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ARTICLE

Paraquat and cold tolerance in doubled haploid maize

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ABSTRACT Paraquat and cold tolerance of doubled haploid maize plants selected and regenerated from microspores using paraquat as ROS progenitor were compared to those of non-selected DH line and the original hybrid. Three of five paraquat tolerant DH lines possess higher cold tolerance than the control DH line and the original hybrid during the germination period. On the other hand the low temperature stress (T8°C) exposed the plants at the early autotrophic phase of developments resulted in a higher cold tolerance in all of the five paraquat-selected DH lines. Our results demonstrated that the microspore-selected DH lines using paraquat as a ROS progenitors resulted not only higher tolerance against the paraquat-mediated oxidative damage, but help in the protection against the low temperature stress.
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KEY WORDS

cold stress,
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Maize is generally considered as a chilling sensitive species with a relatively high temperature optimum for germination, development, and dry matter accumulation. The current agronomic trend is the early spring planting to maximize the duration of the growing season, thereby maximizing yields. But in the early sowing, it has increased the probability that the young plants will spend some portion of early development under suboptimal temperature conditions.

The suboptimal temperature under germination reduces the number of germinated seeds, delays their developments. Amongst the various effects of low temperature on the maize developments, the high susceptibility of the photosynthetic apparatus to low temperature is considered to be of particular importance (Baker 1994). Leaves of maize, which develop at a temperature of 15°C or below are often characterized by a lower photosynthetic capacity, lower quantum efficiency of CO_2-fixation, and lower quantum efficiency of electron transfer at PS II than leaves develop under more favourable conditions (Leipner et al. 1999).

Due to the subtropical origin of maize, it shows a little capacity to acclimate to low growth temperature and therefore it is prone to physiological damage during non-freezing, suboptimal temperatures by formation of reactive oxygen species, such as superoxide (O_2^{-*}), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical (OH^*), lipidperoxides (Jahnke et al. 1991; Apel and Hirt 2004). Therefore, an improvement of the oxidative stress tolerance of maize may as well enhance the protection against the cold stress.

In the Agricultural Research Institute of the Hungarian Academy of Sciences, doubled haploid maize plants tolerant of oxidative stress were produced by the *in vitro* selection of microspores in anther cultures with ROS progenitors (Ambrus et al. 2006).

The study presented in this paper was designed to characterize the cold tolerance of the progeny of DH plants selected and regenerated from microspores exposed to paraquat as a ROS producing agent and to determine the relationship between the tolerance of oxidative stress and cold of the selected DH lines.

Materials and Methods

Experiments were performed on the second generation of fertile doubled haploid (DH2) maize *(Zea mays,* L.*)* plants

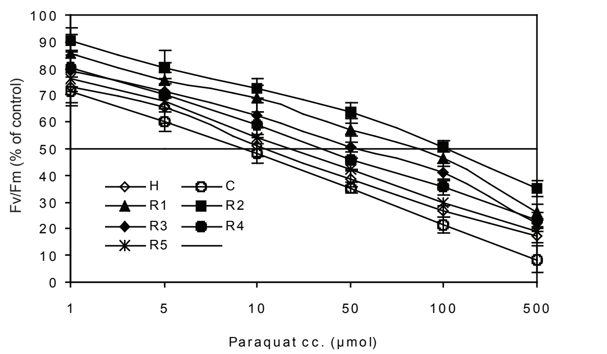

Figure 1. Effect of paraquat on the photosynthetic activity of PS II calculated from Fv/Fm (as a % of values for plants without paraquat stress) in maize leaf discs of DH2 lines derived from paraquat-selected microspores (R_{1-5}), non-selected control DH2 line (C) and the hybrid (H). The leaf discs were floated in different concentration of paraquat solution for 4h in the light (400 µmol m^{-2} s^{-1}).

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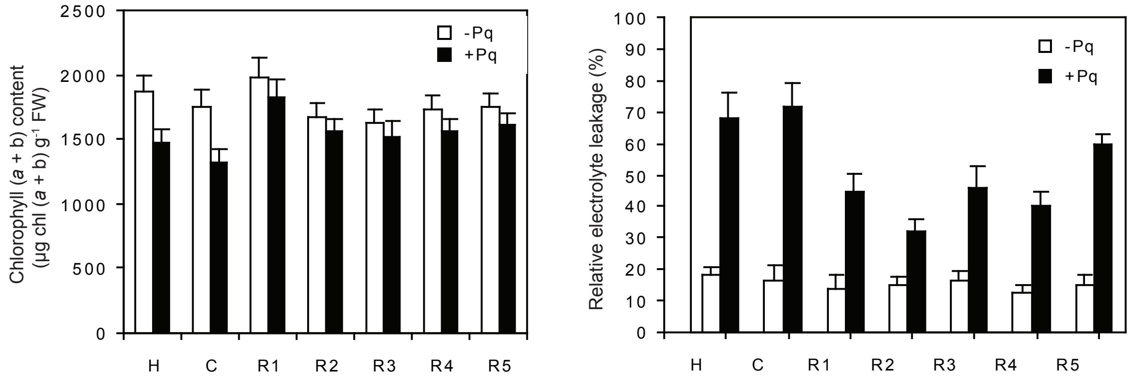

Figure 2. Comparison of the chlorophyll (*a*+b) content (A) and relative electrolyte leakage (B) of maize leaf discs floated on solutions containing 0 or 10 µM paraquat for 24 h in the light. Values are means of 3 independent experiments ± SD. R_{1-5} lines = DH2 lines derived from paraquat-selected microspores, C= non-selected control DH2 lines and H= original hybrid.

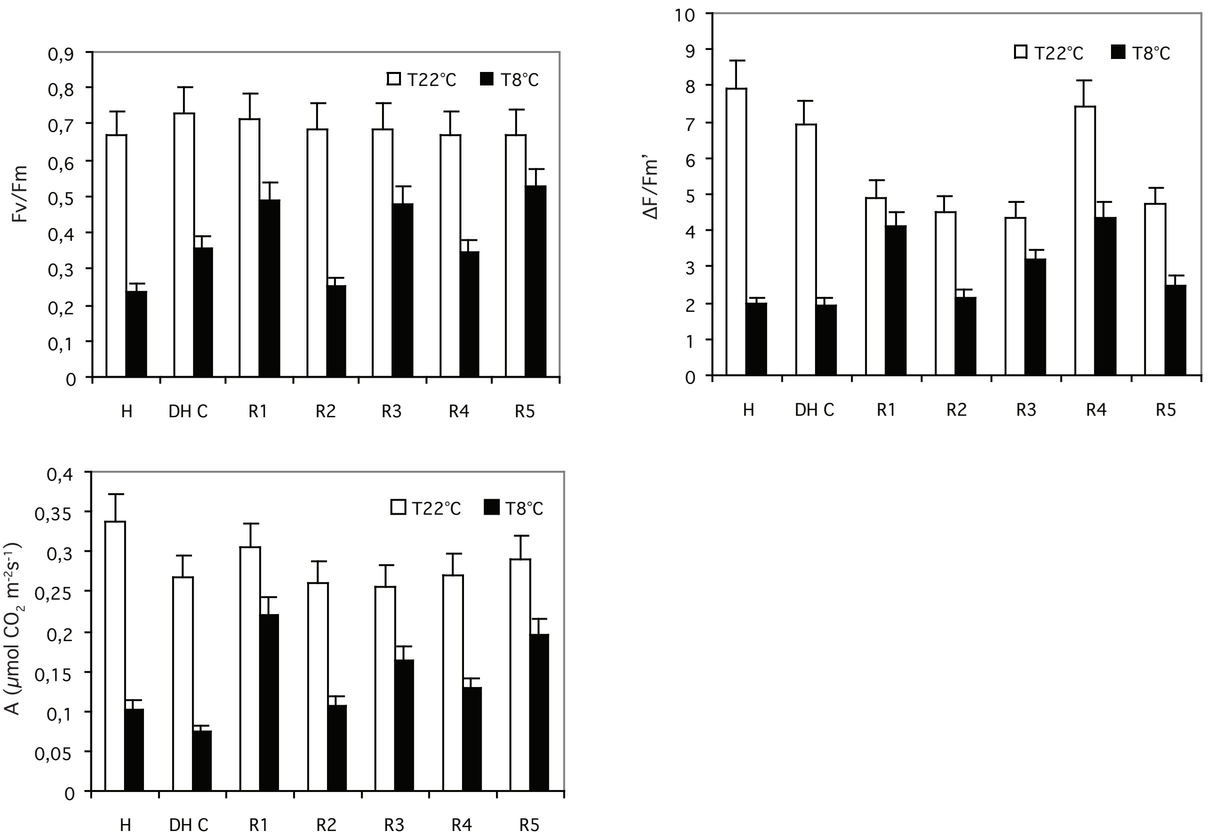

Figure 3. Effect of cold stress on the photosynthetic activity of PS II measured as optimal (Fv/Fm) (A) and the effective quantum yield (ΔF/Fm') (B) of PS II and the rate CO_2 fixation (C). Fv/Fm was determined after 15 min dark adaptation, (ΔF/Fm') was determined after illumination at 340 µmol m^{-2} s^{-1} at steady-state level. The CO_2 fixation was measured at saturated light intensity (700 µmol m^{-2} s^{-1}) without light stress condition and at steady state level (after 15 min).

produced as described by Ambrus et al. (2006). Five paraquat-selected DH2 lines (R1-5) were used and compared to the control, non-selected DH2 line (C) and the original hybrid (H).

The plants were grown in a phytotron chamber (Conviron PGV96) at a light intensity of 200 µmol m^{-2} s^{-1} as described by Tischner et al. 1997.

The oxidative stress tolerance of DH lines and hybrid

Table 1. Paraquat tolerance indicated by tolerance index (PTI) of DH2 lines derived from paraquat-selected microspores (R_{1-5}) and from the hybrid (H) compared to the non-selected control DH2 lines (C). PTI values were calculated as following: $PTI_{Fv/Fm}= Rx_{(Pq\ c.I50)}/ C_{(Pq\ c.I50)}$, $PTI_{chl}= C(Chl_B-Chl_{Pq})/Rx(Chl_B-Chl_{Pq})$ and $PTI_{REL}= C(REL_{Pq}-REL_B)/Rx(REL_{Pq}-REL_B)$, where Pq $c._{I50}$ indicates the paraquat concentrations required to reduce the Fv/Fm parameter by 50%, B indicates the samples without paraquat treatments. Rx =R1-5 lines. REL= relative electrolyte leakage.

	H	C	R1	R2	R3	R4	R5
$PTI_{Fv/Fm}$	1.3	1.0	8.7**	11.8**	5.4**	3.95**	1.6
PTI_{chl}	1.0	1.0	3.2**	3.95**	4.0**	2.57**	3.0**
PTI_{REL}	1.1	1.0	1.8*	3.2**	1.85*	2.02*	1.2

*, ** significantly different from non-selected control (C) at P = 0.05 or P = 0.01 level.

was determined by measurements on photosynthetic activity of PS II via Fv/Fm chlorophyll a fluorescence parameter, by determination of chlorophyll (*a*+b) content and ion conductivity of leaf discs floated in different concentration of paraquat solution (Lichtenthaler et al. 1987; Lehoczki et al. 1992).

The effects of cold stress (T8°C) occurred during germination were characterized by the decrease in number of germinated seeds, by the delay of germination. The cold tolerance of these lines was also determined at the early autotrophic phase of maize developments (on five weeks old plants) by measurements of the quantum efficiency of CO_2-fixation using an infrared gas analyser (LCA-2), and the effective (ΔF/Fm') and optimal (Fv/Fm) quantum yield of PS II photochemistry measured by chlorophyll *a* fluorescence using a PAM 2000 portable Chl fluorometer as described by Molnár et al. (2004).

All experiments were repeated three times under the same conditions. Data were statistically analysed using Student's *t* test. Differences were considered to be significant at *P* = 0.01 or 0.05.

Results

Oxidative stress tolerance of paraquat-selected DH lines

Paraquat-induced oxidative damage was associated with a decrease in the photosynthetic activity of PS II, with chlorophyll bleaching, protein breakdown and increased ion leakage from cells of the leaf discs.

In the absence of paraquat, the optimal quantum yield of PS II (Fv/Fm), reflecting the number of active PS II reaction centres, was 0.78 ± 0.04 and no significant difference was found between the DH2 lines and the hybrid. After the paraquat treatment, Fv/Fm decreased in all of the DH2 lines, especially in DH C and H (Fig. 1).

Exposure of leaf discs to 10 µM paraquat solution for 24 h in the light resulted in chlorophyll (*a*+b) breakdown and ion leakage from the cells of leaf discs in all of the DH2 lines and in the hybrid (Fig. 2). Both of them were more pronounced in control plants (C and H) than in the paraquat-selected DH2 lines, indicating less oxidative damage of these lines (Fig. 2).

To compare the paraquat tolerance of DH2 lines derived from paraquat-selected microspores to those of C and H plants, paraquat tolerance indexes (PTI) were calculated from the values of Fv/Fm, chlorophyll (*a*+b) and electrolyte leakage parameters (Table 1). The paraquat-selected DH2 lines were 2 to 12 times more tolerant to paraquat than the control (C or H) plants. Two genotypes (R1 and R2) exhibit noteworthy paraquat tolerance

Cold tolerance of paraquat-selected DH lines

Since the cold stress generally occurs during germination or at the early autotrophic phase of maize developments, the investigations as dealing with cold stress are focused on these periods.

Under optimal conditions (T22°C), the hybrid, the R1, R2 and R5 DH lines represent good germination potential

Table 2. Effects of low temperature (T= 8°C) on the germination of seeds of DH2 lines derived from paraquat-selected microspores (R_{1-5}), non-selected control DH2 lines (C) and from the hybrid (H). Germination index was calculated as germination %/No. of days till germination according to Herczegh 1978. Values of cold tolerance index (CTI) were calculated as CTI= $R_x(GI_{T8°C}/ GI_{T22°C})/ DHC (GI_{T8°C}/ GI_{T22°C})/$

	Germination % T_{22}°C	No. of day till germination	GI T_{22}°C	Germination % T_8°C	No. of day till germination	GI T_8°C	CTI
H	98	3.5	28.0	53	13	4.0	1.1
DHC	50	6	8.3	15	15	1.0	1.0
R1	90	3.5	25.7	85	13	6.5	2.1*
R2	95	4	23.7	90	13	6.9	2.4*
R3	50	5	10.0	20	18	1.1	1.0
R4	38	6	6.3	10	17	0.6	0.7
R5	75	4	18.7	60	14	4.3	1.9*

*significantly different from non-selected control (C) at P = 0.05

Table 3. Cold tolerance indicated by tolerance index (CTI) of DH2 lines derived from paraquat-selected microspores ($R_{1\text{-}5}$) and from the hybrid (H) compared to the non-selected control DH2 lines (C). TI values were calculated as following: $CTI_{Fv/Fm} = Rx_{(Fv/Fm(T8)/Fv/Fm(T22)}/ C_{(Fv/Fm(T8)/Fv/Fm(T22)}$, $CTI_{\Delta F/Fm'} = Rx_{(Fv/Fm(T8)/Fv/fM(T22)}/C_{(\Delta F/Fm'(T8)/\Delta F/Fm'(T22)}$ and $CTI_A = RX_{(A(T8)/A(T22)}/ C_{(A(T8)/A(T22)}$.

	H	C	R1	R2	R3	R4	R5
$CTI_{Fv/Fm}$	0.73	1.0	1.4*	1.6*	1.1	0.73	1.4*
$CTI_{\Delta F/Fm'}$	1.1	1.0	2.6**	2.5**	1.75*	1.5*	2.4**
CTI_A	0.86	1.0	3.0**	2.1**	1.9**	1.7*	2.6**

*, ** significantly different from non-selected control (C) at P = 0.05 or P = 0.01 level.

as indicated by the high germination index (GI_{T22}; Table 2). The reduction in the number of germination and the delay in germination generally occur in the case of doubled haploid or inbred lines. The low temperature (T8°C) reduced the number of germinated seeds and caused a huge delay in the day of germination resulting in a decrease in GI_{T8} (Table 2). These were pronounced in hybrid (H), DH C, R3 and R4 lines. To compare the genotypes, cold tolerance indexes (CTI) were determined as the ratio of GI_{T8} and GI_{T22}. CTI is significantly higher in R1, R2 and R5 DH lines than in H and DH C (Table 3).

Under non-stressed conditions (T22°C) the optimal and the effective quantum yield of PS II (Fv/Fm), which reflect the number of active PS II reaction centres and the quantum efficiency of PS II photochemistry did not differ in the DH2 lines and the hybrid. However, the CO_2 fixation capacity is lower in R1, R2, R3 and R5 DH lines than in hybrid (H) and DH C and R4 lines.

The low temperature stress (T8°C) given to 5 weeks old plants for 24 h results in a decrease in the efficiency of CO_2-fixation (A), in the optimal (Fv/Fm) and effective (ΔF/Fm') quantum efficiency of electron transfer at PS II (Fig. 3). The decreases in these parameters are marked in hybrid and non-selected DH line. The paraquat-selected DH lines produced a higher cold tolerance in the early autotrophic phase of maize developments than the control plants (DH C and H). Significant cold tolerance was observed for R1, R3 and R5 DH lines (Table 3).

Discussion

Several environmental stresses, such as cold, drought, pathogen infection and herbicide action promote excess formation of reactive oxygen species (Apel and Hirt 2004). The common feature of these environmental stresses suggests a cross-tolerance between them, as demonstrated in several papers (Baker 1994; Leipner et al. 1999).

In the present paper, the hypothesis that an improved paraquat tolerance induces the tolerance against cold was tested in doubled haploid maize plants selected and regenerated from microspores exposed to paraquat. The paraquat tolerance of the progenies of selected lines was characterized by the loss of photosynthetic activity of PS II, chlorophyll bleaching and ion leakage from the cells of leaf discs treated with paraquat. The cold tolerance was determined during germination and at the early autotrophic phase of maize developments by measurements of germination properties of seeds and by the decrease in the photosynthetic activity of leaves exposed to low (T8°C) temperature.

As indicated by the decrease in Fv/Fm (Fig. 1), chlorophyll (*a*+b) content of leaves (Fig. 2A) and the ion conductivity of the solution in which the leaf discs were floated (Fig. 2B) lower rates of chlorophyll bleaching and membrane damage, but higher photosynthetic activity were detected upon exposure to paraquat stress in the leaves of paraquat-selected DH2 lines than either in the leaves of non-selected DH2 lines or in the original hybrid. The paraquat tolerance of the progenies of microspore-selected DH lines was 2-12 times higher than the control plants. However, higher paraquat tolerance is unlikely to be developed during *in vitro* selection due to either the genetic and methodological backgrounds of the DH technology or to the fact that the further increase in the selection pressure during the androgenic development of microspores results in high abortion of microspores and a decrease in the regeneration potential of microspore derived structures (Ambrus et al. 2006).

At low temperature, the germination potential of seeds of three paraquat-selected DH lines (R1, R2 and R5) was significantly higher than that of the control, non-selected DH plants and the hybrid (Table 2). This may result advantage during an early spring sowing. Moreover, as demonstrated by the lower decrease in the photosynthetic parameters (Fv/Fm, ΔF/Fm' and A) during the low temperature stress, the plantlets of paraquat-selected DH lines possess higher cold tolerance than control plants. This may as well contribute to the better development of maize at the early autotrophic phase under suboptimal temperature. However, these results, which were obtained under phytotron growing conditions, should be confirmed under field conditions.

In summary, our results demonstrated that the microspore-selected DH lines using paraquat as a ROS progenitors resulted not only higher tolerance against the paraquat-mediated oxidative damage, but help in the protection against the low temperature stress.

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