Eur Food Res Technol (2016) 242:1267–1276
DOI 10.1007/s00217-015-2630-8

ORIGINAL PAPER

The effects of cold-hardening and *Microdochium nivale* infection on oxidative stress and antioxidative protection of the two contrasting genotypes of winter triticale

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Received: 9 July 2015 / Revised: 17 December 2015 / Accepted: 24 December 2015 / Published online: 13 January 2016

Abstract Pink snow mould resulting from *Microdochium nivale* (Samuels and I.C. Hallett) regularly infects winter triticale (× *Triticosecale*, Wittm.). Therefore, in the present paper we aimed to identify biochemical markers potentially involved in the increased resistance *against M. nivale* of this valuable crop. In our experiment, we used two doubled haploid (DH) lines with the highest and the lowest cold-induced snow mould resistance, previously identified in cold chamber tests. The impact of cold-hardening (4 weeks at 4 °C) as well as covering plants with an artificial system to mimic snow covers and inoculation with *M. nivale* mycelium in leaves of seedlings, different genotypes on the thiobarbituric acid-reactive substance (TBARS) level and superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activities was assessed. Our studies indicate that, indeed, various genotypes differ in their response to the treatments in regard to the measured parameters. The leaf TBARS content was always lower in seedlings of snow mould-resistant DH1 line in comparison with susceptible DH92 ones, suggesting that tissue damage was more pronounced in plants of the second genotype. The activity of SOD and CAT had no correlation with the level of snow mould resistance. The cold-enhanced POX I activity was higher in DH1 seedlings under the snow-imitating covers with or without *M. nivale* inoculation in comparison with DH92 plants. Moreover, POX II activity was higher after cold-hardening in the resistant genotype. POX III activity was enhanced by abiotic stress in leaves of DH1 seedlings, while it was not detected in susceptible genotype in any experimental term.

Keywords Cereals · Cold-hardening · Fungal infection · Cross-tolerance · Antioxidant enzymes

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
Cu/ZnSOD	Copper/zinc superoxide dismutase
DH	Doubled haploid
MnSOD	Manganese superoxide dismutase
PAGE	Polyacrylamide gel electrophoresis
PR	Pathogenesis-related protein
POX	Peroxidase
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances

Introduction

Cereal's overwinterness depends on resistance to many stresses; among others, the tolerance to snow moulds is one of the most important factors determining survival in cold and moderate climates. *Microdochium nivale* (Samuels and I.C. Hallett) is a widely spread fungus causing pink snow mould of cereal seedlings [1]. It infects winter triticale (× *Triticosecale*, Wittm.), a man-made hybrid crop [2] with great yielding potential particularly in severe culture conditions. Variable levels of *M. nivale* infection in triticale were reported from field experiments and from testing at control

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conditions [3–5], but no fully resistant genotype was found. The fungicides used so far against *M. nivale* are very harmful to the environment and not sufficiently effective [6–8]. Only cold-hardening is regularly reported to induce increased resistance against pink snow mould [4, 5, 9–14], but its effect is genotype-dependent.

Despite many efforts made for dissecting the mechanisms underlying increased pink snow mould resistance, it is still uncertain which molecule serves as a cogent and easily measurable marker for screening a wide pool of cereal genotypes. A potential role in the defence response against *M. nivale* infection was previously proposed for unspecific catalase and peroxidase [10] as well as chitinase [14] in winter triticale; chitinase, endochitinase and 1,3–β glucanase in winter rye [15, 16]; and chitinase, endochitinase, 1,3–β glucanase, PR1-a protein, peroxidase [17] and thaumatin-like proteins in winter wheat [18]. Gołębiowska and Wędzony [4] proposed two model winter triticale cultivars for triticale–fungus interaction research: partly pink snow mould-resistant Hewo and susceptible Magnat. Without hardening, seedlings of both cultivars were completely damaged by *M. nivale* mycelium; when exposed to low temperature in cold chamber (4 weeks at 4 °C), they exhibited different levels of resistance. From F_1 hybrids of the described previously cultivars, a population of 92 doubled haploid (DH) lines was developed by an anther culture method [19]. Subsequently, we selected DH lines exhibiting transgression of resistance in relation to parental lines in three separate cold chamber tests, for further studies.

As reported by many authors, an excess of reactive oxygen species (ROS) produced in plant tissues subjected to abiotic and/or biotic stresses could be scavenged by their antioxidant system, consisting of non- and enzymatic antioxidants. Among them, several antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) were documented to facilitate cereal survival during both cold [20–26] and pathogen infection [9, 10, 27, 28]. The oxygen scavenging systems activated by cold-hardening play a key role in enhancing cold tolerance [29], especially those in chloroplasts and mitochondria, the major sites of ROS production in plant cells [30, 31]. Nevertheless, little is known about the responses of antioxidant systems in cereals infected by snow moulds and their role in improving *M. nivale* cold-induced tolerance in triticale. Therefore, in the present study we aimed to dissect the initial differences as well as the role of cold- and inoculation-induced changes in the activity of SOD, CAT and POX in leaves of pink snow mould-resistant triticale seedling and susceptible triticale seedling. As was suggested by Reuveni [32], the differences between susceptible and resistant plants are related to the rapidity and magnitude of the response rather than to the specificity of one or several possible defence mechanisms. In addition to that, we have attempted to identify a possible enzymatic marker for disease resistance.

Materials and methods

Plant material

Two winter hexaploid triticale (× *Triticosecale* Wittm., $2n = 6\times = 42$) doubled haploid (DH) lines: DH1, with the highest cold-induced snow mould resistance (the mean level of infection index $P = 38 \pm 10$ %) and DH92 with the lowest resistance ($P = 71 \pm 19$ %), were used in this study. They were selected according to the results of the preceding three cold chamber *M. nivale* resistance tests as described in Gołębiowska and Wędzony [4], performed in the frame of National Science Centre project No. N N310 140239. Additionally, 90 other DH lines were derived at the Department of Cell Biology of IPP PAS from F_1 hybrid of cross between model [4] winter triticale cultivars: snow mould-resistant Hewo (Strzelce Plant Breeding—IHAR Group Ltd., Poland) and susceptible Magnat (Danko Plant Breeders Ltd., Poland) by the anther culture method according to Wędzony [19]. Their homozygosity was confirmed with DArT and SSR markers as described in Tyrka et al. [33].

Plant growth conditions

The effect of cold-hardening

Well-formed kernels of DH1 and DH92 lines were surface-sterilized as described in Gołębiowska and Wędzony [4] and grown on filter paper soaked with sterile water in plastic Petri dishes at 26 °C in darkness for 2 days. Subsequently, healthy seedlings (24 per genotype) were planted in sterile mixture of soil, turf substrate and sand (2:2:1) in multipots. On day 7, plants reached one leaf trough coleoptile—growth stage Z10 in the Zadoks' scale [34], and they were supplemented once with Hoagland and Arnon's [35] sterile medium, 0.5 L per pot.

The six control seedlings (control) of each genotype were grown for 21 days in optimal conditions in a climatic chamber: light of 100 ± 10 μmol (photon) m^{-2} s^{-1} PAR, 8 h/16 h (day/night) photoperiod, temperature 16/12 °C and RH = 60–67 %. After this period, they reached the Z22 growth stage in the Zadoks' scale (seven leaves and two tillers). The remaining seedlings (18 per genotype), after 7 days in the above conditions, were subjected to the prehardening at 12/12 °C with 8/16 h (day/night) photoperiod for 14 days, followed by cold-hardening at 4/4 °C for 28 days in the same light regime. Plants after prehardening

had two unfolded leaves on main shoot, and a third leaf appeared (growth stage Z13 in the Zadoks' scale). After 49 days of growth, cold-hardened plants reached the same developmental stage considering the number of leaves and tillers as the control—non-hardened plants after growing in optimal conditions for 21 days.

The effect of Microdochium nivale infection

Hardened plants (12 per genotype) from the first part of the experiment were covered with moistened cellulose wadding on rolls and black plastic bags to imitate conditions occurring under a snow cover and then grown in darkness in the cold chamber at 4 °C for 21 days. One half of those plants was inoculated with fungal mycelium from monosporal isolate of *Microdochium nivale* 38z/5a/01 derived and used as described earlier [4], before applying the cover. The control plants were treated and grown under artificial covers as the inoculated ones except inoculation.

Plant sampling

For the present analyses, leaves of six seedlings from each genotype/treatment were collected. This included: (1) non-hardened, control plants; (2) cold-hardened plants; (3) cold-hardened plants with the artificial snow-imitating cover; (4) cold-hardened, inoculated plants with the artificial snow-imitating cover. Leaves were immediately frozen in liquid nitrogen and stored at −80 °C. Analysis was performed in three biological replicates for gel activity assays and four biological replicates for spectrophotometric analysis, consisting of four leaves each in a single growth experiment.

Determination of thiobarbituric acid-reactive substances (TBARS)

The extent of lipid peroxidation was assessed according to Gawronska et al. [36]. Leaf tissue (0.1 g) was homogenized in 1 mL 0.1 % trichloroacetic acid (TCA). Homogenates were centrifuged at 10 000×*g* for 5 min at 4 °C. The obtained supernatant was mixed 1:4 (v/v) with 0.5 % barbituric acid solution in 20 % TCA and incubated at 95 °C for 30 min, cooled on ice and centrifuged at 10 000×*g* for 5 min. The coloured complexes of TBARS (products of lipid peroxidation) were determined at 532 nm, and the non-specific absorption at 600 nm was subtracted. Extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} was used for TBARS concentration calculations.

Antioxidant enzymes activities

For extraction of SOD and CAT, 0.1 g plant material was homogenized in liquid nitrogen and re-suspended in cold phosphate buffer (0.1 M KH_2PO_4/Na_2HPO_4 pH 7.5, containing 3 mM ethylenediaminetetraacetic acid EDTA and 2 % (w/v) polyvinylpolypyrrolidone) in proportion 3:1 v/FW and 10:1 v/FW, respectively. For extraction of POX, 0.1 g plant material was homogenized in liquid nitrogen and re-suspended in cold 50 mM sodium acetate buffer pH 5.5 in proportion 3:1 v/FW. The homogenate was centrifuged at 12 000×*g* for 5 min at 4 °C, and supernatant was used for measuring antioxidant enzyme activity. Protein concentration was determined according to Bradford [37].

For determining the activity of SOD and POX, the fractions of soluble proteins isolated as described above were analysed using native polyacrylamide gel electrophoresis (PAGE) at 4 °C and a constant voltage 180 V and the Laemmli [38] buffer system without sodium dodecyl sulphate (SDS). Electrophoresis was performed for 60 min with the Bio-Rad Mini-Protean device.

To determine SOD activity, samples containing 6 µg of protein were loaded on 1-mm-width 12 % polyacrylamide gel and were separated by native PAGE in triplicate. We used 10-well combs of the 5.08 mm width and 44 µl maximum sample volume per well. SOD bands were visualized using the activity staining method described by Beauchamp and Fridovich [39]. All gels were incubated in staining buffer (potassium phosphate buffer pH 7.8, 10 mM EDTA, 28 mM tetramethylethylenediamine TEMED, 22 mM riboflavin and 0.25 mM nitro blue tetrazolium NBT) for 30 min in the dark at RT. After incubation, the gels were exposed to white light until SOD activity bands became visible (10 min). Identification of different SOD forms was based on their sensitivity to 3 mM KCN and 5 mM H_2O_2 added during activity staining according to Fridovich [40].

To determine POX activity, samples containing 8 µg of protein were loaded on 8 % polyacrylamide gel and separated by native PAGE. POX bands were visualized using the activity staining method described by Ros Barceló [41]. All gels were incubated in staining buffer (50 mM acetate-phosphate buffer pH 5, 2 mM 3,3′-diaminobenzidine DAB) for 20 min at RT. After incubation, 3 mM H_2O_2 was added and the gels were shaked gently until POX activity bands became visible (30 min).

For densitometric quantification, all gels were scanned under the identical standard settings of scanner (EPSON V7000) and the gel images were analysed using ImageJ program. The activity of all isoforms was expressed in arbitrary units corresponding to the area under the densitometric curve (the mean of three biological replicates ± SD).

CAT activity was determined according to Aebi [42]. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.1 mM EDTA, 0.04 % (v/v) H_2O_2 and 20 µl enzyme extract in 1 ml total volume. Decomposition of H_2O_2 was measured with 1 min at 240 nm. As a unit of enzyme activity, a decrease in absorbance equal to 0.0145

was assumed (consumption of 1 μM of H_2O_2). Extinction coefficient of H_2O_2 of 42.6 M^{-1} cm^{-1} was used. The spectroscopic analysis was performed using UV–visible recording spectrophotometer (UV–VIS Specord plus 210, Analytic Jena, Germany).

Statistics

Statistical significances were evaluated by analysis of variance and Duncan's test. Additionally, correlations between the antioxidants and infection index were calculated. Calculations were performed with STATISTICA® version 10.0 software at $P \leq 0.05$. When necessary, values were transformed logarithmically before applying a selected test.

Results

The products of lipid peroxidation were measured via a TBARS assay. In leaves of non-hardened plants of the resistant DH1 line (the mean level of infection index $P = 38 \pm 10$ %), the mean TBARS level was lower in comparison with values obtained for susceptible ($P = 71 \pm 19$ %) DH92 line (Fig. 1). After cold-hardening, the increase in TBARS level was observed in leaves of both genotypes studied, but it was higher in tissues from the susceptible line. Covering the plants with artificial snow-imitating covers did not change the TBARS level in leaves of both lines with respect to cold-hardened plants before covering (Fig. 1). However, inoculation caused the increase in the TBARS level in both genotypes, with a significantly higher rate in DH92 seedlings. The highest experimental level of oxidative damage (3.98 nmol TBARS/mg FW) was observed in leaves of DH92-inoculated seedlings (Fig. 1).

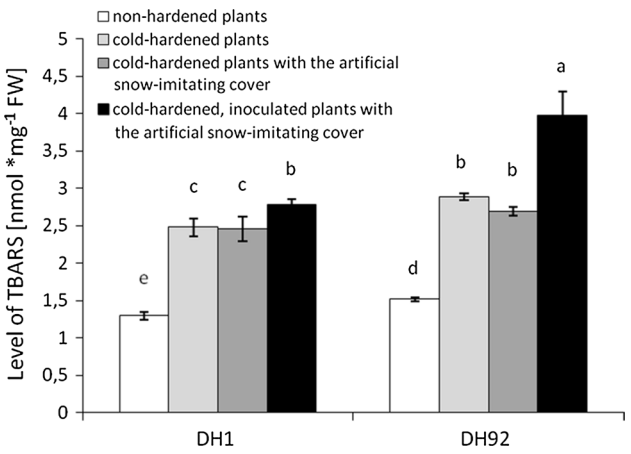

Fig. 1 Level of oxidative damage in leaves of the two genotypes (resistant DH1 and susceptible DH92) subjected to the three kinds of treatment: cold, cold + artificial system to mimic the snow cover and cold + artificial system to mimic the snow cover + inoculation with *M. nivale*. Values represent mean ± SD ($n = 4$). Letters above the *bars* indicate significant differences, according to the Duncan's test at $P \leq 0.05$

Two forms of SOD: manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (Cu/ZnSOD), were identified in extracts from leaves of both winter triticale DH lines (Fig. 2a, b). MnSOD activity was not found in non-hardened plants (Figs. 2, 3a). The exposure to low temperature led to an increase in MnSOD activity in both analysed genotypes, which was more pronounced in susceptible DH92 line (Fig. 3a). In hardened and covered plants, the MnSOD activity increased in comparison with cold-hardened plants before covering in leaves of both genotypes, but more pronounced in DH92 seedlings. Plant inoculation with fungal mycelium decreased MnSOD activity in both genotypes, in relation to a non-inoculated control. The activity of this SOD form was significantly lower in the leaves of resistant DH1 line in comparison with susceptible DH92 line in all treatments (Fig. 3a).

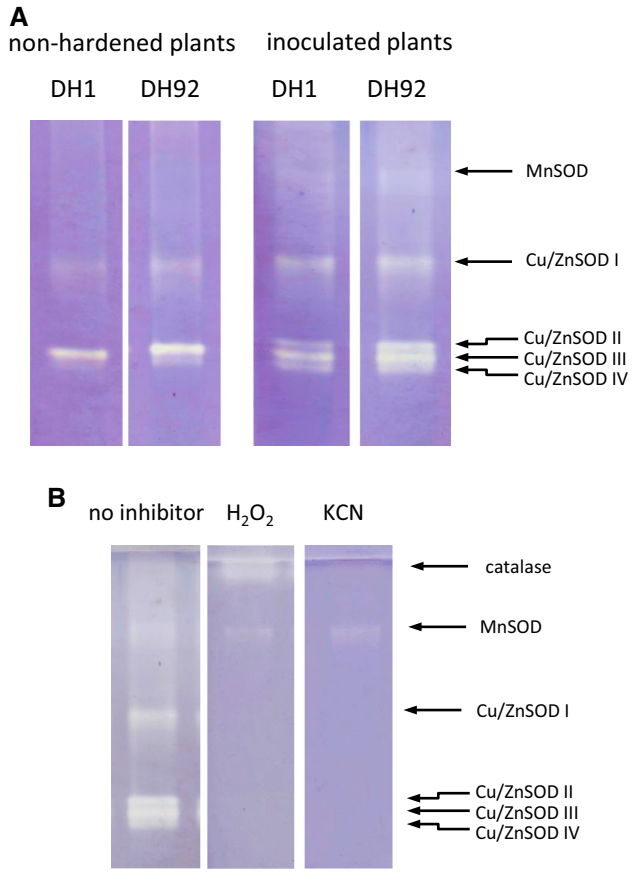

Fig. 2 SOD activity bands, as visualized by native PAGE, in resistant (DH1) and susceptible (DH92) genotype of winter triticale. Extracts containing 6 μg of soluble protein were loaded to each well (**a**). Identification of SOD forms stained with NBT in the presences of 5 mMH_2O_2 or mM KCN (**b**)

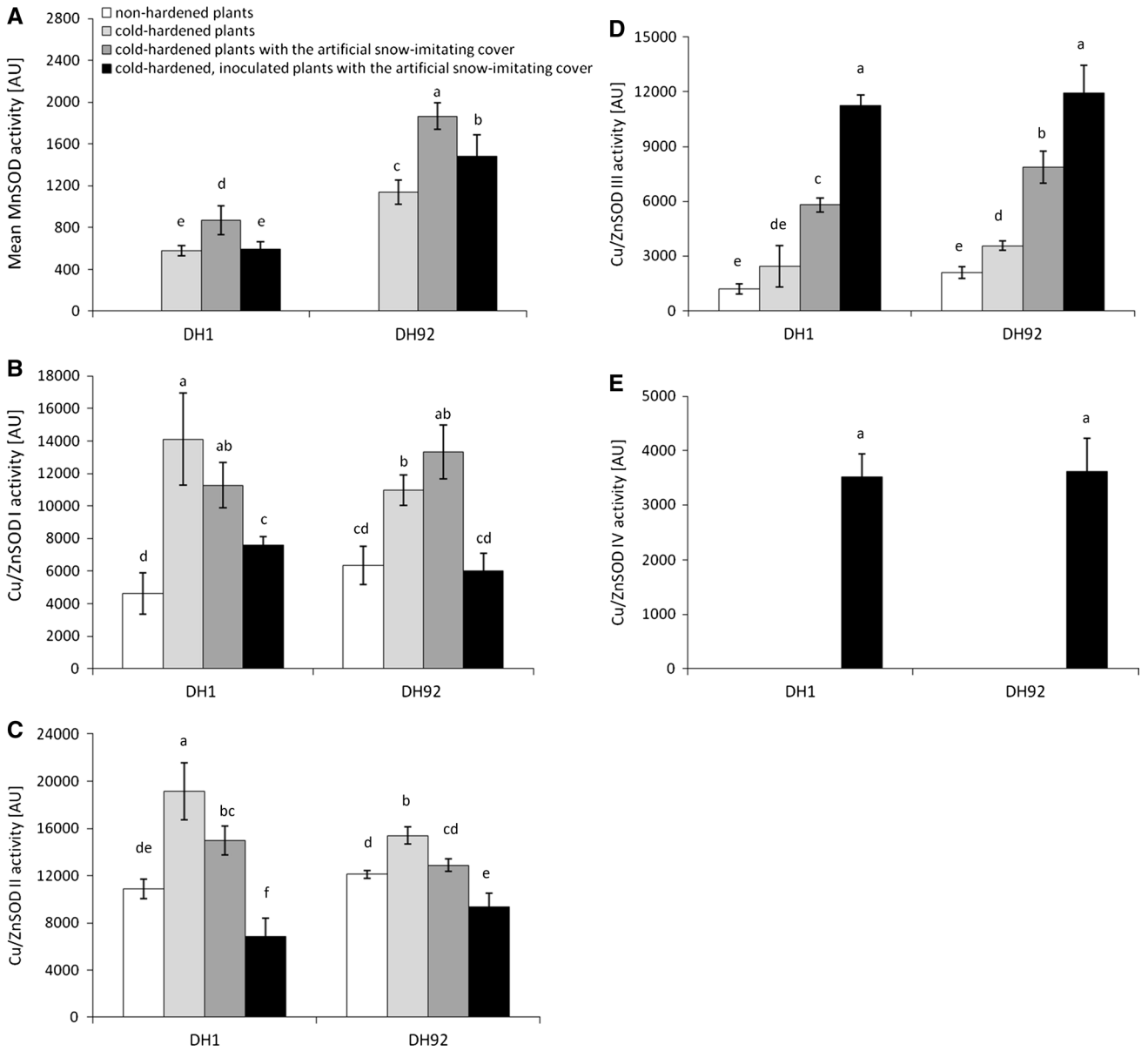

Fig. 3 Quantification of MnSOD (**a**), Cu/ZnSOD I (**b**), Cu/SOD II (**c**), Cu/SOD III (**d**) and Cu/SOD IV (**e**) activities in the two genotypes (resistant DH1 and susceptible DH92), cold + artificial system to mimic the snow cover + inoculation with *M. nivale*. Values represent mean ± SD ($n = 3$). Letters above the *bars* indicate significant differences, according to the Duncan's test at $P \leq 0.05$

In leaves of non-hardened, cold-hardened and covered plants of both DH lines, Cu/ZnSOD was represented by three isoforms, while in leaves of inoculated plants of both DH lines, Cu/ZnSOD was represented by four isoforms (Fig. 2a, b). In leaves of non-hardened plants of both lines, the mean activity of Cu/ZnSOD I was equal and increased after the exposition to low temperature in comparison with the non-hardened control (Fig. 3b). This increase was more prominent in resistant DH1 line. The Cu/ZnSOD I activity was not changed after plant covering, but the decrease after the exposition to biotic stress of *M. nivale* infection was noted in leaves of both DH lines with respect to cold-hardened and covered hardened plants (Fig. 3b).

Without hardening, the activity of Cu/ZnSOD II was equal in both genotypes studied (Fig. 3c). After cold-hardening, it increased in comparison with non-hardened plants of both genotypes. That change was more distinct in resistant DH1 line. Plant covering combined with inoculation caused a decrease in the activity of Cu/ZnSOD II in resistant genotype DH1 as well as in susceptible genotype DH92. In inoculated plants, the activity of Cu/ZnSOD II was higher in leaves of susceptible DH92 than in the resistant DH1 line (Fig. 3c).

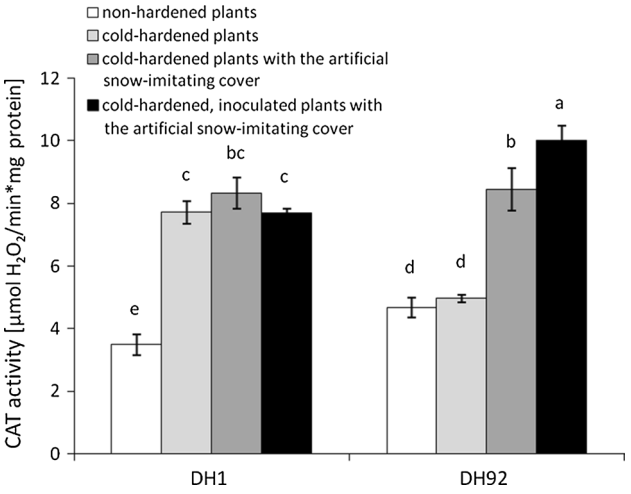

Fig. 4 CAT activity in leaves of the two genotypes (resistant DH1 and susceptible DH92) subjected to the three kinds of treatment: cold, cold + artificial system to mimic the snow cover and cold +artificial system to mimic the snow cover + inoculation with *M. nivale*. Values represent mean ± SD ($n = 4$). Letters above the *bars* indicate significant differences, according to the Duncan's test at $P \leq 0.05$

In leaves of non-hardened and cold-hardened plants, the activity of Cu/ZnSOD III was similar in both genotypes studied (Fig. 3d). Plant covering caused an increase in the activity of Cu/ZnSOD III in comparison with cold-hardened plants in leaves of both genotypes, but more prominent in DH92 seedlings. The highest activity of Cu/ZnSOD III was observed in leaves of inoculated seedlings of both genotypes (Fig. 3d). The Cu/ZnSOD IV activity was not found in extracts from leaves of non-hardened, cold-hardened and cold-hardened plants with the artificial snow-imitating cover (Fig. 3e). It was noted only in leaves of inoculated plants and was equal in both genotypes studied.

Differential induction of CAT activity was noticed between resistant and susceptible genotypes of winter triticale. In non-hardened plants of resistant DH1 line, the CAT activity was lower in comparison with values obtained for DH92 line (3.49 and 4.67 µmol H_2O_2 decomposed/minute/mg protein, respectively) (Fig. 4). Enzyme activity was enhanced with cold treatment for the resistant genotype, but not for the susceptible ones. Covered plants of the resistant DH1 line showed similar catalase activity. In contrast, plant covering combined with plant inoculation caused an increase in the activity of catalase in susceptible genotype DH92. The increase observed after inoculation in DH92 line was twofold in comparison with cold-hardened plants of this genotype before covering (Fig. 4).

Three isoforms of POX were identified in extracts from leaves of the resistant line, and two isoforms of POX were identified in extracts from leaves of the susceptible line of winter triticale (Fig. 5a). The investigated genotypes significantly differed in the activity of POX isoforms. Even in control conditions (non-hardened plants), the mean POX I activity was higher in leaves of resistant DH1 line than in DH92 ones (Fig. 5b). In extracts from leaves of both resistant DH1 and susceptible DH92 lines, POX I activity increased after cold-hardening. There was no change in POX I activity after both plant covering and inoculation of resistant DH1 line (Fig. 5b). In contrast, in leaves of the susceptible DH92 line, the decrease in activity of the enzyme was noted in covered cold-hardened plants. The activity was decreased after infection, in comparison with cold-hardened plants before covering. After plant covering, the POX I activity was higher in leaves of resistant DH1 line than in susceptible DH92 ones (Fig. 5b). In non-hardened and cold-hardened plants, the activity of POX II was higher in leaves of resistant DH1 than in the susceptible DH92 line (Fig. 5c). The activity of POX II did not significantly change after covering and inoculation in leaves of the resistant DH1 line. It was also not changed in DH92 leaves after inoculation, but it was increased in non-inoculated covered plants of this genotype in relation to the cold-hardened individuals (Fig. 5c). The POX III activity was not found in extracts from leaves of susceptible DH92 line (Fig. 5a, d). It was noted only in leaves of resistant DH1 line, in which was enhanced by cold and decreased by plant inoculation to the level observed in optimal conditions.

Discussion

In this study, we documented that both triticale genotype and seedling treatment influence the level of TBARS. Even in optimal conditions, the leaf TBARS level was lower in seedlings of snow mould-resistant DH1 line in comparison with susceptible DH92 ones. Also, cold- and inoculation-driven accumulation of TBARS was stronger in plants of the second genotype. This suggests that tissue damage was more pronounced in seedlings of the susceptible line, especially in leaves of infected seedlings. The level of TBARS is broadly used as a marker of oxidative stress in response to different abiotic and biotic factors [43–45]. The studies of fungal resistance of parental cultivars have shown that *M. nivale* behaves as a biotroph in the resistant triticale cv. Hewo and as a necrotroph in susceptible tissue of cv. Magnat [46]. Here, we suggest that a similar relationship may occur for the tested genotypes. The increase in the level of TBARS observed in seedlings of the susceptible line after infection may directly result from higher levels of ROS. Other authors also indicate that ROS are a virulence factor for necrotrophs [47, 48]. However, ROS are toxic not only to the fungal pathogen, but also to the plant cell structures. The imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates

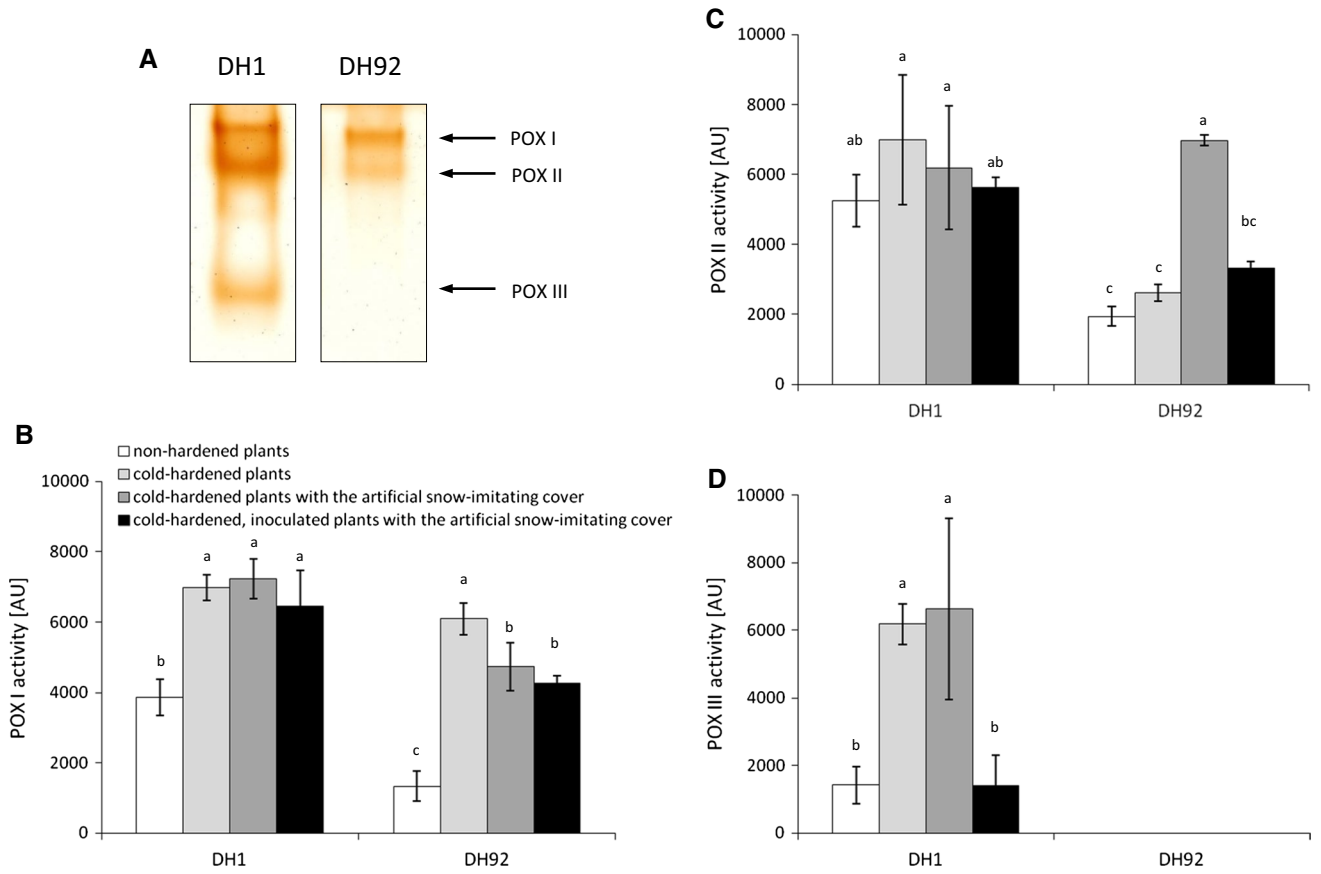

Fig. 5 POX activity bands, as visualized by native PAGE, in resistant (DH1) and susceptible (DH92) genotypes of winter triticale. Extracts containing 8 μg of soluble protein were loaded to each well (**a**). Quantification of POX I (**b**), POX II (**c**) and POX III (**d**) activities in the two genotypes (DH1 and DH92) subjected to the three kinds of treatment: cold, cold + artificial system to mimic the snow cover and cold +artificial system to mimic the snow cover + inoculation with *M. nivale*. Values represent mean ± SD ($n = 3$). Letters above the *bars* indicate significant differences, according to the Duncan's test at $P \leq 0.05$

results in oxidative stress and may lead to excessive molecular damage and tissue injury [49].

A significant role in the restriction of the peroxidation of membrane lipids is played by antioxidants, which remove ROS. In this study, we documented changes in SOD, CAT and POX activity in response to cold, covering with the artificial snow-imitating cover and infection of *M. nivale* in resistant and susceptible triticale seedlings. SOD is one of the most important enzymatic antioxidants in first line of defence against the toxic effects of ROS. In plants, three forms of SOD (MnSOD, FeSOD and Cu/ZnSOD) have been described and are classified according to the metal at the catalytic site and their subcellular location [50, 51]. In our research, two forms of SOD: manganese and copper/zinc superoxide dismutase, were identified in extracts from leaves of both winter triticale DH lines, but only the activity of MnSOD was induced by stress. The direction of changes in SOD activity of all SOD isoforms was similar in seedlings of both genotypes, but we observed statistically significant differences in activity of individual SOD isoforms between seedlings with different resistance to *M. nivale*. The activity of MnSOD isoform was higher in the leaves of susceptible DH92 line in comparison with resistant DH1 line in all treatments. This indicates higher participation of the mitochondrial ROS detoxifying pathways in the leaves of susceptible DH92 line. Cold-hardening resulted in increased activity of all Cu/ZnSOD isoforms in both genotypes studied. However, in leaves of seedlings of the resistant DH1 line the Cu/ZnSOD I and II activity was significantly higher after cold-hardening in comparison with DH92 ones. This result may suggest better adaptation to low temperature of the snow mould-resistant triticale genotype. Other authors also documented an increase in the SOD activity after cold acclimation of plants, e.g. in wheat [26] and tomato seedlings [52]. The Cu/ZnSOD III activity was enhanced by plant inoculation. Additionally, in seedlings of both genotypes the Cu/ZnSOD IV activity was recorded only in extracts of infected plants. This points out that these two isoforms may be involved in general defence against the pathogen.

According to the literature, increase in catalase activity may provide protection from oxidative damage by rapid removal of H_2O_2. Differential induction of CAT activity was noticed in resistant and susceptible genotypes of winter triticale. The CAT activity was enhanced with cold treatment for the DH1 genotype, but not for the DH92, what suggests a better antioxidative efficiency in the more resistant cultivar under the low temperature. This is in agreement with earlier results showing higher total CAT activity after 4 weeks of cold acclimation in parental cultivar Hewo, in contrast to Magnat [10]. The increase in CAT activity in cold-hardened plants of the frost resistant wheat genotype was evidenced also in Janda et al. [23] studies. In contrast, in our experiments, plant covering with the snow-imitating cover and when combined with inoculation caused an increase in the activity of catalase only in the susceptible genotype DH92. In infected hardened plants of this genotype, we also observed a significantly higher activity of the MnSOD isoform in comparison with resistant genotype.

Since CAT activity was not enhanced during infection in the resistant genotype DH1, other enzymes involved in free radical scavenging, such as peroxidases, were studied. Peroxidases are oxido-reductive enzymes that are implicated in several plant defence mechanisms. One of the important physiological roles of POXs is the synthesis of cell-wall polymers (lignin and suberin), which constitute physical barriers for both biotic and abiotic stresses [53, 54]. We observed a positive relationship between resistance and peroxidase activity in genotypes of winter triticale. Even in control—optimal conditions, the activity of all POX isoforms was higher in leaves of resistant DH1 line than in DH92 ones, in contrast to the relation observed for SOD and CAT activity. POX I activity was also higher in DH1 seedlings after infection with *M. nivale*; however, it was equal after cold-mediated increase, in comparison with DH92 plants. Enhanced POX activity has been correlated with resistance in winter triticale [10], winter wheat [17], common bean [55], rice [56] and tomato [57] following inoculation with pathogens. The activity of POX was also used as a marker for localized and systemic acquired responses in plants challenged by a pathogen [58]. The total unspecific POX activity was higher both after 4 weeks of hardening in 4 °C and 10 days post-*M. nivale* inoculation in leaves of resistant triticale seedlings compared to the susceptible ones [10]. Our results seem to be different from those cited above. In the resistant genotype, POX I activity was significantly enhanced only by cold and later maintained on the same level under the artificial snow-imitating covers with or without plant inoculation. Moreover, POX II activity was similar in all experimental terms in the leaves of resistant DH1 line and it was higher after cold-hardening with respect to susceptible DH92 line. POX III activity was increased only by abiotic stress in leaves of DH1 seedlings. Stress did not affect POX activity in susceptible genotype.

Most of higher plants possess a large number of POX isoenzymes, which are encoded by multigene families [59]. A diversity of reactions catalysed by POXs as well as a large number of their genes suggests a possible functional specialization for each isoform [60]. The earlier results of studies carried out by Reuveni and co-workers [32, 61] suggested that POX activity is a biochemical marker, which may or may not be part of the resistance mechanism, but can be used to predict resistance to disease. In Takashima et al. [62] research, cationic peroxidase was related to basal resistance of *Betula platyphylla* var. *japonica* against canker-rot fungus *Inonotus obliquus*. POX activity was increased in wheat cultivars expressing resistance to the leaf rust fungus [63]. Here, we propose that POX isoform activity is worth to be evaluated in a larger set of lines varying in their level of resistance/susceptibility in a follow-up study, complemented with analysis with different number of hours/days post-inoculation.

Acknowledgments The authors are grateful to E. Niewiadomska (The Franciszek Górski Institute of Plant Physiology at the Polish Academy of Sciences in Kraków) for helpful discussions and critical reading of this manuscript. This work was financed from the Polish National Science Centre Project No. N N310 140239.

Compliance with ethical standards

Conflict of interest Both authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

References

1. Tronsmo AM, Hsiang T, Okuyama H, Nakajima T (2001) Low temperature diseases caused by *Microdochium nivale*. In: Iriki N, Gaudet DA, Tronsmo AM, Matsumoto N, Yoshida M, Nishimune A (eds) Low temperature plant microbe interactions under snow. Hokkaido National Agricultural Experimental Station, Sapporo, pp 75–86
2. Ryöppy PH (1997) Haploidy in triticale. In: Mohan Jain S, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, Vol. 4 - Cereals (5). Springer, Netherlands, pp 117–131
3. Hudec K, Bokor P (2002) Field patogenicity of *Fusarium culmorum*, *Fusarium equiseti* and *Microdochium nivale* on triticale. Physiol Plantarum 115:101–110

4. Gołębiowska G, Wędzony M (2009) Cold-hardening of winter triticale (× *Triticosecale* Wittm.) results in increased resistance to pink snow mould *Microdochium nivale* (Fr., Samuels and Hallett) and genotype-dependent chlorophyll fluorescence modulations. Acta Physiol Plant 31:1219–1227
5. Szechyńska-Hebda M, Wędzony M, Tyrka M, Gołębiowska G, Chrupek M, Czyczyło-Mysza I, Dubas E, Żur I, Golemiec E (2011) Identifying QTL for cold-induced resistance to *Microdochium nivale* in winter triticale. Plant Genet Resour 9:296–299
6. Nielsen LK, Justesen AF, Jensen JD, Jørgensen LN (2013) *Microdochium nivale* and *Microdochium majus* in seed samples of Danish small grain cereals. Crop Prot 43:192–200
7. Leroux P, Lanen C, Fritz R (2006) Similarities in the antifungal activities of fenpiclonil, iprodione and tolclofos-methyl against *Botrytis cinerea* and *Fusarium nivale*. Pestic Sci 36:255–261
8. Bateman GL (2008) Control of seed-borne *Fusarium nivale* on wheat and barley by organomercury seed treatment. Ann Appl Biol 83:245–250
9. Szechyńska-Hebda M, Wasek I, Gołębiowska-Pikania G, Dubas E, Żur I, Wędzony M (2015) Photosynthesis-dependent physiological and genetic crosstalk between cold acclimation and cold-induced resistance to fungal pathogens in triticale (*Triticosecale* Wittm.). J Plant Physiol 177:30–43
10. Gołębiowska G, Wędzony M, Płażek A (2011) The responses of pro- and antioxidative systems to cold-hardening and pathogenesis differs in triticale (× *Triticosecale* Wittm.) seedlings susceptible or resistant to pink snow mould (*Microdochium nivale* Fr., Samuels & Hallett). J Phytopathol 159:19–27
11. Dubas E, Golebiowska G, Zur I, Wedzony M (2011) *Microdochium nivale* (Fr., Samuels and Hallett): cytological analysis of the infection process in triticale (× *Triticosecale* Wittm.). Acta Physiol Plant 33:529–537
12. Gaudet DA, Wang Y, Frick M, Puchalski B, Penniket C, Ouellet T, Robert L, Singh J, Laroche A (2011) Low temperature induced defence gene expression in winter wheat in relation to resistance to snow moulds and other wheat diseases. Plant Sci 180:99–110
13. Nakajima T, Abe J (2011) Environmental factors affecting expression of resistance to pink snow mold caused by *Microdochium nivale* in winter wheat. Can J Bot 74:1783–1788
14. Żur I, Gołębiowska G, Dubas E, Golemiec E, Matušíková I, Libantová J, Moravčiková J (2013) β-1,3-glucanase and chitinase activities in winter triticales during cold hardening and subsequent infection by *Microdochium nivale*. Biologia 68:241–248
15. Hiilovaara-Teijo M, Hannukkala A, Griffith M, Tu XM, Pihakaski-Maunsbach K (1999) Snow-mould-induced apoplastic proteins in winter rye leaves lack antifreeze activity. Plant Physiol 121:665–673
16. Yeh S, Moffatt BA, Griffith M, Xiong F, Yang DSC, Wiseman SB, Sarah F, Danyluk J, Xue YQ, Hew CL, Dotherty-Kirby A, Lajoie G (2000) Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiol 124:1251–1263
17. Ergon Å, Klemsdal S, Tronsmo AM (1998) Interactions between cold hardening and *Microdochium nivale* infection on expression of pathogenesis-related genes in winter wheat. Physiol Mol Plant P 53:301–310
18. Kuwabara C, Takezawa D, Shimada T, Hamada T, Fujikawa S, Arakawa K (2002) Abscisic acid an antifungal activity against snow mould, *Microdochium nivale*. Physiol Plantarum 115:101–110
19. Wędzony M (2003) Protocol for anther culture in hexaploid triticale (× *Triticosecale* Wittm.). In: Małuszynski M et al (eds) Doubled haploid production in crop plants. A manual. Kluwer Academic Publisher, Dordrecht, pp 123–128
20. Scebba F, Sebustiani L, Vitagliano C (1999) Protective enzymes against activated oxygen species in wheat (*Triticum aestivum* L.) seedlings: responses to cold acclimation. J Plant Physiol 155:762–768
21. Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K (2002) Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot 53:1305–1319
22. Baek KH, Skinner DZ (2003) Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. Plant Sci 165:1221–1227
23. Janda T, Szalai G, Leskó K, Yordanova R, Apostol S, Popova LP (2007) Factors contributing to enhanced freezing tolerance in wheat during frost hardening in the light. Phytochemistry 68:1674–1682
24. Janmohammadi M, Enayati V, Sabaghnia N (2012) Impact of cold acclimation, de-acclimation and re-acclimation on carbohydrate content and antioxidant enzyme activities in spring and winter wheat. Icel Agric Sci 25:3–11
25. Sasheva P, Yordanova R, Janda T, Szalai G, Maslenkova L (2013) Study of primary photosynthetic reactions in winter wheat cultivars after cold hardening and freezing. Effect of salicylic acid. Bulg J Agric Sci 19:45–48
26. Li X, Cai J, Liu F, Dai T, Cao W, Jiang D (2014) Cold priming drives the sub-cellular antioxidant systems to protect photosynthetic electron transport against subsequent low temperature stress in winter wheat. Plant Physiol Bioch 82:34–43
27. Ivanov S, Miteva L, Alexieva V, Karjin H, Karanov E (2004) Alterations in some oxidative parameters in susceptible and resistant wheat plants infected with *Puccinia recondita* f.sp. *tritici*. J Plant Physiol 162:275–279
28. Király L, Barna B, Király Z (2007) Plant resistance to pathogen infection: forms and mechanisms of innate and acquired resistance. J Phytopathol 155:385–396
29. Thakur P, Nayyar H (2013) Facing the cold stress by plants in the changing environment: sensing, signalling, and defending mechanisms. In: Tuteja N, Gill SS (eds) Plant acclimation to environmental stress. Springer Science + Business Media, New York, pp 29–69
30. Jacoby RP, Li L, Huang S, Pong Lee C, Millar AH, Taylor NL (2012) Mitochondrial composition, function and stress response in plants. J Integr Plant Biol 54:887–906
31. Crosatti C, Rizza F, Badeck FW, Mazzucotelli E, Cattivelli L (2013) Harden the chloroplast to protect the plant. Physiol Plantarum 147:55–63
32. Reuveni R (1995) Biochemical markers for disease resistance. Molecular methods in plant phathology. CRC Press, Boca Raton, p 523
33. Tyrka M, Bednarek PT, Kilian A, Wędzony M, Hura T, Bauer E (2011) Genetic map of triticale compiling DArT SSR and AFLP markers. Genome 54(5):391–401
34. Zadoks JC, Chang TT, Konzak CF (1974) A decimal code for the growth stages of cereals. Weed Res 14:415–421
35. Hoagland DR, Arnon DI (1938) A water culture method for growing plants without soil. California Agricultural Experiment Station, Circular 347
36. Gawronska K, Romanowska E, Miszalski Z, Niewiadomska E (2013) Limitation of C3–CAM shift in the common ice plant under high irradiance. J Plant Physiol 170(2):129–135
37. Bradford MM (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
38. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
39. Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287
40. Fridovich I (1986) Superoxide dismutases. Adv Enzymol RAMB 58:61–97

41. Ros Barceló A, Muñoz R, Sabater F (1987) Lupin peroxidases. I. Isolation and characterization of cell wall-bound isoperoxidase activity. Physiol Plantarum 71:448–454
42. Aebi H (1984) Catalase in vitro. In: Packer L (ed) Methods in Enzymology, 105. Academic press, New York, pp 121–126
43. Salehi M (2013) Antioxidant activity and oxidative stress due to salinity in triticale and wheat lines in field condition. J Plant Process Funct 1(2):38–49
44. Chandra N, Pandey N (2014) Sulfur stress-induced antioxidative responses in leaves of *Triticum aestivum* L. Acta Physiol Plant 36:2079–2089
45. Sheng H, Zeng J, Yan F, Wang X, Wang Y, Kang H, Zhou Y (2015) Effect of exogenous salicylic acid on manganese toxicity, mineral nutrients translocation and antioxidative system in polish wheat (*Triticum polonicum* L.). Acta Physiol Plant 37(2):1–11
46. Szechyńska-Hebda M, Hebda M, Mierzwiński D, Kuczyńska P, Mirek M, Wędzony M, Karpiński S (2013) Effect of cold-induced changes in physical and chemical leaf properties on the resistance of winter triticale (× *Triticosecale*) to the fungal pathogen *Microdochium nivale*. Plant Pathol 62(4):867–878
47. Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature 411(6839):826–833
48. Mengiste T (2012) Plant immunity to necrotrophs. Annu Rev Phytopathol 50:267–294
49. Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930
50. Slesak I, Libik M, Karpinska B, Karpinski S, Miszalski Z (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochimica Polonica-English Edition 54(1):39
51. Szechyńska-Hebda M, Karpiński S (2013) Light intensity-dependent retrograde signalling in higher plants. J Plant Physiol 170(17):1501–1516
52. Zhou J, Wang J, Shi K, Xia XJ, Zhou YH, Yu JQ (2012) Hydrogen peroxide is involved in the cold acclimation-induced chilling tolerance of tomato plants. Plant Physiol Biochem 60:141–149
53. Roberts E, Kutchan T, Kolattukudy PE (1988) Cloning and sequencing of cDNA for a highly anionic peroxidase from potato and the induction of its mRNA in suberizing potato tubers and tomato fruits. Plant Mol Biol 11:15–26
54. Quiroga M, Guerrero C, Botella MA, Barcelo Â, Amaya I, Medina MI, Alonso FJ, de Forchetti SM, Tigier H, Valpuesta V (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiol 122:119–1127
55. Leite ME, Bosco dos Santos J, Ribeiro PM Jr, Araujo de Souza D, de Castro A, Lara L, Vilela de Resende ML (2014) Biochemical responses associated with common bean defence against *Sclerotinia sclerotiorum*. Eur J Plant Pathol 138:391–404
56. Joe MM, Islam MR, Karthikeyan B, Bradeepa K, Sivakumaar PK, Sa T (2012) Resistance responses of rice to rice blast fungus after seed treatment with the endophytic *Achromobacter xylosoxidans* AUM54 strains. Crop Prot 42:141–148
57. Nikraftar F, Taheri P, Rastegar MF, Tarighi S (2013) Tomato partial resistance to *Rhizoctonia solani* involves antioxidative defense mechanisms. Physiol Mol Plant Pathol 81:74–83
58. Mohammadi M, Kazemi H (2002) Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. Plant Sci 162:491–498
59. Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. Plant Cell Physiol 42:462–468
60. Chang IH, Cheng KT, Huang PC, Lin YY, Cheng LJ, Cheng TS (2012) Oxidative stress in greater duckweed (*Spirodela polyrhiza*) caused by long-term NaCl exposure. Acta Physiol Plant 34:1165–1176
61. Shimoni M, Bar-Zur A, Reuveni R (1991) The association of peroxidase activity and resistance of maize to *Exserohilum turcicum*. J Phytopathol 131(4):315–321
62. Takashima Y, Suzuki M, Ishiguri F, Iizuka K, Yoshizawa N, Yokota S (2013) Cationic peroxidase related to basal resistance of *Betula platyphylla* var. *japonica plantlet* No. 8 against canker-rot fungus *Inonotus obliquus* strain IO-U1. Plant Biotech 30:199–205
63. Southerton SG, Deverall BJ (1990) Changes in phenylalanine ammonia-lyase and peroxidase activities in wheat cultivars expressing resistance to the leaf-rust fungus. Plant Pathol 39:223–230