

# The relationship of antioxidant enzymes and some physiological parameters in maize during chilling

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## ABSTRACT

The changes in some physiological parameters of maize seedlings in response to chilling were studied. The emphasis was laid upon their relationship to chilling induced alterations in antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) activity. The exposure of maize seedlings to chilling caused substantial defects in the 4-day-old seedlings and the seedlings with two fully developed leaves, respectively. The membrane semipermeability perturbations and the loss of viability in the young seedlings were observed. Similarly, we found a decrease of chlorophyll content, appearance of necrotic lesions and inhibition of growth in older plants. The measurements of superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase activities provide an evidence of reactive oxygen species formation, that is assumed to be a reason of the found damages. Significant differences between two cultivars were found in the studied parameters. The electrolyte leakage and viability test provided effective methods for the characterization of the chilling tolerance-level in maize cultivars.

**Keywords:** chilling; maize (*Zea mays* L.); antioxidant enzymes; cell viability; electrolyte leakage; growth inhibition; chlorophyll

Due to its tropical origin, maize is sensitive to chilling temperatures. In higher geographical latitudes of cultivation where maize is a crop of increasing economic importance, maize seedlings are injured by cold air waves with temperatures below about 10°C, which often occur in the spring shortly after sowing.

There is a large amount of studies about the importance of chilling induced genes, hormones, enzymes and other organic substances in chilling tolerance (Allen and Ort 2001, Browse and Xin 2001). Accordingly, the plant chilling tolerance is a complex, based on a simultaneous and cumulative action of different biochemical mechanisms.

Chilling has been shown to induce many physiological symptoms, among them a decrease in photosynthetic activity (Leipner and Stamp 1998), growth inhibition, wilting, chlorosis and necrosis, in severe cases even plant death (Lee and Hwang 2003). The induction of reactive oxygen species (ROS) was observed during chilling stress (Leipner and Stamp 1998). Unless these ROS are efficiently metabolized, they rapidly oxidize and disintegrate membrane lipids, proteins, and other cellular components in stress conditions. This leads to cellular dysfunction, swelling and disorganiza-

tion of mitochondria and chloroplasts and can ultimately cause cell death, which is manifested by the appearance of necrotic lesions (Scandalios 1993, Kratsch and Wise 2000). On the other hand, stress induced lipid peroxidation and membrane perturbations were recently shown to have important roles in signalling, leading to expression of late embryogenesis abundant proteins and heat shock proteins (Blokchina et al. 2003).

The role of antioxidant defence in the chilling tolerance of maize has been intensively discussed in recent literature. And additionally, we showed that activities of some antioxidant enzymes partially correlate with the chilling sensitivity of maize cultivars and possess a significant importance in the chilling tolerance of maize (Takáč et al. 2003).

Therefore we found important to analyze the cell injury induced by chilling in association with antioxidant enzymes on 4-day-old seedlings and also the later stage of development.

The aim of our study was to clarify the relationship of chilling induced alterations in some physiological parameters of two maize cultivars. The association of these effects of chilling with our recent results dealing with antioxidant enzymes response could be beneficial.

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## MATERIAL AND METHODS

### Plant material

The seeds of two maize cultivars (*Zea mays* L., Ultra – cold sensitive and X0954D – cold tolerant, obtained from Pioneer) were germinated in rolls of moist filter paper for 4 days in the dark at 25°C.

One group of the seedlings was exposed to 6°C for 2 days in the dark and two-cm apical segments of primary roots were used for the analysis. Another group of the seedlings was cultivated in the plant growth chamber CONVIRON in the Hoagland nutrient solution at 22/18°C with the 16/8-hour light/dark periodicity, 70% air relative humidity, PAR 200  $\mu\text{mol}/\text{m}^2/\text{s}$  until the second leaf stage. Then the plants were subjected to 2 or 5 days of stress at 6/6°C with the 12/12-hour light/dark periodicity. The recovery treatment followed for another 4 days at 22/18°C.

### Assessment of chilling injury

The leakage of electrolytes from 2 cm root apical segments of 4-day-old seedlings was measured according to the modified method of Pinhero et al. (1999) using the conductivity meter, type OK-102/1. The viability of the root cells of maize seedlings was performed by immersing the root segments into the Evans blue solution (50 mg/100 ml of distilled water), according to Baker and Mock (1994).

The chlorophyll content and growth parameters were measured before the plants in the second leaf stage were transferred to chilling, two days and five days after their exposure to low temperature and after the recovery of the plants for four days in optimal temperatures. Total leaf chlorophyll content was determined by the method of Erdelský and Frič (1979) in the second fully developed leaf. The measurements of fresh (FW) and dry (DW) weight of the shoot and roots were used for the growth characterisation.

### Enzyme analyses

To measure the antioxidant enzymes activity of primary roots of 4-day-old maize seedlings, two-cm apical segments were harvested, grounded with a mortar and pestle in 0.1M sodium phosphate buffer (pH 7.5) containing 1mM EDTA, filtered and centrifuged at 15 000 g for 20 min. Supernatant was used as an enzymes source.

**Superoxide dismutase** (SOD, EC 1.15.1.1) activity was determined by monitoring the inhibition of the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at

560 nm as described by Madamanchi et al. (1994). One unit of SOD is defined as the extract volume required for 50% inhibition of MTT reduction. **Catalase** (EC 1.11.1.6) activity was measured by following the decrease in absorbance of  $\text{H}_2\text{O}_2$  at 240 nm (Aebi 1984), **ascorbate peroxidase** (APX, EC 1.11.1.11) activity similarly by following the decrease in the ascorbate concentration at 290 nm (Hossain and Asada 1984). To determine the activity of **glutathione reductase** (GR, 1.6.4.2) the increase of absorbance at 412 nm due to reaction of 5,5'-dithiobis 2-nitrobenzoic acid with GSH, as described by Smith et al. (1988), was followed. The results were expressed per protein content.

The protein content was determined according to Bradford (1976) using BSA as a standard.

The values are means of three independent experiments, each of the three replicates. The sample variability is given as the standard deviation of the mean. Significant differences between the means were calculated according to the Student's *t*-test.

## RESULTS AND DISCUSSION

The electrolyte leakage determination and viability test were used for the chilling injury quantification in 4-day-old seedlings. Both parameters showed substantial changes already in response to 2 days of chilling in the root segments of the maize seedlings (Table 1).

The leakage of ions from the root apical segments of the maize seedlings was nearly the same in both cultivars prior to the chilling. After two days of chilling the leakage increased 2-fold in Ultra and only slightly in X0954D (Table 1) indicating changes in the membrane semipermeability. Electrolyte leakage measurements are generally regarded as a useful tool for plants' cold tolerance estimation (Pinhero et al. 1999). Accordingly, the significant difference between cultivars in conductivity of root surrounding medium shows that cv. Ultra is more sensitive to chilling than cv. X0954D.

The alterations in membrane permeability involve changes in chemical composition of membranes including lipid peroxidation caused by ROS (De Santis et al. 1999). On the other hand, lipid peroxidation and membrane perturbations were recently shown to have important roles in signalling, leading to an expression of late embryogenesis abundant proteins and heat shock proteins (Blokhina et al. 2003). Additionally, Sangwan et al. (2002) showed that cold-activation of SAMK (stress induced MAP-kinase) in alfalfa cell cultures is mediated by membrane rigidification.

The measurements of cell viability showed a similar picture as an electrolyte leakage test (Table 1). The exposure of the seedlings to 2 days of chilling

Table 1. Leakage of ions (%) and viability (absorbance) of root apical segments of 4-day-old maize seedlings (cultivars Ultra and X0954D) exposed to 25 and 6°C

		Ultra	X0954D
Electrolyte leakage (% of total conductivity)	25°C	12.08 ± 0.25	11.66 ± 0.50
	6°C	26.47 ± 0.11**	13.15 ± 0.23
Viability (Abs)	25°C	0.13 ± 0.03	0.16 ± 0.06
	6°C	0.32 ± 0.01**	0.17 ± 0.04

\* $p \leq 0.05$ , \*\* $p \leq 0.01$

led to a significant increase in amount of dead cells in the root apical segments. Our results showed that a substantially higher volume of Evans blue was eluted from the root segments of the sensitive cultivar Ultra (Table 1). This is another evidence of different sensitivity of studied maize cultivars to chilling.

Lee and Hwang (2003) reported that except for the biotic stress, abiotic stresses induce the programmed cell death (PCD), too. Induction of ROS was shown to be in close association with signalling pathways and PCD in response to pathogens (Fath et al. 2001). Due to its complexity it is not clear on the base of our findings if the programmed cell death occurred in our experiment.

The formation of active oxygen species seems being a cause of the damage to the photosynthetic apparatus during the exposure to chilling in the light (Wise 1995, Leipner et al. 2000). If this is a matter also in the roots, we measured the changes in the activities of some antioxidant enzymes involving

superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase.

The activity of SOD significantly increased during the chilling, leading to generation of hydrogen peroxide in the Mehler reaction (Figure 1). Because of its destructive effect, hydrogen peroxide must be inevitably detoxified. Different mechanisms are known for  $H_2O_2$  scavenging, including catalase and enzymes of ascorbate-glutathione cycle (Willekens et al. 1997). However, we found that catalase and APX activities were not induced by chilling in the root segments of the young seedlings. The activity of GR substantially decreased in response to chilling. The low activity of APX and GR during the chilling could indicate that ascorbate-glutathione cycle is not induced in roots of young maize seedling in response to chilling. This indicates that in the roots of seedlings exposed to chilling for 2 days rapid accumulation of hydrogen peroxide appeared in agreement with Anderson et al. (1995). We assume that accumulation of hydrogen

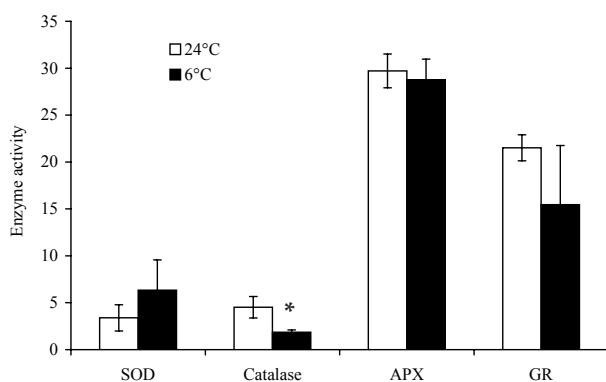


Figure 1. Activities of superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX) and glutathione reductase (GR) in primary root of young maize seedlings (cv. Ultra); SOD activity is expressed as U (1U = amount of sample required for 50% of NBT reduction), catalase ( $\mu\text{mol } H_2O_2/\text{min}/\text{mg}$  protein), APX ( $\mu\text{mol asc}/\text{min}/\text{mg}$  protein. $10^2$ ), GR ( $\mu\text{mol red. GSH}/\text{min}/\text{mg}$  protein. $10^3$ )

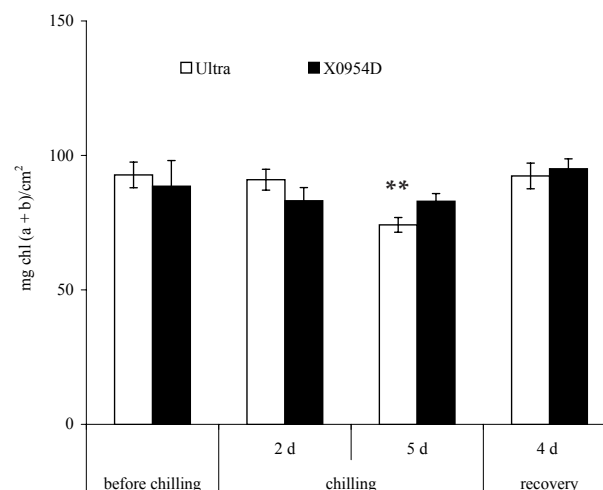


Figure 2. Total chlorophyll content ( $\text{mg}/\text{cm}^2$ ) of the second leaf of two maize cultivars Ultra (white bars) and X0954D (black bars) during chilling and recovery treatments

\*values are significantly different at the  $p \leq 0.05$   
\*\*values are significantly different at the  $p \leq 0.01$

Table 2. Shoot and root fresh (FW) and dry (DW) weights (g) of control and chilled maize cultivars (Ultra and X0954D) during chilling and recovery treatments

	Ultra				X0954D			
	control		chilled plants		control		chilled plants	
	FW	DW	FW	DW	FW	DW	FW	DW
<b>Shoot</b>								
Before chilling	1.31 ± 0.09	0.10 ± 0.01	1.34 ± 0.11	0.10 ± 0.05	0.82 ± 0.05	0.06 ± 0.00	0.81 ± 0.10	0.06 ± 0.01
2 days of chilling	1.83 ± 0.22	0.12 ± 0.01	1.47 ± 0.20	0.11 ± 0.02	1.19 ± 0.11	0.08 ± 0.01	0.99 ± 0.07	0.08 ± 0.01
5 days of chilling	2.79 ± 0.71	0.18 ± 0.04	1.87 ± 0.15	0.16 ± 0.01	1.62 ± 0.14	0.11 ± 0.01	1.05 ± 0.15	0.09 ± 0.01
Recovery	4.32 ± 0.82	0.27 ± 0.05	2.95 ± 0.43	0.21 ± 0.03	2.14 ± 0.28	0.15 ± 0.01	1.25 ± 0.16	0.10 ± 0.01
<b>Roots</b>								
Before chilling	0.82 ± 0.12	0.05 ± 0.01	0.88 ± 0.09	0.05 ± 0.01	0.54 ± 0.04	0.03 ± 0.00	0.52 ± 0.06	0.03 ± 0.00
2 days of chilling	1.18 ± 0.33	0.06 ± 0.01	0.95* ± 0.16	0.06 ± 0.01	0.77 ± 0.02	0.04 ± 0.00	0.65 ± 0.1	0.04 ± 0.00
5 days of chilling	1.60 ± 0.30	0.08 ± 0.02	1.12** ± 0.12	0.08 ± 0.01	0.90 ± 0.07	0.05 ± 0.00	0.64* ± 0.11	0.04 ± 0.01
Recovery	2.27 ± 0.39	0.11 ± 0.02	1.48 ± 0.19	0.09 ± 0.01	1.28 ± 0.12	0.06 ± 0.00	0.73 ± 0.07	0.05 ± 0.00

\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$

peroxide cause a membrane structure deprivation documented by electrolyte leakage and cells viability measurements.

It is generally known that plant tolerance to environmental factors increases during the plant development. We found that 2 days of chilling already caused a substantial damage to root segments in 4-day-old chilling-sensitive maize seedlings, while longer exposure is required for a considerable damage of tissues in older plants.

We recently reported that the ROS induction occurred also in latter stages of development (Takáč et al. 2003). The intensive accumulation of ROS that occurred after 5 days of chilling resulted in a decrease in the chlorophyll content (Figure 2), appearance of necrotic lesions and in inhibition of growth of plants.

Antioxidant enzymes protect the pigment apparatus at photosystem I from photooxidation during chilling (Tjus et al. 1998). Total chlorophyll content decreased mostly after 5 days of chilling in the leaves of both cultivars, but more intensively in Ultra seedlings (Figure 1). However, the chlorophyll content in both cultivars recovered attaining near the control levels within 4 days of post chilling. It seems that the induction of antioxidant enzymes activity in the leaves of both cultivars was insufficient for the protection of the photosynthetic pigment apparatus from photooxidation.

It is known that chilling in the light sensitize photosynthesis to photoinhibition (Xu et al. 1999), which results in bleaching of photosynthetic pigments (Nie and Baker 1991). A decrease in the chlorophyll content was suggested to reduce the absorption of potentially damaging light and therefore the risk of singlet oxygen formation is relatively lower (Leipner et al. 2000). Our results do not propose a regulative function for this reduction, because a reduction of chlorophyll content occurred in the leaf of the sensitive cultivar. An advanced research is necessary for definite statements. It seems that the induction of antioxidant enzymes activity in the leaves of the sensitive cultivar was insufficient for such protection.

Consequently, we wanted to elucidate the effects of chilling to the growth of maize seedlings.

Chilling substantially influenced the growth parameters of the shoot and roots in both cultivars. The shoot fresh and dry weight accumulation was inhibited already after 2 days of chilling mainly in the sensitive cultivar (Table 2). In addition, necrotic lesions were observed on the apical (distal) part of the leaf blade of both cultivars after 5 days of exposure to 6°C, but the extent of the lesions was markedly higher in the leaves of the sensitive cultivar (data not shown). Despite of the full recovery of total chlorophyll content, the accumulation of shoot and root fresh and dry weight did not fully

recover during the 4-day-long re-exposure of plants to the optimal grow temperature.

Thus the full recovery of the chlorophyll content did not affect the regeneration of growth after 4-day-long re-exposure to the optimal temperatures. Nie and Baker (1991) reported that although re-exposure to the optimal temperatures caused a rapid synthesis and accumulation of pigments and many proteins to levels close to those found in the leaves grown at 25°C, the leaves do not attain similar levels of photosynthetic competence. It is possible that the exposure to low temperature during early stages of leaf development restricts the synthesis of components essential for the thylakoid development, which cannot be made at later stages of leaf development when the temperature increases. Leipner et al. (2000) found a lower recovery of photosynthetic activity in maize seedlings after chilling exposition compared to the Fv/Fm ratio, chlorophyll content, and the deepoxidation status of the xanthophyll cycle. They suggest that a disturbance of the photosynthetic apparatus downstream of PSII might occur during the recovery phase. This observation, except for other factors influenced by chilling, might cause the incomplete recovery of growth parameters.

In conclusion we could summarize that chilling caused injury of membranes is an important factor limiting the growth of maize. The generation of ROS closely correlates with the electrolyte leakage, viability and total chlorophyll content and is an important component of the chilling tolerance in young and older maize seedlings respectively.

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## ABSTRAKT

### Vztah aktivity antioxidantických enzymů a některých fyziologických parametrů kukuřice během působení chladu

Studovali jsme vliv působení nízkých teplot na některé fyziologické parametry rostlin kukuřice a jejich vztah ke změnám aktivity antioxidantických enzymů (superoxiddismutázy, katalázy, askorbátperoxidázy a glutathionreduktázy). Z výsledků vyplývá, že chlad způsobil značné poškození 4-denních klíčenců a podobně i rostlin s plně vyvinutým druhým listem. Měřením úniku elektrolytů buněk apikálních kořenových segmentů a jejich viability jsme v podmínkách nízkých teplot zjistili změny v semipermeabilitě membrán a ztrátu viability buněk kořenových segmentů mladých klíčenců kukuřice po 2 dnech expozice klíčících rostlin. Byla pozorována nekrotická poškození listové čepele, pokles obsahu chlorofylu a inhibice akumulace čerstvé i suché hmotnosti nadzemní části a kořenů starších rostlin. Stanovení aktivity antioxidantických enzymů ukázalo, že v buňkách apikálních kořenových segmentů klíčenců došlo ke zvýšené akumulaci peroxidu vodíku, což pravděpodobně způsobilo jejich poškození. Výsledky ukázaly úzkou souvislost enzymatické antioxidantické obrany s pozorovanými alteracemi ve starších rostlinách.

**Klíčová slova:** chlad; kukuřice (*Zea mays* L.); antioxidantické enzymy; viabilita buněk; únik elektrolytů; inhibice růstu; chlorofyl

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