitive to several environmental stresses. Chlorophyll fluorescence technique gives information about the state of PS2 and points us how PS2 is using the energy absorbed by chlorophyll complexes and the extent to which it is being damaged by an excess of radiation (Maxwell and Johnson 2000). PS2 appears to be preferentially affected by chilling stress (Bolh  r-Nordenkamp and   quist 1993, Agati *et al.* 1996). Changes in F_v/F_m and F_0/F_m are accepted as reliable diagnostic indicators of alterations in PS2 (Maxwell and Johnson 2000). One of the early events in chilling stress is a light-dependent inactivation of the primary photochemistry: a sustained decrease in dark-adapted F_v/F_m and in F_v/F_0 ratios (Greer 1990, Roh   ek and Bart  k 1999).

In our experiments, chilled plants showed lower F_v/F_m and F_v/F_0 ratios than control plants. So, these data are indicators of sensitivity of the PS2 complexes to low temperatures in the three genotypes. Altered photochemical efficiency of the PS2 reaction centres was inferred from the low value of F_v/F_m ratios as can be expected when a significant fraction of the reaction centres is transformed to the photochemically inactive state by chilling stress (Maxwell and Johnson 2000). Similar results were reported in *Phaseolus vulgaris* L. (Agati *et al.* 1996) or maize (Koscielniak and Biesaga-Koscielniak 1999). Furthermore, the higher reduction of the F_v/F_m ratio in maize and eastern gamagrass chilled plants in relation to their hybrid (after 2 or 3 d at 5 °C, see Fig. 1) supports our conclusion that there is a genotypic difference in susceptibility to the inactivation of PS2.

During the chilling treatment, data recorded from the leaves of seven plants in each genotype showed changes of the F_v/F_0 ratio. This parameter is a good indicator of changes in the maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PS2 related to a leaf in a dark adapted state (Roh   ek 2002). It is affected by chilling and also, by other factors, *e.g.* chlorophyll content (Csap   *et al.* 1991). Decline in F_v/F_0 started in the three genotypes in the first day at 5 °C and was more pronounced in both parents during the second and third day of chilling (Table 1). These results are in accordance with tendencies mentioned above in relation to the F_v/F_m ratios.

Differences in susceptibility of PS2 to low temperatures were also reflected in the negative slopes of the regression equations of F_v/F_m *vs.* days of chilling

treatment. For this limited period of chilling treatment, the linear relationship may be an adequate description of the functional dependence of F_v/F_m on number of days at 5 °C in the three genotypes. Maize and *Tripsacum* showed slopes 78 and 55 % more negatives than the hybrid (Fig. 1). These tendencies might be in agreement with the tolerance of plants studied to low temperatures during prolonged periods of chilling. After 1 d at 10 °C and 3 d of continuous growth at 5 °C, low temperatures produced irreversible damages in maize seedlings, similarly to results of Koscielniak and Biesaga-Koscielniak (2000), and in the shoot of eastern gamagrass while their hybrid was less susceptible and had the capacity for recovery. After chilling treatments, all chilled plants were moved to 25 °C. Only F₁ chilled plants recovered back in a healthy state condition. This behavior could be related to a higher photochemical efficiency capacity of PS2 in the hybrid if compared to its progenitors. Fluorescence data in our assay were early predictors of leaves injuries in maize and eastern gamagrass as Agati *et al.* (1996) reported in *Phaseolus* and Koscielniak and Biesaga-Koscielniak (2000) in maize. For instance, linear fitting curve for F_v/F_m ratios versus time at 4 °C on the chilling sensitive *Phaseolus vulgaris* L. (Agati *et al.* 1996) was similar to our eastern gamagrass straight line (Fig. 1).

Graphical relationship between F_v/F_0 *vs.* F_v/F_m (Fig. 2) is similar as the one reviewed by Roh   ek (2002). Correlation analysis indicates that both parameters are interdependent with a high degree of association ($r = 0.925$; $P < 0.001$).

The electrolyte leakage from leaves, measured as the electric conductivity of washed leaves, showed higher values for plants under the low temperature (3 d at 5 °C) in comparison with controls at 25 °C indicating that membrane permeability was affected by chilling treatment (Markowski and Skrudlik 1995, Koscielniak and Biesaga-Koscielniak 1999). Differences in electrical conductivity values were found among species under the low temperature: the F₁ plants less affected and the maize ones the most susceptible to the chilling treatment. Expressed as stressed plants/control plants ratio, differences among genotypes were not significant. Under the conditions applied herein, these results indicate that the electrical conductivity is not a sufficiently sensitive parameter to find out differences in susceptibility to chilling injuries among genotypes as the F_v/F_m ratio can be for chilling-induced damage to PS2.

Differences in leaf chlorophyll content were found

among the three genotypes grown at 25 °C. It is known that slightly reduced chlorophyll content per leaf area does not affect leaf photosynthesis per area in full sunlight as the light-gathering ability of normal leaves exceeds their photo- and dark-reaction capabilities (Gutschick 1988). As a result of continuous chilling of maize seedlings the content of chlorophyll drops (Haldimann *et al.* 1996). According to Kaniuga *et al.* (1999) chlorophyll content of maize seedlings was slightly affected in chilling and dark conditions but a large decrease was observed upon rewarming under 16-h photoperiod. In our chilling conditions, maize and the hybrid showed a decrease in the chlorophyll content,

similarly to Haldimann *et al.* (1996) results but, after 24-h of post-chilling rewarming, the chlorophyll content also decreased in eastern gamagrass. After 3 d of post-chilling rewarming, only the hybrid plants increased their chlorophyll content to normal values (Fig. 3).

Conclusion: This study confirms that the hybrid *Zea* × *Tripsacum* is more tolerant to chilling temperatures than its parents, both in the field and in controlled conditions. The maximum photochemical efficiency of PS2, expressed as the F_v/F_m ratio, is a good an early indicator of the different growth behavior of the hybrid and its ancestors.

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