Seed Germination and Seedling Emergence of Blackgrass (*Alopecurus myosuroides*) as Affected by Non–Target-Site Herbicide Resistance

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Seedling emergence traits of susceptible (S) and resistant (R) blackgrass subpopulations isolated from a single non–target-site resistant (NTSR) population were studied in controlled conditions. The seedling emergence of the R subpopulation was lower and slower than that of the S subpopulation, especially at low temperature and deep burial. The burial depth inhibiting final emergence by 50% for the R subpopulation was significantly lower than that of the S subpopulation at low temperature. The present study revealed that under suboptimal conditions the NTSR loci conferring herbicide resistance were correlated with a fitness cost in relation to seedling emergence traits. The results suggest that deep soil cultivation and delayed sowing of autumn-sown crops can hamper germination of the R more than of the S subpopulation and thus potentially reduce the prevalence of the R subpopulation in the blackgrass population.

Nomenclature: Blackgrass, *Alopecurus myosuroides* Huds. ALOMY.

Key words: Final seed germination, final seedling emergence, fitness penalty, NTSR.

Herbicide resistance is a major threat to the sustainability of modern cropping systems, with 471 unique cases now being reported worldwide (Heap 2016). Herbicide resistance can be broadly divided into target-site resistance (TSR) and non–target-site site resistance (NTSR) (Délye et al. 2013a; Powles and Yu 2010). TSR is monogenically inherited and is caused by mutations in target sites of the herbicide, while NTSR is generally assumed to be polygenically inherited, although monogenic NTSR does exist (Délye et al. 2013a; Yuan et al. 2007). Both TSR and NTSR have been reported in blackgrass populations (Brazier et al. 2002; Délye 2005).

As one of the most common grass weeds, blackgrass can cause up to 45% yield reduction in winter wheat (*Triticum aestivum* L.) (Vizantinopoulos and Katranis 1998). Importantly, it is ranked among the 15 most important herbicide-resistant weeds worldwide (Heap >2016). In Europe, blackgrass is the number one herbicide-resistant weed, with confirmed resistance to many herbicides, including, chlorotoluron, isoproturon, fenoxaprop-P, clodinafop-propargyl, flupyrsulfuron-methyl-sodium, mesosulfuron + iodosulfuron, fluazifop-P, propaquizafop, and cycloxydim (Lutman et al. 2013). Recently, the resistance status of Danish resistant blackgrass populations collected during the last decade were tested, and both TSR and NTSR mechanisms were found within populations, with NTSR being most prevalent (Keshtkar et al. 2015). One of the NTSR populations was used in the present study.

According to evolutionary theory, it is assumed that herbicide resistance, as an adaptive defense mechanism, is often associated with a fitness cost in the absence of herbicide (the so-called cost of adaptation) (Vila-Aiub et al. 2009, 2011). Therefore, the frequency of resistant alleles within a population would be expected to decline after some generations in the absence of selection pressure caused by herbicide applications. Nevertheless, a long-term field experiment showed that resistance to acetyl-CoA carboxylase (ACCase) inhibitors was present in a resistant rigid ryegrass (*Lolium rigidum* Gaudin) population 7 yr after the use of ACCase-inhibitor herbicides was abandoned (Collavo et al. 2013). Herbicide resistance fitness costs are not universal, and their expression depends on particular plant mutations and may only be expressed in certain environments (Lehnhoff et al. 2013a; Vila-Aiub et al. 2009). This means it is necessary to quantify the fitness of each herbicide resistance case individually (Lehnhoff et al. 2013a).

In 75% of published fitness studies, genetic background of populations was not taken into account (Vila-Aiub et al. 2009). The fitness studies comparing susceptible (S) and resistant (R) populations originating from different geographical locations are imperfect, as different populations

might reveal high genetic diversity at a number of loci in addition to a resistant locus or loci. These results cannot be clearly interpreted from a scientific standpoint or a weed management point of view. It should, however, be noted that even though selecting R and S plants with close genetic background is one of the most important steps in fitness studies (Holt 1990; Menchari et al. 2008; Vila-Aiub et al. 2005a), results of studies using isogenic R and S lines may not be representative of fitness of resistant phenotypes in natural conditions, as genetic diversity is common in weed field populations (Lehnhoff et al. 2013b). Therefore, when the aim of a fitness study is to detect differences between R and S populations and apply the results in an herbicide-resistant weed management program, it may be more appropriate to select R and S plants within the target field, as we did in the present study.

In fitness studies comparing the performance of S and R phenotypes under competitive conditions, the two parameters most frequently recorded are biomass and seed production. We did not, however, find any vegetative and fecundity fitness cost with an NTSR blackgrass phenotype, whether grown alone or in competition with winter wheat in a replicated greenhouse experiment (Keshtkar 2015). This prompted us to study other plant physiology processes in more detail, in particular seed germination and seedling emergence. Despite its importance, germination has only been studied in 7% of the published fitness studies (Vila-Aiub et al. 2009). Seedling emergence can, among other things, be affected by burial depth, soil temperature, and seed size (Keshtkar et al. 2009), but the effects of these factors on germination and seedling emergence of R and S plants have rarely been studied. To our knowledge, only one study evaluated effects of burial depth and temperature regimes on NTSR and S plants with a closely related genetic background, and this study showed that seedling emergence was lower in NTSR rigid ryegrass (Vila-Aiub et al. 2005b).

The objective of this study was to assess germination and seedling emergence in an NTSR blackgrass population resistant to several herbicides and not bearing any of the known TSR mutations under contrasting temperature regimes and different sowing depths.

Materials and Methods

Plant Material. A fenoxaprop-P resistant blackgrass population (ID914) that originated in the central part of Denmark, Funen (54.956°N, 10.611°E), was used in this study. In our previous study, it was confirmed that this population not only was highly resistant to fenoxaprop-P (resistance index [RI] > 64), but also showed various levels of resistance to pendimethalin (RI > 10), prosulfocarb (RI > 2.5), and flupyrsulfuron-methyl-sodium (RI > 10.5) (Keshtkar et al. 2015). As herbicides with four different sites of action failed to control the plants and, importantly, genotyping did not confirm known mutations in the ACCase gene and acetolactate synthase (ALS) gene, the resistance mechanism involved was assumed to be NTSR (Keshtkar et al. 2015).

The issue with herbicide-resistant blackgrass was detected some years before the experiment was initiated. The seed sample was collected in 2011 from untreated plots within a winter wheat field experiment in which plants survived herbicide treatments with prosulfocarb, flupyrsulfuron-methyl-sodium + diflufenican, clodinafop-propargyl, pyroxsulam, and mesosulfuron + iodosulfuron. Seeds were collected from approximately 20 individual plants within 20 m². The seeds were used to produce S and R plants.

In November 2011, around 400 seeds of the parent population were grown in plastic boxes (45 by 45 by 12 cm) filled with a potting mixture consisting of soil (sandy loam with 12% clay, 6.5% silt, 80% sand, 1.5% organic matter), peat (Kekkilä, Finland), and sand (Dansand, Denmark) (2:1:1 w/w) containing all necessary micro- and macronutrients (0.11% nitrogen, 0.04% phosphorus, and 0.07% potassium).

The plants were kept in a greenhouse at $17/10 \pm 2$ C and 14/10-h day/night cycle with supplemental light (180 µmol m^{-2} s^{-1}). A plant-cloning technique, the single-population protocol described by Pedersen et al. (2007) and Vila-Aiub et al. (2005b), was used to select S and R plants within the population. Briefly, at the 2- to 3-tiller stage, seedlings were excavated from the boxes and one tiller (clone) was carefully excised from the parent plant. The clones were trimmed to 2 cm of shoot and 2 cm of root and then were separately transplanted into 1-L pots filled with the potting mixture described earlier. The parent plants were also transplanted into 2-L pots. Parent plants and their corresponding clones were tagged to identify origin. When the clones reached the 3- to 4-leaf stage, they were sprayed with the field recommended rate of fenoxaprop-P (69 g ai ha^{-1}). Four weeks after herbicide treatment, the clones were classified as S (dead plants), partly resistant (PR, alive but no

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regrowth), and R (alive and vigorously regrowing) with frequencies of 1.5% (S), 14.0% (PR), and 84.5% (R), respectively.

The parent plants were kept in a cold greenhouse (2 C) for vernalization (Chauvel et al. 2002), and they were identified according to their corresponding clones. The PR parent plants were discarded, while the S and R parent plants were used for seed multiplication. To prevent cross-pollination between S and R parent plants, each subpopulation was surrounded by a polyethylene pollen-proof enclosure until seed maturity in mid-July 2012.

As pointed out by Vila-Aiub et al. (2005b), the often unavoidable smaller sample size of the S phenotype, which may result in a more narrow genetic background, will not affect the results, as the main objective of the present study was to detect fitness cost that potentially could be used as a herbicide-resistance management tool. However, to increase the uniformity of the genetic background within each subpopulation and to obtain sufficient seeds, F_1 seeds of each subpopulation ($n = 16$ plants) were sown in November 2012, grown in the greenhouse to maturity, and bulk crossed in pollen-proof plastic enclosures to produce F_2 seeds. Seeds were harvested in mid-July 2013. To increase germinability and break primary seed dormancy, the seeds were subjected to a high temperature (35 ± 2 C) for 6 wk. Then they were stored dry for 7 mo at a constant temperature of 4 C until the onset of the experiment.

Resistance Patterns of the Subpopulations. A dose–response experiment was conducted to determine resistance status of the two subpopulations. Seeds of each subpopulation were sown separately in 2-L pots. Pots were filled with the potting mixture described earlier. The pots were arranged in a completely randomized design with three replications per treatment in a greenhouse at $17/10 \pm 2$ C and 14/10-h day/night cycle with supplemental light ($180\,\mu$mol m^{-2} s^{-1}). The pots, each containing 6 seedlings, were sprayed at the 3- to 4-leaf stage with three ACCase-inhibitor herbicides and one ALS-inhibitor herbicide. Different dose ranges of herbicides were used for the S and R subpopulations based on previous experience (Table 1; data not shown). All spray solutions were prepared in deionized water and applied using a laboratory pot sprayer fitted with two Hardi® ISO F-110-02 nozzles (Hardi, Nørre Alslev, Denmark). The nozzles were operated at a pressure of 310 kPa and a speed of 5.5 km h^{-1} delivering a spray volume of

Table 1. Herbicides and rates applied in the dose–response experiment. The S and R subpopulations were isolated within a non–target-site resistant blackgrass population (ID914).

Common name	Trade name	Safener	Formula	Herbicides rates for R and S subpopulations g ai ha^{-1}	Manufacturer	Manufacturer's website
Fenoxaprop-P	Primera Super	Mefenpyr-diethyl (75 g L^{-1})	OD[a]	S: 0, 4.35, 8.62, 17.25, 34, 69, 138 R: 0, 17.25, 34, 69, 138, 276, 552	Bayer CropScience, Copenhagen, Denmark	www.cropscience.bayer.dk
Cycloxydim	Focus® Ultra	—	EC	S: 0, 125, 2.5, 5, 10, 19, 38 R: 0, 125, 2.5, 5, 10, 19, 38	BASF A/S, Copenhagen, Denmark	www.agro.basf.dk
Clodinafop-propargyl	Topik®	Cloquintocet-mexyl (25 g L^{-1})	EC	S: 0, 1.9, 3.8, 7.5, 15, 30, 60 R: 0, 3.8, 7.5, 15, 30, 60, 120	Syngenta Nordics A/S, Copenhagen, Denmark	www.syngenta.dk
Flupyrsulfuron-methyl-sodium	Lexus® 50WG	—	WG	S: 0, 0.315, 0.625, 1.25, 2.5, 5, 10 R: 0, 0.625, 1.25, 2.5, 5, 10, 20	DuPont, Copenhagen, Denmark	www.dupont-danmark.com

[a] OD, oil dispersion; EC, emulsifiable concentrate; WG, wettable granules.

150 L ha^{-1}. The plants were harvested 3 wk after treatment, and fresh weight of aboveground plant material was recorded. The plant material was dried at 80 C for 48 h, and the dry weight was recorded.

Molecular Characterization of the Subpopulations.

Twenty seeds of each of the two subpopulations and their parent populations were grown as described earlier. Leaf material from 8 individual plants from the plants that grew from the twenty seeds was analyzed for the five ACCase mutations identified in blackgrass (Ile-1781-Leu, Trp-2027-Cys, Ile-2041-Asn, Asp-2078-Gly, and Gly-2096-Ala) (Délye et al. 2007) and the two known mutation sites of the *ALS* gene conferring resistance to ALS inhibitors (Pro-197 and Trp-574) (Tranel et al. 2015). The genotyping was done twice for the two subpopulations and once for the parent populations for the *ACCase* gene, as described in Beffa et al. (2012). For ALS genotyping, leaf samples from the 8 individual plants from each population were harvested for DNA extraction as described by Beffa et al. (2012), with the following modifications. Leaf tissue (20 mg) was disrupted in 400 µl 100 mM Tris (HCl) and 1 M KCl (pH 9.5) with stainless-steel beads in a swing mill (Retsch 300MM). The 164-bp fragment of the *ALS* gene containing the sequence for Pro-197 was amplified using the forward and reverse primers F1 (F1: 5′-TCTGCGTCGCCACCTCCG-3′) and R1 (R1: 5′-GGAGCGGGTGACCTCTACTAT-3′), respectively, at a 60 C annealing temperature. After amplification of the above mentioned 164-bp fragment, the pyrosequencing was performed using the primers P1 (primer for codon position 1: 5′-ATGGTCGCCATCACGGGGCAGGTT-3′) and P2 (primer for codon position 2: 5′-ATGGTCG CCATCACGGGGCAGGTT-3′). Similarly, in order to detect a mutation at Trp-574, the 376-bp fragment of ALS was amplified using the forward and reverse primers, F2 (F2: 5′-ATTCAGGAGTTGGCACT GATT-3′) and R2 (R2: 5′-AAGAAACCCTGCCA TCACCG-3′) at an annealing temperature of 57 C, which was followed by pyrosequencing using the primer 5′-CAACATCTAGGAATGGTGGTGCAG-3′.

Effect of Temperature on Seed Germination.

Fifty uniformly sized seeds of the S and R subpopulations (1,000-kernel weight [$n = 400$ seeds lot^{-1}]: S = 1.9 ± 0.06 g; R = 1.8 ± 0.05 g) were selected and placed in 9-cm petri dishes containing three layers of cellulose filter papers (Whatman No. 1) covered by one glass-fiber filter paper (Whatman GF/A). The petri dishes were placed in zipped polythene bags to avoid loss of water by evaporation. The petri dishes were arranged in a completely randomized design with four replicates in a growth chamber at 14/10-h day/night photoperiods with a photosynthetic photon flux density of 175 µmol m^{-2} s^{-1}. Germination rate of seeds was evaluated under two alternating temperature regimes: 17/10 C and 10/5 C (day/night). The experiment was conducted twice. The two temperature regimes mimic the range of soil temperatures corresponding to an early (September) and late (October) sowing of winter wheat in Denmark, respectively. Germinated seeds were recorded at consecutive inspection times until no further germination was observed. The intervals between monitoring ranged from 8 to 96 h, because germination was low at the beginning and final part of the experiments, while germination was high in the middle of the study. The seeds were considered germinated when the radicle was visible.

Effect of Sowing Depth and Temperature on Seedling Emergence.

Thirty-six uniformly sized seeds of each subpopulation were sown in square pots (9 by 9 by 9.5 cm) filled with a potting mixture consisting of soil (sandy loam with 12% clay, 6.5% silt, 80% sand, 1.5% organic matter), peat (Kekkilä, Finland), and sand (Dansand, Denmark) (4:1:1 w/w/w). All the bulk particles were removed from the growing medium using a 4-mm sieve. The seeds were seeded at four sowing depths (0, 1, 3, and 6 cm) with a similar distance between seeds within each depth. Seedling emergence of seeds was evaluated under the two alternating temperature regimes described above (17/10 C and 10/5 C (day/night)). The pots were arranged in a completely randomized design with factorial arrangement of treatments (i.e., two subpopulations by four sowing depths by two temperature regimes). There were four replicates per treatment. The pots were subirrigated whenever needed. The seeds on the soil surface (0-cm treatment) were covered with a moistened filter paper to promote imbibition. The moistened filter paper was discarded after the first seedlings emerged. Subsequently, 2 ml of water was added carefully to the soil surface every third day. The pots were grown in a growth chamber as described earlier. Seedling emergence was recorded at consecutive inspection times until no further emergence was observed. The plants were considered emerged when the leaf tip was visible. The experiment was conducted twice.

Statistical Analysis

Resistance Patterns of the Subpopulation. The dry weight for each dose was expressed as a percentage of

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the untreated control. A four-parameter log-logistic model (Equation 1), as described by Streibig et al. (1993), was fit to the data:

$$Y = c + \frac{d - c}{1 + \exp[b(\log(x) - \log(ED_{50}))]} \quad [1]$$

where Y is the dry weight (percentage of untreated), x represents herbicide dosage (g ai ha^{-1}), c is the lower limit (asymptote) of the response curve at high herbicide doses, d is upper limit when herbicide doses are zero, ED_{50} is the dosage (g ai ha^{-1}) that reduced dry weight by 50% (midway between the d and c values), and b is the slope of the curve around ED_{50}. Wherever the homogeneity of variance was not met for dry weight data, a transform-both-sides technique (Box-Cox data transformation) was used (Carroll and Ruppert 1988; Ritz et al. 2016).

Where possible, an RI was calculated as the ratio of the ED_{50} of the resistant plants to the corresponding S subpopulation (ED_{50R}/ED_{50S}), and the ratio (RI) was compared to 1 using a t-test.

Seed Germination and Seedling Emergence. Analysis by means of nonlinear mixed-effects regression models is challenging (Ritz et al. 2015), and it was not possible to use such an analysis for the event-time data (Ritz et al. 2013). Data were therefore analyzed in a two-step procedure as described and applied in previous studies (Gundel et al. 2008; Mennan et al. 2012; Ritz et al. 2013).

First, a three-parameter log-logistic model (Equation 2) was fit to the cumulative seedling emergence according to the event-time approach (Ritz et al. 2013).

$$E(t) = \frac{d}{1 + \exp[b(\log(t) - \log(T_{50}))]} = \frac{d}{1 + \left(\frac{t}{T_{50}}\right)^b} \quad [2]$$

where E is the cumulative seedling emergence or germination at time t, d is the upper limit parameter representing the percentage germination of the total number of seeds present, T_{50} is the time to reach 50% of the maximum seedling emergence or germination (d), and b is the slope of the curve at T_{50} denoting the rate of increase in emergence.

Second, estimates of the two biologically meaningful parameters, d and T_{50}, obtained for each replicate from the event-time model were analyzed using a linear mixed model in which experiments were considered as random effects, while phenotypes, temperature regimes, and sowing depths were considered fixed effects. The estimated standard errors of the d and T_{50} parameters obtained for each replicate were also used as weights in the linear mixed model. Based on the fitted mixed model, relevant pairwise comparisons were made (estimated differences were compared to 0 by means of post hoc t-tests).

All statistical analysis and plots were done using R statistical software (R Core Team 2014) with the add-on 'drc' (Ritz and Streibig 2005), 'lme4' (Bates et al. 2014), and 'multcomp' (Hothorn et al. 2008) packages for event-time models, linear mixed models, and multiple comparisons, respectively.

Results and Discussion

Resistance Pattern of the Subpopulations. Different responses to the four herbicides were observed between the R and S subpopulations (Table 2; Figure S1A–D). The S subpopulation was susceptible to all herbicides. As expected, the R subpopulation was highly resistant to fenoxaprop-P, and it was not possible to estimate an ED_{50} dose (Figure S1A). At the field recommended rate of fenoxaprop-P (i.e., 69 g ha^{-1}), dry weight of the S subpopulation was reduced by up to 97%, while the corresponding value for the R subpopulation was only 16%.

Both subpopulations were controlled by the three other herbicides; however, they showed varying susceptibility. The R subpopulation was significantly more tolerant to clodinafop-propargyl in comparison to the S subpopulation, with a RI value equal to 1.9 (P < 0.001) (Table 2; Figure S1B). No significant differences were found in the response of the S and R subpopulations to cycloxydim and flupyrsulfuron-methyl-sodium (Table 2; Figure S1C and D).

Molecular Characterization of Subpopulations. None of the known mutations conferring TSR to ALS- and ACCase-inhibiting herbicides were detected in the two subpopulations or their parental populations, suggesting that the mechanism of resistance is NTSR. A similar result was observed in a previous experiment with seedlings of the parent ID914 population (Keshtkar et al. 2015).

Effect of Temperature on Seed Germination
Final Germination (G_{max}). The two subpopulations showed similar final germination (the d parameter; hereinafter referred to as G_{max}) at both temperature regimes. This means there were no statistically significant differences in G_{max} between the R and S

Table 2. The estimated parameters by the log-logistic model (Equation 1) for herbicide-resistant (R) and herbicide-susceptible (S) subpopulations selected within a non–target-site resistant blackgrass population.[a,b]

Herbicide	Lower limit (c) S	R	Upper limit (d) S	R	Slope (b) S	R	ED_{50} (g ai ha^{-1}) S	R	RI	R^2
Fenoxaprop-P	4.3 (1.14)	nd	93.0 (7.77)	nd	2.8 (0.73)	nd	17.8 (2.96)	>552	nd	0.85
Clodinafop-propargyl	6.4 (1.84)	6.4 (1.84)	99.4 (6.88)	99.4 (6.88)	2.8 (0.76)	2.8 (0.76)	5.0 (0.62)	9.5 (1.28)	1.9 (0.28)**	0.93
Cycloxydim	6.0 (0.63)	6.0 (0.63)	98.7 (4.01)	98.7 (4.01)	24.0 (84.06)	24.0 (84.06)	3.7 (4.14)	4.6 (1.48)	1.2 (1.21) NS	0.96
Flupyrsulfuron-methyl-sodium	5.2 (1.05)	5.2 (1.05)	98.7 (7.15)	98.7 (7.15)	1.6 (0.21)	1.6 (0.21)	0.63 (0.09)	0.77 (0.12)	1.2 (0.16) NS	0.95

a Abbreviations and values: c, lower limit (asymptote) of the response curve at high herbicide doses; d, upper limit when herbicide doses are zero; b, the slope of the curve around ED_{50}; ED_{50}, dosage (g ai ha^{-1}) that reduced dry weight by 50%; RI, resistance index, the ratio of the ED_{50} of the R plants to the S subpopulation (ED_{50R}/ED_{50S}); nd, not possible to determine; NS, nonsignificant.
b RI and R^2 are presented. Standard errors are in parentheses.
**Significant at 0.01 level.

subpopulations in any of the temperature regimes (Table 3).

Germination Timing (T_{G50}). Time to reach 50% of maximum germination (T_{50} parameter; hereinafter referred to as T_{G50}) was significantly ($P < 0.001$) shorter at optimal compared with low temperature for both subpopulations. On average, T_{G50} at the low temperature was around two times higher than at the high temperature (approximately 200 and 100 h, respectively). As with the G_{max} parameter, there were no significant differences in T_{G50} between the R and S subpopulations (Table 3).

Several studies have investigated the correlation between herbicide resistance and seed germination traits but have reached different conclusions (Délye et al. 2013b; Park et al. 2004; Vila-Aiub et al. 2005b; Wang et al. 2010). This was partly due to the fact that different resistance allele(s) were studied, but the lack of control of the genetic background of the S and R phenotypes also explains this inconsistency (Vila-Aiub et al. 2011). Studies with R and S plants with closely related genetic backgrounds showed lower germination rates or delayed germination in foxtail millet [*Setaria italic* (L.) Beauv.], blackgrass, and rigid ryegrass plants carrying the Leu-1781 ACCase mutation versus the wild-type gene (Délye et al. 2013b; Vila-Aiub et al. 2005b; Wang et al. 2010). In contrast, the Asn-2041 mutation had no effect, while the Gly-2078 mutation increased the speed of germination of blackgrass (Délye et al. 2013b). Ashigh and Tardif (2009) did not find any differences between four S and four R eastern black nightshade (*Solanum ptychanthum* Dunal) populations carrying a mutation in an *ALS* gene (Ala-205-Val). All the studies mentioned investigated the possible effects of TSR (monogenic R) on seed germination and seedling emergence traits.

As pointed out by Délye et al. (2011), more attention should be directed toward NTSR, due to its unpredictable resistance patterns. However, only one study has evaluated other related phenotypic characteristics of NTSR (pleiotropic effects) using plant materials with almost similar genetic background in an herbicide-resistant weed (rigid ryegrass; Vila-Aiub et al. 2005b). Vila-Aiub et al. (2005b) found that germination of NTSR rigid ryegrass plants resistant to diclofop was lower compared with the S phenotype at low constant temperatures (5 and 8 C), while at higher constant and alternating temperatures, no difference was found between S and R phenotypes in their study, which is similar to our results.

Table 3. Germination parameters of herbicide-resistant (R) and herbicide-susceptible (S) subpopulations under different temperature regimes were estimated by the event-time model (Equation 2), then the estimated parameters were subjected to a linear mixed model.[a]

| Subpopulation | G_{max}[b] | | T_{G50}[c] | |
	10/5 C	17/10 C	10/5 C	17/10 C
	———————%———————		———————h———————	
S	97.1 (0.71) ab	97.4 (0.72) a	213.0 (2.67) a	111.6 (1.99) b
R	94.8 (0.87) b	96.4 (0.91) ab	222.0 (2.92) a	116.2 (2.22) b

[a] Standard errors are in parentheses. Different letters show statistically significant differences between mean values of each parameter.
[b] Final germination = d parameter ×100.
[c] Germination timing: time to reach 50% of the final germination.

Effect of Sowing Depth and Temperature on Seedling Emergence

Final Seedling Emergence (E_{max}). Final seedling emergence (d parameter; hereinafter referred to as E_{max}) decreased significantly with increasing sowing depths at both temperature regimes for both subpopulations (Table 4). Decreasing seedling emergence with increasing sowing depth has been reported for many weed species (Bello et al. 2017; Benvenuti et al. 2001). Similar to the results for the petri dish experiments, E_{max} was not affected by temperature.

However, statistically significant (P < 0.01) differences were only noted between the E_{max} of R and S subpopulations at low temperature across all sowing depths, except for the seeds placed on the soil surface (0 cm). The differences were more pronounced under stressful conditions (i.e., at low temperature and 6-cm sowing depth) than under more favorable conditions (i.e., reduced burial depths: 1 and 3 cm). The E_{max} of the R subpopulation was around 5-fold lower than that of the S subpopulation at low temperature and 6-cm sowing depth. Vila-Aiub et al. (2005b) reported that the final seedling emergence of NTSR rigid ryegrass was similar to S phenotypes at all sowing depths less than 8 cm, but at 8-cm soil depth, NTSR plants showed lower emergence compared with S plants. Our results confirmed the results found in rigid ryegrass (Vila-Aiub et al. 2005b), as seedling emergence of the R subpopulation was significantly lower (i.e., lower E_{max}) than the S subpopulation, especially under suboptimal conditions.

Seedling Emergence Timing (T_{E50}). The time to reach 50% of the maximum emergence (T_{50} parameter; hereinafter referred to as T_{E50}) increased for both subpopulations with sowing depth greater than 1 cm (Table 5). The T_{E50} for seeds placed on the soil surface (0 cm) was generally higher than for buried seeds. As in the petri dish experiments, low temperature significantly delayed seedling emergence of both subpopulations. On average, the T_{E50} was 550 and 300 h at low and optimal temperatures, respectively.

Table 4. Final seedling emergence for herbicide-resistant (R) and herbicide-susceptible (S) subpopulations selected within a non–target-site resistant blackgrass population under increasing sowing depths and two temperature regimes. The parameters were estimated by fitting the event-time model (Equation 2), then the estimated parameters were subjected to a linear mixed model.[a]

| Sowing depth | E_{max}[b] | | | |
| | 10/5 C | | 17/10 C | |
	S	R	S	R
—cm—	————————————————————%————————————————————			
0	92.18 (2.69) a	80.11 (3.55) ab	83.49 (4.44) ab	68.84 (4.84) b
1	77.64 (3.44) a	47.78 (3.93) c	69.92 (3.69) ab	58.33 (3.94) bc
3	72.46 (3.62) a	49.78 (3.99) b	70.04 (3.70) a	57.47 (4.01) ab
6	37.36 (3.85) a	8.01 (2.76) b	21.03 (4.68) ab	13.26 (4.06) b

[a] Standard errors are in parentheses. Different letters within each row show statistically significant differences between mean values of each parameter.
[b] Final seedling emergence = d parameter × 100.

Table 5. Time to reach 50% of seedling emergence for herbicide-resistant (R) and herbicide-susceptible (S) sub-populations selected within a non–target-site resistant blackgrass population under increasing sowing depths and two temperature regimes. The parameters were estimated by fitting the event-time model (Equation 2), then the estimated parameters were subjected to a linear mixed model.[a]

	T_{E50}[b]			
	10/5 C		17/10 C	
Sowing depth	S	R	S	R
—cm—	—————————————————h—————————————————			
0	651.8 (13.02) a	671.8 (12.63) a	378.4 (10.71) b	377.5 (12.34) b
1	475.2 (11.14) a	498.0 (14.03) a	213.1 (6.10) b	224.0 (6.46) b
3	465.5 (10.51) b	514.5 (11.90) a	211.7 (6.02) d	224.9 (6.10) c
6	572.6 (12.49) a	599.5 (25.47) a	277.6 (10.64) b	298.1 (21.36) b

[a] Standard errors are in parentheses. Different letters within each row show statistically significant differences between mean values of each parameter.
[b] Seedling emergence timing: time to reach 50% of seedling emergence.

Importantly, statistically significant differences between the two subpopulations were observed when the seeds were buried at a 3-cm depth. At 3-cm sowing depth, the R subpopulation emerged around 50 and 13 h later than the S subpopulation under suboptimal and optimal temperature regimes, respectively. However, no statistically significant differences were found between subpopulations in other treatments, except at the 3-cm sowing depth.

Overall, similar trends were observed in the petri dish experiments and pot experiments, with G_{max} and E_{max} of the R subpopulation tending to be lower than those of the S subpopulation and T_{G50} and E_{G50} of the R subpopulation also having a tendency to be higher than those of the S subpopulation. However, E_{max} at all sowing depths, and especially at deep burial depth, was lower than G_{max}. This could be due to a lower water potential in the soil, a lower light intensity, or a reduced oxygen content in the pots compared with the petri dishes. However, significant differences in E_{max} were only observed in pot experiments (buried seeds under low-temperature regimes), which could be attributed to environmental conditions amplifying the difference in seedling emergence between the R and S plants.

Seedling Emergence Inhibition (D_{50}). As previously mentioned, the E_{max} of both subpopulations decreased with increasing sowing depth. To estimate
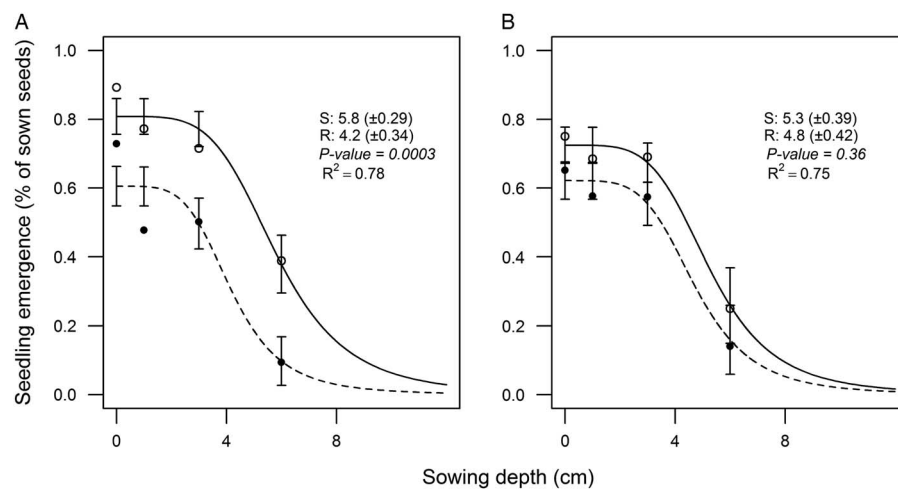

Figure 1. Influence of sowing depth on final emergence for herbicide-resistant (R; ●) and herbicide-susceptible (S; ○) subpopulations selected within a non–target-site resistant blackgrass population. The accumulated seedling emergence (%) of subpopulations in response to sowing depth at (A) suboptimal (10/5 C) and (B) optimal (17/10 C) temperature regimes estimated by the log-logistic model (Equation 1). Values indicate the estimated D_{50} (50% reduction of final emergence) for subpopulations. Values in parentheses and vertical bars represent standard errors.

the sowing depth resulting in a 50% reduction of final emergence (D_{50}) the accumulated number of seedlings emerging (percentage of sown seeds) at all sowing depths were fit against sowing depth using a log-logistic model (Equation 1). As there were no differences between experiments, the data were pooled.

The temperature did not change the D_{50} parameter for any of the subpopulations (Figure 1A and B). The D_{50} for the R subpopulation (4.2) was significantly lower than for the S subpopulation (5.8) at low temperature (Figure 1A). Our study is the first to report a lower D_{50} (higher sensitivity to soil burial) of the R subpopulation compared with the S subpopulation, particularly at suboptimal temperatures.

To our knowledge, the report by Vila-Aiub et al. (2005b) was the first study on NTSR fitness in a population with closely related genetic background. The present study is only the second study comparing the germination and seedling emergence of S and NTSR weeds and the first study on blackgrass using a methodology (single-population protocol) attempting to ensure comparable genetic backgrounds. Obviously, the genetic backgrounds of NTSR and S plants cannot be fully controlled.

It is generally agreed that weed seeds collected directly from a field may possess high genetic diversity. But this may not be completely true in the present case, because the parent seeds were collected from 20 individual plants within an experimental field where the herbicide selection pressure had been the same for many years and the occurrence of resistant blackgrass had been known for several years. More importantly, the seeds were collected from an untreated small plot, where random mating in the year of collection added to the homogeneity of the genetic background. It should also be noted that the maximum distance for pollen spread of blackgrass has been found to be 4 m (Petersen et al. 2010). Adopting the plant-cloning technique to select the R and S subpopulations within a parent population collected within a well-defined area in the field, we claim that the subpopulations will have comparable genetic backgrounds. However, some genetic diversity may still exist, and we therefore referred to the selected R and S clones as "subpopulations," in contrast to Vila-Aiub et al. (2005b) and Pedersen et al. (2007), who used the terms "phenotype" and "line." Nevertheless, as pointed out by Lehnhoff et al. (2013b), the remaining diversity within the selected R and S subpopulations mimics the natural field populations.

Accordingly, as we selected R and S plants within a field, our results can be more applicable and reliable for resistance management programs than results obtained with selected R and S populations from different geographical locations.

A recent review concluded that moldboard plowing and delayed drilling in autumn can reduce blackgrass population by approximately 69% and 50%, respectively (Lutman et al. 2013). The present study showed that deep sowing and a low-temperature regime mimicking deeper soil tillage and delayed drilling of autumn-sown crops not only influence seedling emergence traits of both sub-populations but also have a potential to increase differences in seedling emergence between R and S subpopulations to the detriment of the R plants.

This implies that postponing the drilling of autumn-sown crops until soil temperatures have decreased not only can lead to lower and later seedling emergence of the R subpopulation, but can also increase the sensitivity of the R subpopulation to burial depth (lower D_{50}), and this can contribute to the fitness penalty of the R subpopulation. It is generally agreed that fitness cost associated with adaptive alleles (e.g., herbicide-resistance alleles) can be increased under stressful conditions (Vila-Aiub et al. 2009). In the absence of herbicides, the lower E_{max}, D_{50}, and speed of emergence of the R subpopulation can be expected to favor the S subpopulation and eventually result in an increased frequency of the wild-type loci in the population.

Owen et al. (2011) concluded that lower germination of herbicide-resistant rigid ryegrass populations is not the direct effect of resistance alleles. Instead, they attributed it to cropping-system selection pressure. However, Délye et al. (2013b) and Vila-Aiub et al. (2005b) attributed low germination in ACCase TSR phenotypes of rigid ryegrass and blackgrass to the Leu-1781 ACCase allele (i.e., herbicide selection pressure) in studies where the genetic background of the phenotypes was controlled. From an herbicide-resistance manage-ment point of view, no matter which type of selection pressure—cropping system or herbicide resistance—causes a fitness penalty, lower fitness can be used as a resistance management tool.

Further studies are needed, in particular inclusion of additional populations in greenhouse studies and, ultimately, in field studies like the one done by Collavo et al. (2013). The results should be implemented in simulation models to predict the long-term impact of time and depth of soil cultivation on the frequency of resistant individuals

in the population. Finally, our results suggest that seed germination and seedling emergence should be assessed in future fitness studies to fully elucidate the differences between S and R plants, a pertinent factor in developing integrated weed management strategies specifically targeting herbicide resistance.

Acknowledgments

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2017.44

Literature Cited

Ashigh J, Tardif FJ (2009) An amino acid substitution at position 205 of acetohydroxyacid synthase reduces fitness under optimal light in resistant populations of *Solanum ptychanthum*. Weed Res 49:479–489

Bates D, Maechler M, Bolker B, Walker S (2014) lme4: Linear mixed-effects models using Eigen and S4. R Package v. 1.1-7. http://CRAN.R-project.org/package=lme4. Accessed June 7, 2017

Beffa R, Figge A, Lorentz L, Hess M, Laber B, Ruiz-Santaella JP, Strek H (2012) Weed resistance diagnostic technologies to detect herbicide resistance in cereal growing areas. A review. Julius-Kühn-Archiv 434:75–80

Bello IA, Hatterman-Valenti H, Owen MDK (2017) Factors affecting germination and seed production of *Eriochloa villosa*. Weed Sci 48:749–754

Benvenuti S, MacChia M, Miele S (2001) Quantitative analysis of emergence of seedlings from buried weed seeds with increasing soil depth. Weed Sci 49:528–535

Brazier M, Cole DJ, Edwards R (2002) *O*-glucosyltransferase activities toward phenolic natural products and xenobiotics in wheat and herbicide-resistant and herbicide-susceptible black-grass (*Alopecurus myosuroides*). Phytochemistry 59:149–156

Carroll RJ, Ruppert D (1988) Transformation and Weighting in Regression. New York: Chapman and Hall. 264 p

Chauvel B, Munier-Jolain NM, Grandgirard D, Gueritaine G (2002) Effect of vernalization on the development and growth of *Alopecurus myosuroides*. Weed Res 42:166–175

Collavo A, Strek H, Beffa R, Sattin M (2013) Management of an ACCase-inhibitor-resistant *Lolium rigidum* population based on the use of ALS inhibitors: weed population evolution observed over a 7 year field-scale investigation. Pest Manag Sci 69:200–208

Délye C (2005) Weed resistance to acetyl coenzyme a carboxylase inhibitors: an update. Weed Sci 53:728–746

Délye C, Gardin JAC, Boucansaud K, Chauvel B, Petit C (2011) Non–target-site-based resistance should be the centre of attention for herbicide resistance research: *Alopecurus myosuroides* as an illustration. Weed Res 51:433–437

Délye C, Jasieniuk M, Le Corre V (2013a) Deciphering the evolution of herbicide resistance in weeds. Trends Genet 29:649–658

Délye C, Menchari Y, Guillemin JP, MatÉJicek A, Michel S, Camilleri C, Chauvel B (2007) Status of black grass (*Alopecurus myosuroides*) resistance to acetyl-coenzyme A carboxylase inhibitors in France. Weed Res 47:95–105

Délye C, Menchari Y, Michel S, Cadet E, Le Corre V (2013b) A new insight into arable weed adaptive evolution: mutations endowing herbicide resistance also affect germination dynamics and seedling emergence. Ann Bot 111:681–691

Gundel PE, Martínez-Ghersa MA, Ghersa CM (2008) Dormancy, germination and ageing of *Lolium multiflorum* seeds following contrasting herbicide selection regimes. Eur J Agron 28:606–613

Heap I (2016) The International Survey of Herbicide Resistant Weeds. www.weedscience.org. Accessed: June 7, 2016

Holt JS (1990) Fitness and ecological adaptability of herbicide-resistant biotypes. Pages 419–429 *in* Green MB, LeBaron HM & Moberg WK eds., Managing Resistance to Agrochemicals: From Fundamental Research to Practical Strategies. Washington, DC: American Chemical Society

Hothorn T, Bretz F, Westfall P (2008) Simultaneous inference in general parametric models. Biom J 50:346–363

Keshtkar E (2015) Ecological Fitness, Molecular Basis and Selection of Resistant Black-Grass (*Alopecurus myosuroides*) Biotypes. Ph.D dissertation. Aarhus, Denmark: Aarhus University. 149 p

Keshtkar E, Kordbacheh F, Mesgaran MB, Mashhadi HR, Alizadeh HM (2009) Effects of the sowing depth and temperature on the seedling emergence and early growth of wild barley (*Hordeum spontaneum*) and wheat. Weed Biol Manag 9:10–19

Keshtkar E, Mathiassen SK, Moss SR, Kudsk P (2015) Resistance profile of herbicide-resistant *Alopecurus myosuroides* (black-grass) populations in Denmark. Crop Prot 69:83–89

Lehnhoff EA, Keith BK, Dyer WE, Menalled FD (2013a) Impact of biotic and abiotic stresses on the competitive ability of multiple herbicide resistant wild oat (*Avena fatua*). PLoS ONE 8:e64478

Lehnhoff EA, Keith BK, Dyer WE, Peterson RK, Menalled F (2013b) Multiple herbicide resistance in wild oat and impacts on physiology, germinability, and seed production. Agron J 105:854–862

Lutman PJW, Moss SR, Cook S, Welham SJ (2013) A review of the effects of crop agronomy on the management of *Alopecurus myosuroides*. Weed Res 53:299–313

Menchari Y, Chauvel B, Darmency H, Délye C (2008) Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides*. J Appl Ecol 45:939–947

Mennan H, Streibig JC, Ngouajio M, Kaya E (2012) Tolerance of two *Bifora radians* Bieb populations to ALS inhibitors in winter wheat. Pest Manag Sci 68:116–122

Owen MJ, Michael PJ, Renton M, Steadman KJ, Powles SB (2011) Toward large-scale prediction of *Lolium rigidum* emergence. II. Correlation between dormancy and herbicide resistance levels suggests an impact of cropping systems. Weed Res 51:133–141

Park KW, Mallory-Smith CA, Ball DA, Mueller-Warrant GW (2004) Ecological fitness of acetolactate synthase inhibitor–

resistant and –susceptible downy brome (*Bromus tectorum*) biotypes. Weed Sci 52:768–773

Pedersen BP, Neve P, Andreasen C, Powles SB (2007) Ecological fitness of a glyphosate-resistant *Lolium rigidum* population: growth and seed production along a competition gradient. Basic Appl Ecol 8:258–268

Petersen J, Dresbach-Runkel M, Wagner J (2010) A method to determine the pollen-mediated spread of target-site resistance to acetyl-coenzyme A carboxylase inhibitors in black grass (*Alopecurus myosuroides* Huds.). J Plant Dis Prot 117:122–128

Powles SB, Yu Q (2010) Evolution in action: plants resistant to herbicides. Ann Rev Plant Biol 61:317–347

R Core Team (2014) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ritz C, Baty F, Streibig JC, Gerhard D (2016) Dose-response analysis using R. PLoS ONE 10:e0146021

Ritz C, Kniss AR, Streibig JC (2015) Research methods in weed science: statistics. Weed Sci 63:166–187

Ritz C, Pipper CB, Streibig JC (2013) Analysis of germination data from agricultural experiments. Eur J Agron 45:1–6

Ritz C, Streibig JC (2005) Bioassay analysis using R. J Stat Softw 12:1–22

Streibig JC, Rudemo M, Jensen JE (1993) Dose-response curves and statistical models. Pages 25–55 *in* Streibig JC & Kudsk P, eds. Herbicide Bioassays. Boca Raton, FL: CRC

Tranel PJ, Wright TR, Heap IM (2015). Mutations in Herbicide-Resistant Weeds to ALS Inhibitors. http://www.weedscience.com. Accessed: January 5, 2015

Vila-Aiub MM, Neve P, Powles SB (2005a) Resistance cost of a cytochrome P450 herbicide metabolism mechanism but not an ACCase target site mutation in a multiple resistant *Lolium rigidum* population. New Phytol 167:787–796

Vila-Aiub MM, Neve P, Powles SB (2009) Fitness costs associated with evolved herbicide resistance alleles in plants. New Phytol 184:751–767

Vila-Aiub MM, Neve P, Roux F (2011) A unified approach to the estimation and interpretation of resistance costs in plants. Heredity 107:386–394

Vila-Aiub MM, Neve P, Steadman KJ, Powles SB (2005b) Ecological fitness of a multiple herbicide-resistant *Lolium rigidum* population: dynamics of seed germination and seedling emergence of resistant and susceptible phenotypes. J Appl Ecol 42:288–298

Vizantinopoulos S, Katranis N (1998) Management of blackgrass (*Alopecurus myosuroides*) in winter wheat in Greece. Weed Technol 12:484–490

Wang T, Picard JC, Tian X, Darmency H (2010) A herbicide-resistant ACCase 1781 *Setaria* mutant shows higher fitness than wild type. Heredity 105:394–400

Yuan JS, Tranel PJ, Stewart CN Jr (2007) Non–target-site herbicide resistance: a family business. Trends Plant Sci 12:6–13