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# Germinability and seed biochemical properties of susceptible and non–target site herbicide-resistant blackgrass (*Alopecurus myosuroides*) subpopulations exposed to abiotic stresses

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## Research Article

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## Abstract

Quantifying the level of ecophysiological, biochemical, and agronomical fitness of herbicide-resistant (R) and herbicide-susceptible (S) weeds is useful for understanding the evolutionary development of herbicide resistance, but also for implementing herbicide-resistance management strategies. Although germination is a key fitness component in the life cycle of weeds, germinability of S and R weeds has rarely been evaluated under stressful conditions. Germinability traits of S and non–target site resistant subpopulations of blackgrass (*Alopecurus myosuroides* Huds.) sharing closely related genetic background were tested under salinity, drought stress, and accelerated seed-aging (i.e., exposed to 100% relative humidity at 45 C from 0 to 134 h) conditions. In addition, the activity of three antioxidant enzymes and protein concentration of accelerated aged seeds of the subpopulations were studied. There were no differences in maximum seed germination ($G_{max}$) and time to 50% germination between the two subpopulations under optimum conditions. However, under salinity, drought stress, and accelerated aging conditions, there were differences between the subpopulations. The salinity, drought, and accelerated aging treatments reducing $G_{max}$ of the S subpopulation by 50% were 18 dS $m^{-1}$, 0.75 MPa, and 90 h, respectively, while for the R subpopulation the corresponding values were 15 dS $m^{-1}$, 0.66 MPa, and 67 h. No differences were found in the activity of the antioxidant enzymes and the content of protein between non-aged seeds of the subpopulations. The aging treatments reducing the activity of catalase and superoxide dismutase enzymes by 50% were 118 and 82 h for the S subpopulation, respectively, while they were 54 and 58 h for the R subpopulation. In contrast, there were no differences in the effect of the aging treatments on the peroxidase activity and protein content between subpopulations. The results provided clear evidence that the non–target site resistant loci of blackgrass is associated with fitness costs under environmental stress.

## Introduction

Overreliance on herbicides, the most cost-effective method for weed control (Matzrafi et al. 2016), has resulted in a global problem with herbicide-resistant weed species. From the first reports of herbicide-resistant weed species in 1957 to the 1970s, only three herbicide resistance cases were reported (Hilton 1957; Switzer 1957), but since then, the number of cases has increased dramatically, with about 12 unique herbicide-resistant cases per year (Heap 2019).

Blackgrass (*Alopecurus myosuroides* Huds.) is one of the most common annual grass weeds in winter crops in Northwestern Europe, and has been reported in 37 countries (Holm et al. 1997). Being a very competitive weed species (Maréchal et al. 2012), *A. myosuroides* can reduce yields of winter wheat (*Triticum aestivum* L.) up to 45% (Vizantinopoulos and Katranis 1998). The infestation of farms by *A. myosuroides* has increased due to the adoption of agricultural practices such as reduced tillage or no-till, continuous cropping of winter cereals (Colbach and Dürr 2003), and cessation of stubble burning. It is considered one of the 15 most important herbicide-resistant weeds worldwide (Heap 2019), and the most critical herbicide-resistant weed in Europe (Lutman et al. 2013). Resistance to seven different sites of action (acetyl CoA carboxylase [ACCase] inhibitors [A/1], acetolactate synthase [ALS] inhibitors [B/2], photosystem II [PSII] inhibitors [C1/5 and C2/7], mitosis inhibitors [K1/3 and K3/15], and fatty-acid and lipid biosynthesis inhibitors [N/8]). Both target-site resistance (TSR) and non–target site resistance (NTSR) mechanisms have been reported in *A. myosuroides* populations (Heap 2019; Keshtkar et al. 2015).

According to general ecological theories, it is anticipated that herbicide-resistant weeds will be less fit than the wild type in the absence of herbicide, that is, resistance is associated with ecological and or physiological fitness cost; the so-called costs of adaptation (Vila-Aiub et al. 2009). It is worth noting that resistance to herbicides may not always have a fitness penalty (Ghanizadeh and Harrington 2019). Inconsistent results from studies on the fitness of herbicide-resistant weeds and, in some cases, positive fitness (Wang et al. 2010) and neutral fitness penalties (Vila-Aiub et al. 2014) have been found.

One of the most common flaws in fitness studies is the lack of control of genetic background of plant material, that is, using plant material with dissimilar genetic background in fitness studies, which can lead to inconclusive results (Dang et al. 2019; Keshtkar et al. 2019). Regardless of this flaw, it was reasoned that variations in growth characteristics between resistant (R) and susceptible (S) plants should not be described as fitness cost (Cousens and Fournier-Level 2018), because fitness is determined by the number of offspring a genotype produces and contributes to the next generation or growing season (Cousens and Fournier-Level 2018; Primack and Hyesoon 1989). Plant characteristics that determine fitness, that is, germination, dormancy, phenology, establishment, growth rate, pollination, seed size, seed yield per plant, biomass production, adaptation to the environment, and competition with neighboring plants, are all highly important, as they affect final fitness (Holt 1990; Warwick and Black 1994). Importantly, knowledge about each fitness component can not only be used as a tool in herbicide-resistance management programs but can also be applied to predict frequency of R and S alleles in populations. Hence, it is important to increase our knowledge about fitness components of R and S plants from seed to seed under different environmental conditions, as suggested by Vila-Aiub et al. (2009).

Seed germination is the most sensitive and critical stage in the life cycle of plants, especially for annual weeds (Keshtkar et al. 2009). However, seed germination has rarely been considered in fitness studies (Vila-Aiub et al. 2009). Germination is a complicated process affected by genetic and environmental factors. Soil properties (pH, temperature, light, water, salinity, fertility, air quality), tillage (i.e., burial depth), and surface residue are factors that influence seed germination (Forcella et al. 2000; Tang et al. 2015). In a previous study, the effect of sowing depth and temperature was studied on an NTSR *A. myosuroides* subpopulation, and it was found that seedling emergence of the R subpopulation was lower and slower than that of the S subpopulation at low temperature and deep burial (Keshtkar et al. 2017a). On the other hand, there were no vegetative and fecundity fitness costs in the NTSR *A. myosuroides* subpopulation, either grown alone or in competition with winter wheat (Keshtkar et al. 2017b).

Salinity and drought stress are two major abiotic stresses to seed germination traits (Forcella et al. 2000; Uddin et al. 2016). Seed longevity, seed dormancy, and germination ability of seeds affect the success of annual weed species in agricultural ecosystems (Gundel et al. 2008). Therefore, knowledge about the ecophysiological mechanisms of seed longevity and seed germination traits is essential to predict how long weed seeds can persist in the seedbank (Long et al. 2015). This information will be important for the management of S and R weed biotypes.

Based on a general premise, it is hypothesized that under stressful environmental conditions, fitness costs would be exacerbated. This study evaluated seed biochemical and physiological processes of the same S and NTSR *A. myosuroides* subpopulations under stressful abiotic conditions, including salinity, drought stresses, and accelerated aging.

## Materials and Methods

### Plant Material

A previously characterized NTSR population of *A. myosuroides*, population ID914, that evolved resistance to different herbicide sites of action was used in this study (Keshtkar et al. 2015). The population developed different levels of resistance to different herbicide groups, including groups A/1 (fenoxaprop-P-ethyl), B/2 (flupyrsulfuron-methyl-sodium), K1/12 (pendimethalin), and N/8 (prosulfocarb) (Keshtkar et al. 2015). Neither the parent plants nor the subpopulations carried any of the 7 and 12 known point mutations conferring TSR to ALS and ACCase inhibitors, respectively (Keshtkar et al. 2015, 2017a). The S and R subpopulations were selected within the parent population via a plant-cloning technique called the "single population approach," as previously described (Keshtkar et al. 2017a, 2017b; Pedersen et al. 2007; Vila-Aiub et al. 2005). The single population approach is one of the suggested methods for controlling the genetic background of plant materials (Keshtkar et al. 2019; Vila-Aiub et al. 2011). The subpopulations were grown under the same greenhouse conditions but separated for seed production ($F_1$) by pollen-proof nets. Subsequently, the $F_1$ generations were grown to produce more seeds ($F_2$) under similar conditions. The nondormant $F_2$ seeds were kept at constant low temperature (4 C) until the beginning of the experiments.

### Seed Germination under Salinity and Simulated Drought Stresses

#### Salinity Assay

Fifty similar-sized seeds of both subpopulations were placed on two layers of filter paper (Whatman No. 1) in 9-cm glass petri dishes. To reduce water evaporation, the petri dishes were kept in plastic bags. The petri dishes were placed in a growth cabinet adjusted to 14/10-h day/night photoperiods. A photosynthetic photon flux density of 175 μmol $m^{-2}$ $s^{-1}$ was provided by cool-white fluorescent lamps. The salinity concentrations, including 0, 4, 8, 12, 16, 20, and 24 dS $m^{-1}$, were obtained using sodium chloride. Germination tests were initiated by adding 7 ml of each saline solution to each petri dish. The tests were carried out under two different temperature regimes: 17/10 C and 10/5 C (day/night). The two different temperature regimes represent early (September) and late (October) sowing times for winter cereals in the autumn in northwestern Europe. Seeds having a visible and normal radicle were recorded as germinated and removed from the petri dishes. The number of germinated seeds was recorded until no new germination was observed for three consecutive observation times. There were four petri dishes (replication) per treatment, and the experiment was conducted twice.

#### Simulated Drought Stress Assay

Fifty similar-sized seeds of both subpopulations were exposed to seven water potential (ψ) levels (i.e., drought stress levels) including 0, −0.2, −0.4, −0.6, −0.8, −1, and −1.2 MPa under the same temperature regimes as the salinity experiment. Polyethylene glycol 6000 (PEG 6000) was added to distilled water to establish the ψ treatments (Michel and Kaufmann 1973). The drought stress experiments followed the same protocol described for the salinity experiments.

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### Biochemical and Germination Traits of Seeds Exposed to Accelerated Seed-Aging Test

#### Germination Assay

Twenty-five similar-sized seeds from each subpopulation were exposed to approximately 100% relative humidity at 45 C for different durations: 0 (untreated seeds), 24, 48, 72, 96, 120, 144, and 168 h. The accelerated aging treatments were chosen based on a series of preliminary treatments leading to 100% seed deterioration (0% germination) when seeds were subjected to the aging treatment for 168 h.

Accelerated aging treatments were conducted by placing a 3-L plastic cubic container filled with 2 L of distilled water in an oven set at a temperature of 45 C. Paper bags containing seeds were placed in the container floating on top of the water. Immediately after termination of the artificial aging treatments, the germinability test was started for all treatments. The germination test was carried as described above under 10/17 C. The experiment was repeated three times with four replications per treatment.

### Biochemical Assays

#### Enzyme and Protein Extraction

Seeds of the R and S subpopulations were exposed to four different accelerated aging timings (0, 36, 84, and 132 h) under approximately 100% relative humidity at 45 C. The timings were chosen based on the results of the accelerated aging test. There were three replications per treatment. A combination of methods described by Giannopolitis and Ries (1977), Vanacker et al. (1998), and Siminis et al. (1994) was used to extract protein and enzymes from 200 mg of seed of each of the treated subpopulations. The seeds were ground in a mortar containing liquid nitrogen. Then the samples were homogenized in 1.5 ml of extraction buffer containing 0.1 M Tris (tromethamine salt), 0.23 M sucrose, 1% (w/v) polyvinylpyrrolidone, 4 mM β-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM KCl, 10 mM $MgCl_2$, 0.2% Triton X-100, 1 mM henylmethylsulfonyl fluoride, and 1% PEG 4000. Finally, the homogenized samples were centrifuged at 12,000 rpm for 20 min at 4 C, and the supernatant was used for the antioxidant enzyme assays, including catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), peroxidase (POD, EC 1.11.1.7), and total protein content.

#### CAT Assay

Activity of CAT was measured spectrophotometrically, according to the method described by Beers and Sizer (1952) with minor modifications, where CAT decomposes $H_2O_2$ to water. The crude enzyme extract (supernatant), stored at −80 C, was placed in an ice bath. Then, 130 µl of enzyme extract was added to a reaction mixture consisting of 100 mM phosphate buffer (pH 7.0) and 3.41 mM $H_2O_2$ (3%) in a 3-ml quartz cuvette cell. The absorbance was read at a wavelength of 240 nm to determine the decrease of absorbance.

#### SOD Assay

Activity of SOD was evaluated spectrophotometrically by quantifying the capacity of enzymes inhibiting photochemical reduction of nitroblue tetrazolium (NBT), as suggested by Beauchamp and Fridovich (1971). The crude extract enzyme (supernatant), stored at −80 C, was placed in an ice bath. The reduction of NBT was measured by adding 30 µl of enzyme extract into a cuvette cell filled with a reaction mixture comprising 50 mM potassium phosphate buffer (pH 7.8), 134.03 mM methionine, 5.372 mM EDTA, 2.4 mM NBT, and 2 µM riboflavin. The cuvettes containing the reaction mixture were illuminated by a fluorescent lamp for 16 min. A reaction mixture without the crude enzyme extract was also exposed to light and considered the nontreated control, while the crude enzyme extract kept in the dark was considered a blank. The absorbance was measured against a blank sample using a spectrophotometer (CECIL model 3000, Cambridge, UK) at a wavelength of 560 nm (Shariat et al. 2017). Under illuminated conditions, the reduction of NBT was measured in the presence and absence of the enzyme extract. The SOD activity was calculated by subtracting the absorbance of the nontreated control sample from the absorbance of the sample containing the reaction mixture. The amount of enzyme required for 50% inhibition of NBT photoreduction to blue formazan, that is, 50% reduction in color intensity, was defined as one unit of SOD enzyme (Giannopolitis and Ries 1977).

#### POX Assay

The activity of POX was determined by adding 20 µl of enzyme extract to a reaction mixture consisting of 50 mM phosphate buffer (pH 6.0), 0.5% pyrogallol, and 0.5% $H_2O_2$ (Kar and Mishra 1976). The production of purpurogallin was determined by the increase in absorbance at 420 nm at 25 C.

#### Protein Content

The method described by Bradford (1976) was used to determine the protein concentration of the crude extracts (supernatant). The sample absorbance was read at the wavelength of 595 nm using a spectrophotometer (CECIL model 3000) at a wavelength of 595 nm. Bovine serum albumin was used as a standard.

### Statistical Analysis

#### Germination Assays

A slightly modified version of a new analytical approach, recently described and applied by Jensen et al. (2017), was used to analyze the data. At first, biologically meaningful parameters, including final germination and speed of germination, were calculated using a three-parameter log-logistic model adopting an event-time approach, by fitting the model (Equation 1) to the cumulative germination of each petri dish (replication) (Ritz et al. 2013):

$$E(t) = \frac{d}{1 + \exp[b(\log(t) - \log(T_{50}))]} = \frac{d}{1 + \left(\frac{t}{T_{50}}\right)^b} \quad [1]$$

where $E$ is the accumulative germination at time $t$, $d$ is upper limit parameter representing the germination percent of the total number of seeds (also referred to as $G_{max}$) used in each petri dish, $T_{50}$ is the time to 50% of the maximum germination ($d$), and $b$ is the slope of the curve at $T_{50}$ denoting the rate of increase in emergence.

In a second step, the $d$ (final germination, $G_{max}$) and the $T_{50}$ (speed of germination) parameters, obtained in the first step for each petri dish, were regressed against treatment (drought stress levels, salinity concentrations, and accelerated aging times). For the $d$ parameter, a three-parameter log-logistic model (Equation 2, described by Streibig et al. [1993]) was used:

$$E(X) = \frac{d}{1 + \exp[b(\log(X) - \log(X_{50}))]} \quad [2]$$

where $E$ is the final germination at treatment $X$; $X$ is the treatment level; $d$ is the upper limit parameter representing the final germination of nontreated seeds, that is, at the nonstress condition

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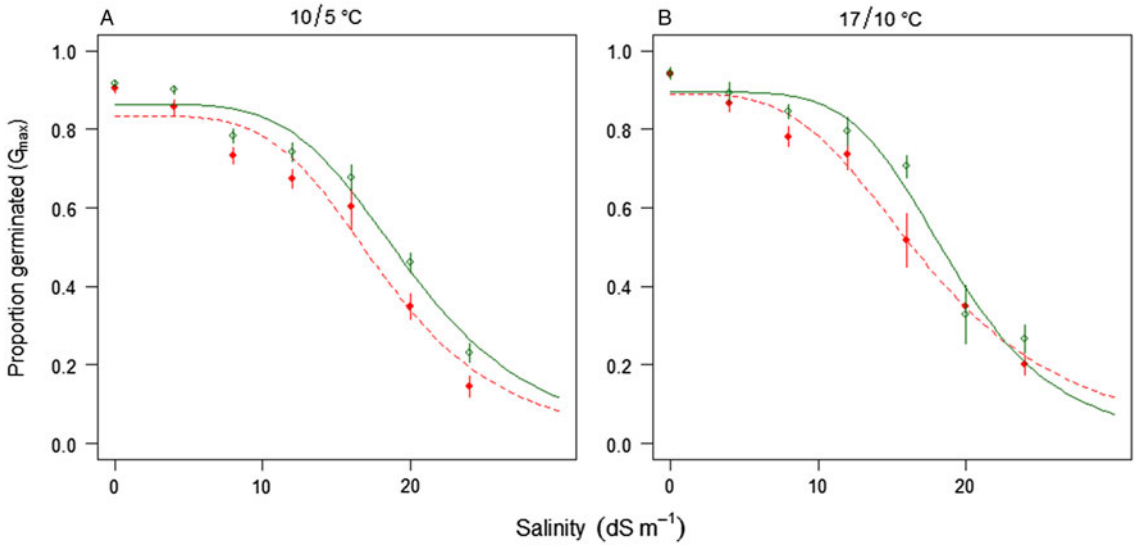


**Figure 1.** Influence of salinity concentrations on germination pattern of herbicide-resistant (R; ···•···) and herbicide-susceptible (S; —◇—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The germination pattern of subpopulations in response to salinity concentrations at (A) suboptimal (10/5 C) and (B) optimal (17/10 C) temperature regimes is shown. The symbols are the mean seed germination at each salinity concentration obtained from two separate experiments. Vertical bars represent standard error of the mean.

($X = 0$); $X_{50}$ is the treatment level reducing the maximum germination ($d$) by 50%; and $b$ is the slope of the curve at $X_{50}$.

For the $T_{50}$ parameter, a linear regression (Equation 3) was fit to the data:

$$E(X) = a + bX \quad [3]$$

where $E$ is the speed of germination at treatment $X$, $X$ is the treatment level, $a$ is the intercept (the speed of germination when $X = 0$), and $b$ is the slope of the line.

Then, the parameters of Equation 3 (a and b) and Equation 2 ($d$ and $X_{50}$), estimated in the second step, were analyzed using a meta-analytic random-effects model (Jensen et al. 2017) in which experiment number was considered as random effect while subpopulations and temperature regimes were considered fixed effects. Finally, as the last step, appropriate pairwise comparisons using Tukey's test were made to compare the parameters where a Bonferroni-type correction was used for multiple comparisons.

### *Biochemical Assays*

The three-parameter log-logistic model (Equation 2) was fit to the data, where $E$ is activity of enzymes and protein content; $d$ is the upper-limit parameter representing the maximum activity of enzymes and protein content of nontreated seeds, that is, at the nonstress condition (no accelerated aging treatment); $X_{50}$ is the accelerated aging time (hours) reducing 50% of the $d$ (i.e., $Aging_{50}$ [hours]); and $b$ is the slope of the curve at $X_{50}$. The estimated parameters for the R and S subpopulations were compared using a $t$-test.

The three-parameter log-logistic model, the linear regression model, the multiple comparisons, and the meta-analytic approach were carried out with the add-on packages DRC (Ritz et al. 2015), STATS, MULTCOMP (Hothorn et al. 2008), and METAFOR (Viechtbauer 2010), respectively, in the R statistical software (R Core Team 2018).

## Results and Discussion

### *Effect of Salinity and Simulated Drought Stresses on Germination Traits*

The final germination (the $d$ parameter; hereafter referred to as $G_{max}$) of both subpopulations decreased with increasing salinity levels under two different temperature regimes (Figure 1A and B). No statistical difference ($P > 0.05$) was found in the $G_{max}$ between the R and S subpopulations in the nonstress condition (i.e., no salinity stress) at either high- or low-temperature regimes (Figure 1; Table 1). However, there were differences ($P < 0.001$) between the two subpopulations at $X_{50}$, also referred to as $Salinity_{50}$ (dS m$^{-1}$), at the high-temperature regime. This means $Salinity_{50}$ for the S subpopulation was significantly higher than for the R subpopulation at the high-temperature regime, while there was no difference at the low-temperature regime (Table 1).

Time to 50% of final germination ($T_{50}$ parameter) increased as the seeds were exposed to increasing salinity levels under both temperature regimes (Figure 2A and B). As expected, the highest germination speed (i.e., lowest $T_{50}$) was observed in the nonstress condition under both temperature regimes. Importantly, no difference ($P > 0.05$) was found in $T_{50}$ between the R and the S subpopulations in the nonstress condition at any of the temperature regimes (Figure 2; Table 2). The temperature significantly increased $T_{50}$ of both subpopulations at the lower temperature ($P < 0.001$). The average $T_{50}$ of the two subpopulations doubles at the low temperature compared with the high temperature (approximately 60 and 135 h, respectively) at no salinity stress.

As shown in Figure 2, there was a linear relationship between $T_{50}$ and the salinity levels for both subpopulations at both temperature regimes. At the low-temperature regime, each unit of increase in salinity level increased the $T_{50}$ of the S subpopulation less (1.9 h) than that of the R subpopulation (3.5 h) (Table 2). However, at the high-temperature regime, the subpopulations showed a similar response to salinity. On average, at high temperature, each unit of increase in salinity level increased $T_{50}$ by 5.8 h.

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**Table 1.** Germination of herbicide-resistant (R) and herbicide-susceptible (S) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population in response to different salinity, drought, and aging treatments under different temperature regimes.[a]

| | $G_{max}$[b] | | | | | | $X_{50}$[c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature 10/17 C | | | Temperature 5/10 C | | | Temperature 10/17 C | | | Temperature 5/10 C | | |
| Treatment | S | | R | S | | R | S | | R | S | | R |
| Salinity | 89 (1.56) | NS[d] | 87 (1.78) | 88 (1.39) | NS | 85 (1.35) | 18 (0.44) | e | 15 (0.46) | 21 (0.46) | NS | 20 (0.44) |
| Drought | 93 (1.29) | NS | 95 (1.44) | 90 (1.12) | NS | 89 (1.15) | 0.75 (0.073) | e | 0.66 (0.073) | 0.91 (0.073) | e | 0.83 (0.073) |
| Aging | 88 (1.34) | NS | 90 (1.58) | nd | | nd | 90 (2.38) | e | 67 (2.37) | nd | | nd |

[a]The values are the mean of parameters measured from two or three experiments and calculated by a meta-analytic random-effects model. Values in parentheses represent standard error of the mean. nd, not determined.
[b]The accumulative germination at nonstress condition, i.e., without salinity, drought, and aging treatment, *d* parameter × 100 (Equation 2).
[c]The treatment level, i.e., salinity, drought, and aging reducing 50% of the parameter *d*.
[d]Abbreviation: NS, nonsignificant.
[e]Significant at 0.001 level.

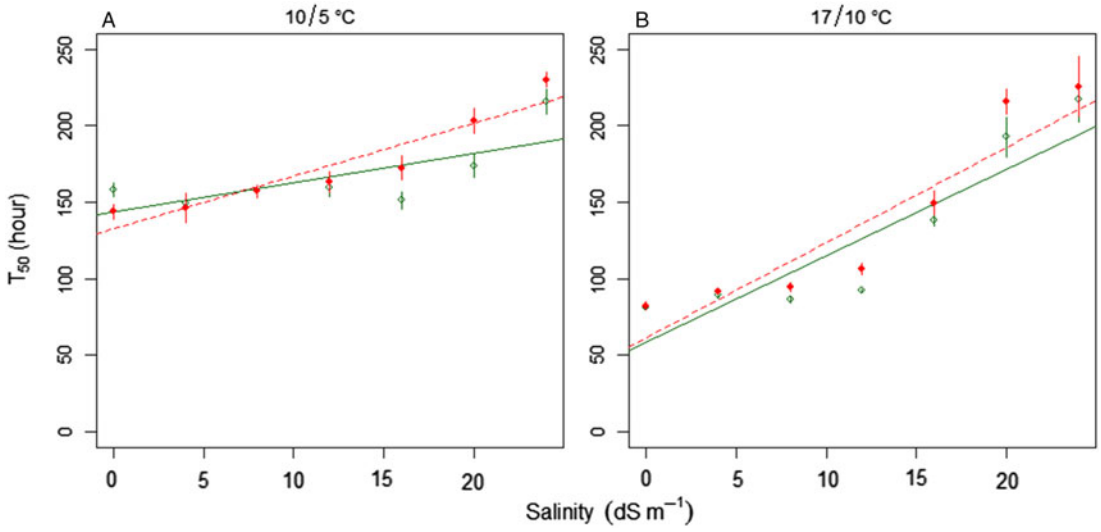


**Figure 2.** Influence of salinity concentrations on the speed of germination ($T_{50}$, time to 50% of final germination) for herbicide-resistant (R; ···•···) and herbicide-susceptible (S; —○—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The $T_{50}$ of subpopulations in response to salinity concentrations at (A) suboptimal (10/5 C) and (B) optimal (17/10 C) temperature regimes is shown. The symbols are the mean of germination speed at each salinity concentration obtained from two separate experiments. Vertical bars represent standard error of the mean.

Similar to the salinity experiment, the $G_{max}$ of both subpopulations declined with increasing drought stress at the two temperature regimes (Figure 3A and B). The $G_{max}$ values for the subpopulations were similar under the nonstress condition ($\psi = 0$) at both temperature regimes (Figure 3; Table 1). No seed germination was observed at the $\psi = -1.2$ MPa for any of the subpopulations. At low temperature, the subpopulations had a lower $G_{max}$ than at the higher temperature, but similar to the salinity experiment, there were no differences ($P > 0.05$) between temperature regimes. More importantly, at the $\psi$ level reducing the $G_{max}$ by 50%, also referred to as $Drought_{50}$ or $\psi_{50}$ MPa, a difference ($P < 0.05$) was observed between the two subpopulations. The S subpopulation tolerated drought stress better ($\psi_{50} = -0.91$ MPa) than the R subpopulation ($\psi_{50} = -0.83$ MPa) (Table 1). This trend was also observed in the high-temperature regime (Table 1).

Similar to the results of the salinity experiment, the time to 50% of final germination ($T_{50}$ parameter) increased as drought stress increased at both temperature regimes (Figure 4A and B). A linear relationship was observed between $T_{50}$ and the $\psi$ levels. The $T_{50}$ could not be estimated at $\psi = -1.2$ MPa, as no germination was recorded in any scenario. As expected, the lowest $T_{50}$, that is, the highest germination speed, was observed at optimal conditions at both temperature regimes. Importantly, no differences ($P > 0.05$) were found between the a and *b* regression parameters of the R and the S subpopulations at any of the temperature regimes (Figure 4; Table 2). Hence, the R and the S subpopulations showed similar responses to the drought stress. It should, however, be noted that the R subpopulation tended to be more sensitive to drought stress than the S subpopulation. For instance, at the low temperature, the slope of the curve was 94 and 65 for the R and S subpopulations, respectively. On the other hand, the R subpopulation germinated around 30 h later than the S subpopulation at $\psi = -1$ MPa. The low-temperature regime reduced speed of germination by 50% (Figure 4A and B). On average, for the two subpopulations under the nonstress condition, $T_{50}$ was ca. 70 and 130 h for the low- and high-temperature regimes, respectively.

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**Table 2.** Germination speed (time to 50% of final germination) of herbicide-resistant (R) and herbicide-susceptible (S) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population in response to different salinity, drought, and aging treatments under different temperature regimes.[a]

| | *a*[b] | | | | | | *b*[c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Temperature 10/17 C | | | Temperature 5/10 C | | | Temperature 10/17 C | | | Temperature 5/10 C | | |
| Treatment | S | | R | S | | R | S | | R | S | | R |
| Salinity | 61 (9.3) | NS[d] | 63 (9.2) | 143 (8.3) | NS | 131 (8.0) | 5.6 (0.50) | NS | 6.0 (0.51) | 1.9 (0.38) | e | 3.5 (0.34) |
| Drought | 68 (5.2) | NS | 71 (6.9) | 133 (5.5) | NS | 131 (6.6) | 65 (9.6) | NS | 94 (12.8) | 77 (9.8) | NS | 103 (11.7) |
| Aging | 83 (10.4) | NS | 87 (7.6) | nd | | nd | 1.4 (0.18) | NS | 1.5 (0.16) | nd | | nd |

[a]The values are the mean of parameters measured from two or three experiments and calculated by a meta-analytic random-effects model. Values in parentheses represent standard error of the mean.
[b]Speed of germination (hour) under nonstress conditions (i.e., without salinity, drought, and aging), i.e., intercept of linear model (Equation 3).
[c]Slope of the linear model estimated under different levels of salinity, drought, and aging stresses.
[d]Abbreviations: NS, nonsignificant; nd, not determined.
[e]Significant at 0.01 level.

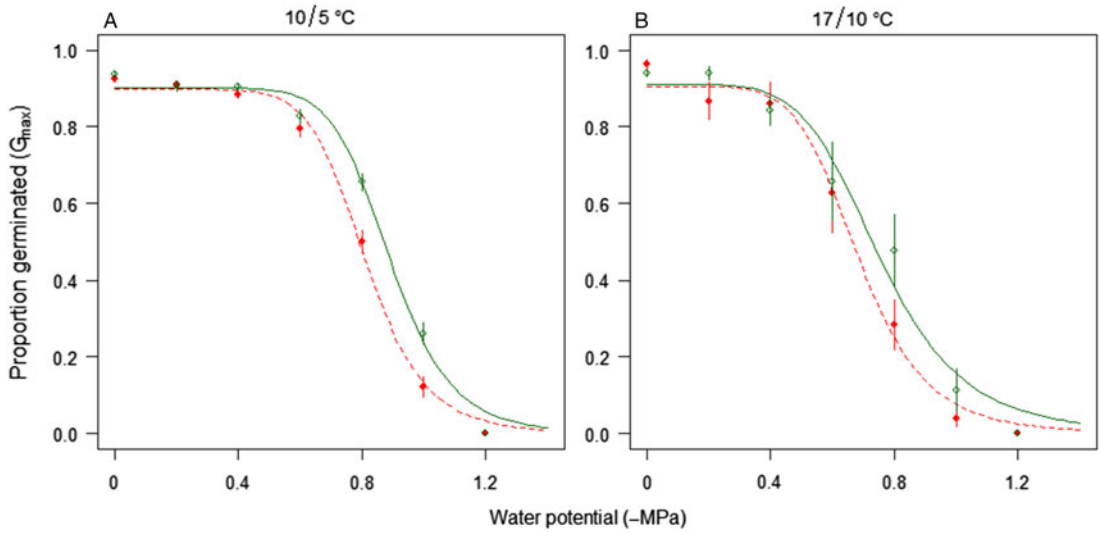


**Figure 3.** Influence of water potential (ψ) levels on germination pattern of herbicide-resistant (R; ···●···) and herbicide-susceptible (S; —○—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The germination pattern of subpopulations in response to ψ at (A) suboptimal (10/5 C) and (B) optimal (17/10 C) temperature regimes is shown. The symbols are the mean seed germination at each ψ level obtained from two separate experiments. Vertical bars represent standard error of the mean.

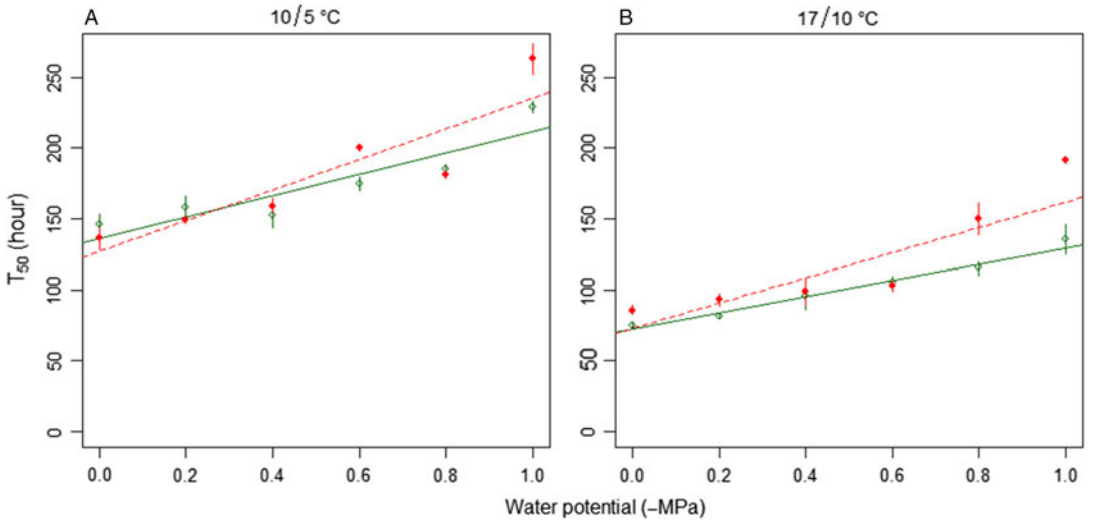


**Figure 4.** Influence of water potential (ψ) levels on the speed of germination ($T_{50}$, time to 50% of final germination) for herbicide-resistant (R; ···●···) and herbicide-susceptible (S; —○—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The $T_{50}$ of subpopulations in response to ψ levels at (A) suboptimal (10/5 C) and (B) optimal (17/10 C) temperature regimes is shown. The symbols are the mean of germination seed at each ψ level obtained from two separate experiments. Vertical bars represent standard error of the mean.

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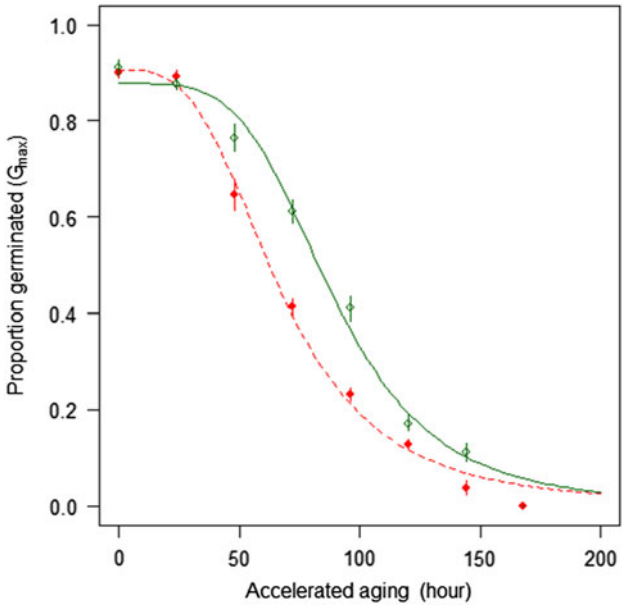


**Figure 5.** Influence of accelerated aging treatments on germination pattern of herbicide-resistant (R; ···•···) and herbicide-susceptible (S; —○—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The symbols are the mean seed germination in response to each artificial aging treatment obtained from three separate experiments. Vertical bars represent standard error of the mean.

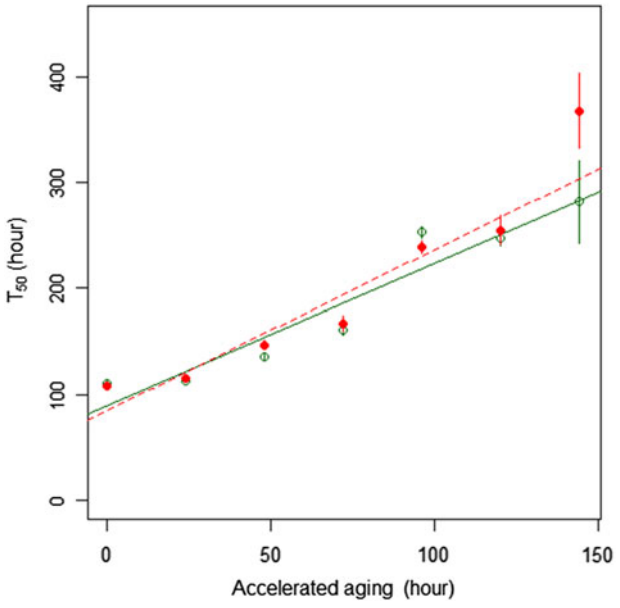


**Figure 6.** Influence of accelerated aging treatments on the speed of germination ($T_{50}$, time to 50% of final germination) for herbicide-resistant (R; ···•···) and herbicide-susceptible (S; —○—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The symbols are the mean of germination seed in response to each accelerated aging treatment obtained from three separate experiments. Vertical bars represent standard error of the mean.

Osmotic and salinity stresses are common environmental stressors that negatively affect seed germination and plant growth (Lee et al. 2010). Plant responses to both osmotic and salinity stress are often similar (Uddin et al. 2016), leading to reduced water uptake. Salinity affects seed germination via ion-specific effects, osmotic stress, and oxidative stress (Ibrahim 2016). The S subpopulation showed better tolerance to both salinity and drought stresses, reflected in higher $Salinity_{50}$ and $Drought_{50}$ values compared with the R subpopulation under the high-temperature regime. Effects of simulated drought and salinity stress on germinability and seedling emergence have been reported for many weed species (e.g., Ebrahimi and Eslami 2012; Florentine et al. 2018; Hoveland and Buchanan 1973; Li et al. 2011; Shrestha et al. 2018; Stéphane et al. 2018); however, germinability of R and S biotypes with a shared genetic background have very rarely been studied.

Recently, Wu et al. (2016) studied the germinability of TSR fenoxaprop-P-ethyl resistant and susceptible biotypes of Japanese foxtail (*Alopecurus japonicus* Steudel) with controlled genetic background under different salinity and drought stress conditions. Wu et al. (2016) found that the $G_{max}$ of R and S biotypes of *A. japonicus* was similar, while the osmotic and salinity levels reducing $G_{max}$ by 50% was higher for the R biotype, as we also found in this study. In contrast to our results and the results of Wu et al. (2016), Shrestha et al. (2018) found that a glyphosate-resistant jungle rice [*Echinochloa colona* (L.) Link] biotype was more tolerant to salinity and drought stresses than a S biotype with a different genetic background. The contrasting result reported by Shrestha et al. (2018) may be attributed to uncontrolled genetic variations. Also, Du et al. (2017) obtained inconsistent results in a study in which germinability of American sloughgrass [*Beckmannia syzigachne* (Steud.) Fernald] populations possessing three different ACCase target-site mutations with shared genetic background was studied under different salinity and osmotic levels. The inconsistency was attributed to genotype, that is, the type of mutation. The higher $\psi_{50}$ and $Salinity_{50}$ of the S subpopulation in comparison with the R subpopulation observed in our study are in accordance with the hypothesis that components of fitness cost may be increased under stress conditions.

In this study, we found that not only the final germination, but also the speed of germination ($T_{50}$) was affected by salinity and water deficiency, and that there was a positive relationship between stress levels and $T_{50}$. However, the only difference between the subpopulations was found for $T_{50}$ when exposed to salinity at low-temperature conditions. The differences in speed of germination might affect the competitive ability of weed species (Cousens et al. 1997) and hence their success of establishment in the field. Weed species with the ability to cope with high salinity conditions can colonize a larger range of ecological niches (Javaid and Tanveer 2014). Hence, it can be expected that the S subpopulation would be more successful than the R subpopulation under stress caused by salinity or drought.

### *Effect of Accelerated Seed-Aging Test on Germination Traits*

The $G_{max}$ of both subpopulations declined with increasing artificial aging (Figure 5). The $G_{max}$ of both subpopulations was statistically similar under nonstress conditions, as also observed in the salinity and simulated drought stress experiments. As expected, no seed germination occurred when seeds were aged for 168 h. However, there was a difference ($P < 0.001$) between the subpopulations, as the $X_{50}$, also referred to as $Aging_{50}$, was 90 and 67 h for the S and R subpopulations, respectively (Table 1).

Time to 50% of final germination ($T_{50}$ parameter) increased when artificial aging time increased (Figure 6), and a linear relationship was found between $T_{50}$ and the artificial aging treatments. As no germination occurred at highest aging treatment (168 h), $T_{50}$ could not be calculated for this treatment. No difference ($P > 0.05$) was observed in $T_{50}$ between the subpopulations (Table 2).

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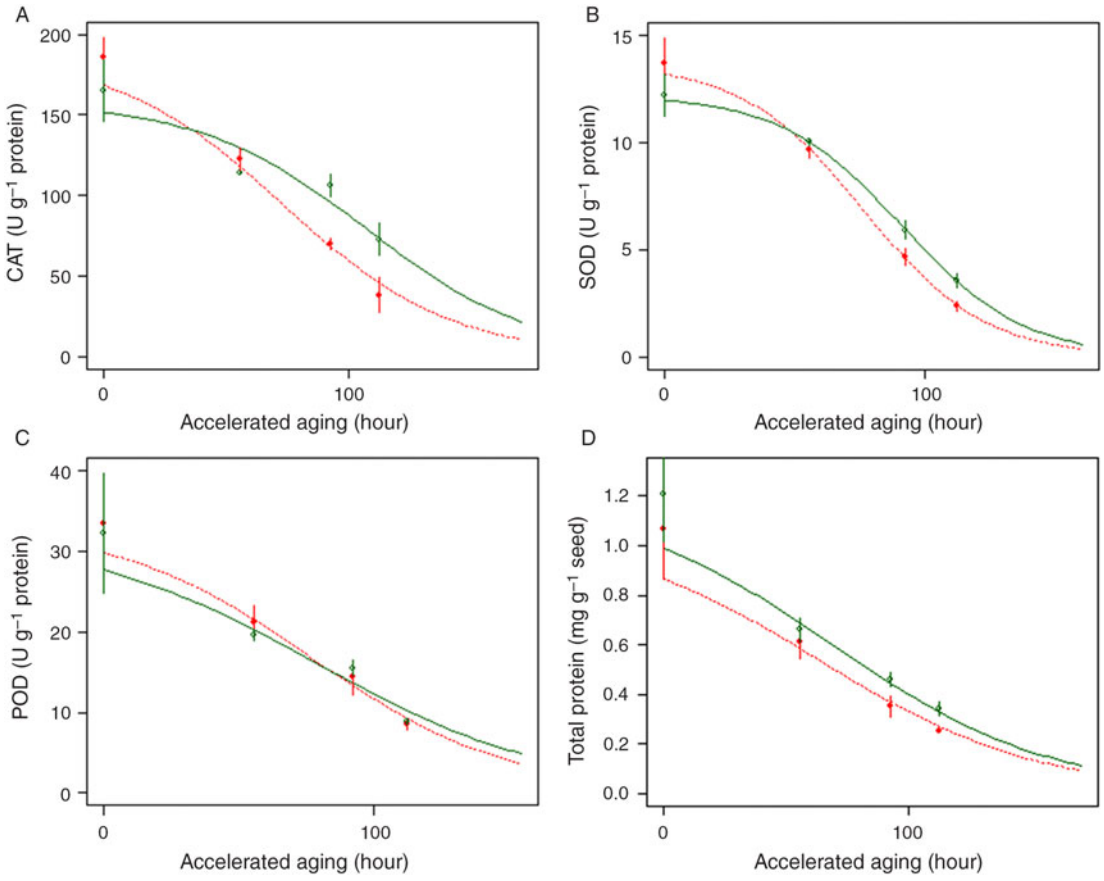


**Figure 7.** Influence of accelerated seed-aging treatments on seed antioxidant enzymes including catalase (CAT; A), superoxide dismutase (SOD; B), peroxidase (POD; C), and seed protein content (D) of herbicide-resistant (R; ···•···) and herbicide-susceptible (S; —○—) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population. The symbols are the mean value of enzymes and seed protein content in response to each accelerated aging treatment. Vertical bars represent standard error of the mean.

Knowledge about seed longevity is critical for developing weed management strategies (Long et al. 2015). Seed persistence permits dispensing genetic diversity over time, but it depends on both environmental factors and the genetic basis of the seeds. The accelerated aging test is used to assess inherent seed persistence, which is the determinant of seed longevity (Long et al. 2008, 2015). As an ex situ technique, it can also be a useful indicator of seed persistence in field soil (Long et al. 2008). A higher $Aging_{50}$ index, an indicator of seed vigor and persistence (Du et al. 2017), may reflect a longer persistence of seeds in the soil seedbank, silage, livestock rumen, and manure (Aper et al. 2014).

Our accelerated seed-aging test clearly showed a slower seed deterioration of the S subpopulation with an $Aging_{50}$ about 20 h longer than the R subpopulation, suggesting that different relative longevity might be expected within two subpopulations. Thus, it is expected that the soil seedbank of the R subpopulation would be depleted faster than that of the S subpopulation; however, field experiments are needed to confirm this assumption. If the difference in seed longevity is also reflected in the field, a backward selection can be expected in the absence of herbicide selection pressure. In accordance with our results, two glyphosate-resistant populations of kochia [*Bassia scoparia* (L.) A.J. Scott] had reduced seed longevity compared with three S populations (Osipitan and Dille 2017). However, Aper et al. (2014) reported diverging results and showed that accelerated aging rate depends on the type of mutation endowing resistance to PSII-inhibiting herbicides in populations of common lambsquarters (*Chenopodium album* L.). A Swedish metamitron-resistant *C. album* population carrying the Ala-251 mutation had lower $Aging_{50}$ (ca. 3 d) than two metamitron- and atrazine-resistant Belgian populations possessing the Ser-264 mutation and a S population. It should be noted that the genetic background of plant material was not controlled in the study by Aper et al. (2014). Gundel et al. (2008) did not find a consistent relationship between herbicide-resistance level and aging rate in Italian ryegrass [*Lolium perenne* L. ssp. *multiflorum* (Lam.) Husnot]) accessions that had evolved resistant to diclofop-methyl. Another study showed no $Aging_{50}$ fitness cost in ACCase TSR populations of *B. syzigachne* carrying three different mutations (Du et al. 2017).

### *Effect of Accelerated Seed-Aging Test on Seed Biochemical Traits*

The activity of all three antioxidant enzymes and protein content of both subpopulations declined with increasing aging treatments (Figure 7A–D). Under nonstress conditions, no statistical differences ($P > 0.05$) were found in the activity of the enzymes, that is, $Enzyme_{max}$, and content of total soluble protein, that is, $Protein_{max}$, between subpopulations (Table 3).

The length of the accelerated aging treatment to reduce the activity of antioxidant enzymes and protein content by 50% (i.e., $Enzyme_{50}/Protein_{50}$) was different for enzymes and subpopulations.

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**Table 3.** Seed antioxidant enzyme activity and seed protein content of herbicide-resistant (R) and herbicide-susceptible (S) subpopulations selected within a non–target site resistant *Alopecurus myosuroides* population in response to different accelerated seed-aging treatments.[a]

| | $Enzyme_{max}/Protein_{max}$[c] | | | $Enzyme_{50}/Protein_{50}$[d] | | |
|---|---|---|---|---|---|---|
| Biochemical traits[b] | S | | R | S | | R |
| CAT | 158 (10.5) | NS | 188 (10.4) | 118 (23.4) | e | 54 (8.3) |
| SOD | 12.2 (0.51) | NS | 13.7 (0.54) | 82 (7.2) | e | 58 (4.8) |
| POD | 32 (3.0) | NS | 33 (3.0) | 61 (19.5) | NS | 60 (15.7) |
| Protein | 1.2 (0.098) | NS | 1.07 (0.096) | 48 (14.1) | NS | 43 (13.8) |

[a]Values in parentheses represent standard error of the mean.
[b]Abbreviations: CAT, catalase; NS, nonsignificant; SOD, superoxide dismutase; POD, peroxidase.
[c]The maximum activity of CAT, POD, SOD (U $g^{-1}$ protein) and protein content (mg $g^{-1}$ seed) of seeds at nonstress condition, i.e., non-aged seeds, *d* parameter (Equation 2).
[d]The accelerated aging treatment (hour) reducing 50% of CAT, POD, SOD, and protein content, i.e., the *d* parameter.
[e]Significant at 0.05 level.

The aging treatment reducing the activity of the CAT enzyme by 50% ($CAT_{50}$ h) for the S subpopulation (118 h) was significantly ($P < 0.05$) higher than for the R subpopulation (54 h) (Table 3; Figure 7A). A statistical difference ($P < 0.05$) was also observed for the SOD enzyme, where the $SOD_{50}$ values for the S and R subpopulations were 82 and 58 h, respectively (Table 3; Figure 7B). However, there was no difference ($P > 0.05$) between subpopulations regarding POD ($POD_{50}$) and protein content ($Protein_{50}$) (Table 3; Figure 7C and D).

The aging process is associated with exposure to oxidative stress (Clerkx et al. 2004), that is, accumulation of reactive oxygen species (ROS). Due to an increase in ROS under environmental stress, the germinability decreases (Lee et al. 2010). The ROS are the primary molecules reducing seed longevity (Lee et al. 2010). The involvement of proteins and antioxidants in seed longevity has been confirmed (Long et al. 2015). A decrease in soluble protein content was reported in Barbados nut (*Jatropha curcas* L.) seeds due to storage and aging (Moncaleano-Escandon et al. 2013), similar to the findings in the present study. A positive correlation between seed longevity and activity of antioxidant enzymes is usually expected. For instance, enhanced seed longevity in tobacco (*Nicotiana tabacum* L.) was attributed to overexpression of CAT and SOD (Lee et al. 2010; Long et al. 2015). Also, deficiency of CAT resulted in reduced seed longevity in *Arabidopsis thaliana* (Clerkx et al. 2004). Bailly et al. (1996) reported that loss of seed viability in sunflower (*Helianthus annuus* L.) seeds was associated with a reduction of CAT, SOD, and glutathione reductase. Our results are in line with previous results, as aged seeds of both the R and S subpopulations resulted in a reduction of CAT and SOD activity. Our results revealed, however, that aged seeds of the R subpopulation had lower CAT and SOD activity than the S subpopulation, that is, NTSR in *A. myosuroides* was correlated with a greater reduction of $CAT_{50}$, $SOD_{50}$, and seed persistence (i.e., $Aging_{50}$) when exposed to artificial aging conditions.

In summary, germinability traits, antioxidant enzyme activity, and protein content of the R and S seeds were statistically similar under optimum conditions. However, the final germination of the R subpopulation was suppressed more than that of the corresponding S subpopulation under stressful conditions, including salinity, drought, and accelerated seed-aging treatments. The aging treatment reduced the activity of the CAT and SOD enzymes of the R subpopulation significantly more than those of the S subpopulation, while there were no differences in $POD_{50}$ and $Protein_{50}$ between the two subpopulations. In conclusion, the S subpopulation showed a higher tolerance to abiotic stress than the R subpopulation. Thus, it is expected that the effects of global climate change, such as drought and soil salinity stress, may influence germinability of the R subpopulation more than that of the S subpopulation, that is, cause biochemical and germinability fitness cost in the NTSR *A. myosuroides* subpopulation.

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