Research Article

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A dinitroaniline herbicide resistance mutation can be nearly lethal to plants

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Abstract

BACKGROUND: *Lolium rigidum* is the most important weed in Australian agriculture and pre-emergence dinitroaniline herbicides (e.g., trifluralin) are widely and persistently used for *Lolium* control. Consequently, evolution of resistance to dinitroaniline herbicides has been increasingly reported. Resistance-endowing target-site α-tubulin gene mutations are identified with varying frequency. This study investigated the putative fitness cost associated with the common resistance mutation Val–202–Phe and the rare resistance mutation Arg–243–Met causing helical plant growth.

RESULTS: Results showed a deleterious effect of Arg–243–Met on fitness when plants are homozygous for this mutation. This was evidenced as high plant mortality, severely diminished root and aboveground vegetative growth (lower relative growth rate), and very poor fecundity compared with the wild-type, which led to a nearly lethal fitness cost of >99.9% in competition with a wheat crop. A fitness penalty in vegetative growth was evident, but to a much lesser extent, in plants heterozygous for the Arg–243–Met mutation. By contrast, plants possessing the Val–202–Phe mutation exhibited a fitness advantage in vegetative and reproductive growth.

CONCLUSION: The α-tubulin mutations Arg–243–Met and Val–202–Phe have contrasting effects on fitness. These results help understand the absence of plants homozygous for the Arg–243–Met mutation and the high frequency of plants carrying the Val–202–Phe mutation in dinitroaniline-resistant *L. rigidum* populations. The α-tubulin Arg–243–Met mutation can have an exceptional fitness cost with nearly lethal effects on resistant *L. rigidum* plants.

Keywords: trifluralin; resistance cost; α-tubulin gene mutation; Arg–243–Met; Val–202–Phe

1 INTRODUCTION

Herbicides are important tools for the effective control of weeds that infest field crops. Over-reliance on herbicides has resulted in high selection pressure that has inevitably led to the evolution of herbicide resistance and enrichment of adaptive resistance alleles in agroecosystems worldwide.[1–3]

Plants exhibiting gene mutations endowing target-site herbicide resistance in weeds may express a fitness penalty driven by the direct impact of a particular resistance allele on fitness-related traits and/or co-evolution with altered life-history traits, eventually leading to fitness costs under particular ecological conditions.[4–7] Target-site herbicide resistance mutations that alter the geometry of herbicide target enzymes through structural changes in the contact interface between enzyme subunits, and/or between enzyme and substrate, leading to altered kinetic parameters are likely to have detrimental effects on plant fitness, compared with the susceptible wild-type (WT) counterpart.[4]

Trifluralin is a dinitroaniline herbicide categorized under group 3/D. Dinitroaniline compounds with a similar chemical structure (e.g., trifluralin, oryzalin, pendimethalin, ethalfluralin) are soil-active pre-emergent herbicides controlling many germinating weed seeds by inhibiting the assembly of microtubules comprised of α- and β-tubulin subunits.[8] In Australia, trifluralin is a particularly important pre-emergence herbicide and is used in many crops, especially wheat. However, its persistent use has resulted in resistance evolution in *Lolium rigidum* Gaud. (annual or rigid ryegrass). Since the first trifluralin resistance case was reported in South Australia in 1995,[9] the number of *L. rigidum* populations resistant to trifluralin has increased across Australia, particularly in South and Western Australia.[10–12]

Target-site α-tubulin mutation is an important mechanism of resistance to dinitroaniline herbicides.[13] Target-site α-tubulin mutations endowing dinitroaniline resistance have been reported

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in *Eleusine indica* (Thr–239–Ile; Met–268–Thr),[14,15] *Setaria viridis* (Leu–136–Phe; Thr–239–Ile)[16] and *Alopecurus aequalis* (Leu–125–Met; Leu–136–Phe; Val–202–Phe).[17] Recently, the novel α-tubulin mutations Arg–243–Met/Lys, plus Val–202–Phe and Thr–239–Ile, have been characterized in trifluralin-resistant *L. rigidum*.[18–21]

Various α-tubulin mutations (including the above-mentioned mutations at positions 136, 239, 243 and 268) have been shown to confer dinitroaniline resistance in the parasite *Toxoplasma gondii* at a cost to microtubule function.[22] Likewise, a 20% reproductive cost associated with the α-tubulin mutation Thr–239–Ile has been recorded in trifluralin-resistant *Setaria*.[23]

In recent studies, we observed in *L. rigidum* that the frequency of α-tubulin Arg–243–Met mutation is extremely low and *L. rigidum* plants homozygous for the mutation are undetectable in field-resistant populations. When forced to cross between heterozygous resistant Arg–243–Met plants, the resulting homozygous plants exhibited helicoidal leaf shapes and slow seedling growth.[20] For a better understanding of the evolution of α-tubulin target-site dinitroaniline resistance mutations and the potentially associated fitness cost, we assessed growth and reproductive traits under contrasting ecological conditions in *L. rigidum* genotypes sharing a common genetic background except for the Val–202–Phe and Arg–243–Met α-tubulin mutations.

2 MATERIAL AND METHODS

2.1 Plant material

All experiments were conducted at UWA, Crawley campus. Three genotypes, WT, Val–202–Phe (202Phe), Arg–243–Met (243Met), purified from within a single resistant *L. rigidum* population (M4/16), were used in this study.[12,20,21] The genotyped homozygous plants were bulked in isolation under glasshouse conditions to produce seeds that resulted in three purified subpopulations containing plants with homozygous genotypes of WT, 202Phe and 243Met. Progeny plants ($n = 10$) from each of the purified genotypic populations were randomly selected for RNA extraction to confirm the α-tubulin resistance mutations in each characterized genotype in accordance with Chu *et al.*[20] In addition, heterozygous (243Met-RS) plants for the Arg–243–Met mutation were also identified and selected before use in subsequent experiments.

2.2 Seed germination

Seeds of similar size from the purified WT, 202Phe and 243Met plants were pre-germinated on 0.6% (w/v) agar at 4°C in darkness for 5 days and then incubated at room temperature (20°C) under natural photoperiod (12 h). To ensure plant uniformity, seeds from the 243Met genotype were pre-germinated 4 days ahead because of late germination compared with WT and 202Phe genotypes. Wheat seeds were germinated on 0.6% (w/v) agar at room temperature for 3 days before transplanting (see below).

2.3 Effect of Val–202–Phe and Arg–243–Met mutations on relative growth rate

Variations in relative growth rate (RGR) are often positively correlated with variations in plant establishment ability and as such, RGR is a useful eco-physiological parameter in denoting the expression of herbicide resistance costs.[24] Seedlings of similar height from each of the three genotypes were transplanted into pots (17 cm diameter × 17 cm height) containing a substrate of composted fine pine bark (50%), coco-peat (30%) and washed river sand (20%), and were grown under glasshouse conditions (night/day average temperatures were 19.5/23.4°C with a natural photoperiod of 10–11 h). A total of 25 individual plants per genotype were evaluated at each sampling point. Plants were grown in pots and RGR of aboveground (shoot: leaf and stem) and root biomass during the vegetative stage was estimated sequentially over a 10-day interval during a 44-day growth period in which a linear increase in plant biomass is attained. First biomass evaluation was conducted 14 days after seedling transplanting (DAT). Plants were watered daily and rearranged frequently to randomize environmental differences in the glasshouse. The plants were removed from pots and washed gently using tap water. Aboveground and root material was oven-dried at 60°C for 3 days.

The unbiased formula proposed by Hoffmann and Poorter[25] was used to determine plant RGR:

$$\mathrm{RGR} = \left(\overline{\ln W2}\right) - \left(\overline{\ln W1}\right)/t2 - t1 \quad (1)$$

where $\overline{ln(W)_t}$ is the mean of the ln-transformed plant weights at harvest time t.

The variance (V) associated with RGR was estimated using Causton and Venus's formula[26]:

$$V(\mathrm{RGR}) = V\left(\overline{\ln W2}\right) + V\left(\overline{\ln W1}\right)/(t2 - t1)^2 \quad (2)$$

The degree of freedom associated with *RGR* is $n - 2$, where n is the total number of plants in two harvest intervals. One-way analysis of variance (ANOVA) with Tukey's HSD test were performed to compare RGR among WT, 202Phe and 243Met genotypes.

2.4 Effect of temperature and homozygosity of Arg–243–Met mutation on RGR

Seeds were germinated, transplanted and grown as described above. Estimation of RGR associated with WT, homozygous 243Met-RR and heterozygous 243Met-RS genotypes was conducted under glasshouse conditions at mean temperatures of 18°C (15.9°C/19.9°C night/day average temperatures) and 26°C (24.2°C/27.6°C night/day average temperatures). Data were subjected to two-way ANOVA to test for main and interaction effect of genotype homozygosity (243Met-RR versus 243Met-RS) and environmental temperature (18°C versus 26°C) on shoot and root RGR estimates. Tukey's HSD test was used to compare mean values ($\alpha = 5\%$).

2.5 Effect of Val–202–Phe and Arg–243–Met mutations on fitness traits under crop competition

Target-neighborhood competition experiments were conducted under glasshouse conditions and designed to assess the reproductive fitness traits of WT, homozygous 202Phe and 243Met *L. rigidum* genotypes under competitive and non-competitive interactions when growing with a wheat crop. The competitive response of two target plants (enable outcrossing *Lolium* species to produce seeds) of each genotype in competition with five equally distant wheat plants (i.e., 78 plants m^{-2}) was evaluated in pots (28.5 cm diameter × 26 cm height) containing potting soil under glasshouse conditions. Seedlings of the target genotypes and wheat were transplanted at the same time (two-leaf stage) to account for symmetric competition between the species. Plants were watered daily using an automatic irrigation system, fertilized every week and frequently rearranged to randomize environmental differences in the glasshouse. At the end of the growing season, *L. rigidum* target genotypes and neighboring wheat plants

were harvested. Aboveground biomass of target and neighbor plants was oven-dried at 60°C for 3 days. Seed heads produced by each target genotype were threshed and total seed mass and number were determined. There were 25 replicates for each experimental treatment (WT, 202Phe, 243Met; control versus wheat competition).

Plant fitness (*W*) was estimated as the linear combination of the probability of the proportion of plants that survive from seed dispersal to reproduction and the number of offspring produced by adult plants.[27] Thus, the magnitude of the fitness cost (*FC*) was estimated as[28]:

$$FC = 1 - \frac{W_R}{W_S} \quad (3)$$

where *W* is the quantitative estimation of a fitness trait from resistant (W_R: 202Phe or 243Met) and susceptible (W_S: WT) α-tubulin variants. In the case of reproductive traits such as seed mass or seed number, the probability of plant survival, when lower than 1.0, integrates in the estimation of fitness cost.[28] Fitness cost estimates may range from 0.99 to 0 indicative of nearly lethal and negligible costs, respectively.

For those cases in which W_R is higher than W_s ($W_R/W_S > 1$), denoting a fitness advantage of the resistant over the susceptible genotype, the relative fitness (RF) is informed and estimated as: $RF = W_R/W_S$.

Data were subjected to two-way ANOVA to test for main and interaction effects of genotype and crop competition on fitness traits. Tukey's HSD test was used to compare mean values ($\alpha = 5\%$). To comply with the assumptions of normal distribution and homoscedasticity, data for seed mass were log-transformed ($y = \log_{10} x + 1$).

3 RESULTS

3.1 Effect of Val–202–Phe and Arg–243–Met mutations on RGR

During early plant growth, production of aboveground and root biomass was severely limited in plants with the 243Met genotype, compared with WT and the 202Phe genotype (Figures 1 and 2). This was due to a significant effect of the 243Met genotype on shoot and root RGR ($p < 0.0001$). Dwarfism with twisted and helical growth in leaves was observed in plants with the 243Met genotype (Figure 1C).

Seedlings of the 243Met genotype showed a 40% reduction in shoot RGR (0.09 day^{-1}) compared with WT (0.15 day^{-1}) and 202Phe (0.15 day^{-1}) plants during a 6-week growth period after transplanting (Figure 1). The reported mean RGR estimate for WT and 202Phe does not account for the higher RGR associated with the 202Phe genotype compared with WT during the last 34–44-day period (Figure 1, data not shown). This RGR differential is likely to explain the higher cumulative aboveground vegetative biomass of 202Phe plants compared with WT plants on day 44 at the end of the growth period ($p < 0.05$; Figure 1).

A significant reduction of 40% was also observed in root RGR of 243Met plants (0.09 day^{-1}) compared with both WT and 202Phe plants which showed a similar root RGR (0.16 day^{-1}) (Figure 2). Whereas the root RGR estimated during the 44-day growth period was similar between WT and 202Phe plants, the cumulative root biomass of 202Phe at 44 DAT was significantly higher than WT ($p < 0.05$) (Figure 2).

FIGURE 1. (A) Changes in aboveground biomass of homozygous 202Phe (■), 243Met (▲) and wild-type (WT) (○) *Lolium rigidum* genotypes in the absence of herbicide treatment under glasshouse conditions. Data are means ± SE ($n = 25$). Different lower case letters indicate significant differences in the relative growth rate (RGR) of shoots within a 6-week growth period and shoot–leaf biomass among genotypes at 44 day after transplanting according to Tukey's HSD test ($\alpha = 5\%$). (B) Growth of homozygous (RR) 202Phe, 243Met and WT genotypes 44 days after transplanting. (C) Twisted and helicoidal leaves and reduced aboveground growth in homozygous (RR) 243Met plants.

3.2 Effect of temperature and homozygosity of Arg–243–Met mutation on RGR

To further investigate the reduction in shoot and root RGR associated with the 243Met genotype, the effect of environmental temperature (26°C versus 18°C) and homozygosity of the mutant allele (RR versus RS) on growth was assessed. Regardless of the genotype (WT, 243Met-RS, 243Met-RR), shoot RGR was reduced when plants were grown at 26°C rather than at 18°C ($p = 0.02$) (Figure 3A,B). In both temperature conditions, however, the homozygous 243Met-RR genotype had 32% lower shoot RGR than both the heterozygous 243Met-RS and WT plants ($p < 0.0001$) (Figure 3). By contrast, root RGR was not affected by the temperature (26°C or 18°C) at which the plants were grown ($p = 0.11$). However, homozygous 243Met-RR plants also showed

a significant reduction of 30% in root RGR compared with heterozygous 243Met-RS and WT plants at both temperature conditions ($p < 0.0001$) (Figure 4).

No significant differences ($p > 0.05$) in shoot and root RGR were evident between the heterozygous 243Met-RS and WT plants regardless of the temperature conditions during the 3-week growth period (Figures 3 and 4). However, clear differences in final growth were evident between the heterozygous 243Met-RS and WT plants at 44 DAT, with WT always exhibiting higher shoot–leaf and root biomass than 243Met-RS (Figures 3 and 4). These growth differences between the heterozygous 243Met-RS and WT plants, despite no differences in RGR, corresponded to significant differences in plant size at 24 DAT (the harvest before the RGR analysis) between the genotypes, with the heterozygous 243Met-RS exhibiting an intermediate growth response compared with WT and homozygous 243Met-RR (data not shown).

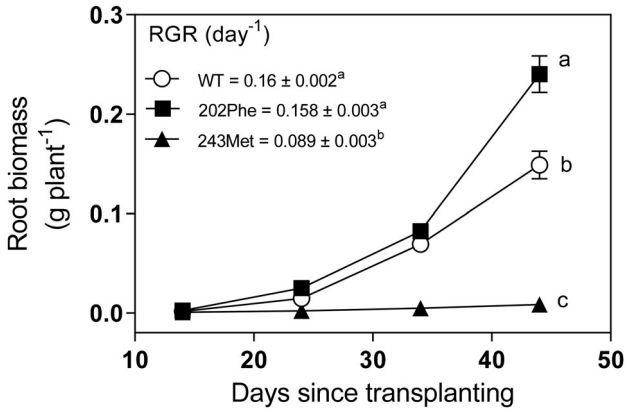

FIGURE 2. Changes in root biomass of homozygous 202Phe (■), 243Met (▲) and wild-type (WT) (○) *Lolium rigidum* genotypes in the absence of herbicide treatment under glasshouse conditions. Data are means ± SE ($n = 25$). Different lower case letters indicate significant differences in the relative growth rate (RGR) of roots within a 6-week growth period and root biomass among genotypes at 44 days after transplanting according to Tukey's HSD test ($\alpha = 5\%$).

3.3 Effect of Val–202–Phe and Arg–243–Met mutations on fitness traits under crop competition

For all evaluated fitness traits, a significant interaction effect of genotype × crop competition was observed ($p < 0.0001$) (Figures 5–7), and this was due to the 243Met genotype being more responsive to the high competition effect from the crop. Without crop competition, plants of the 243Met genotype had 50%, 17% and 24% of the aboveground biomass, seed mass and seed number, respectively, produced by WT plants (Figures 5–7). By contrast, the fitness traits observed in plants of the 202Phe genotype were either similar (seed mass and number) or 20% higher (aboveground biomass) than WT plants in absence of wheat competition (Figures 5–7).

Under wheat competition, 68% of 243Met plants (17 replicates) did not reach and/or completed their reproductive phase and no progeny seed was produced. The 243Met plants that produced seed exhibited 3.8% of the aboveground biomass produced by WT under wheat competition (Figure 5). Similarly, the amount of progeny measured as seed mass and number produced by

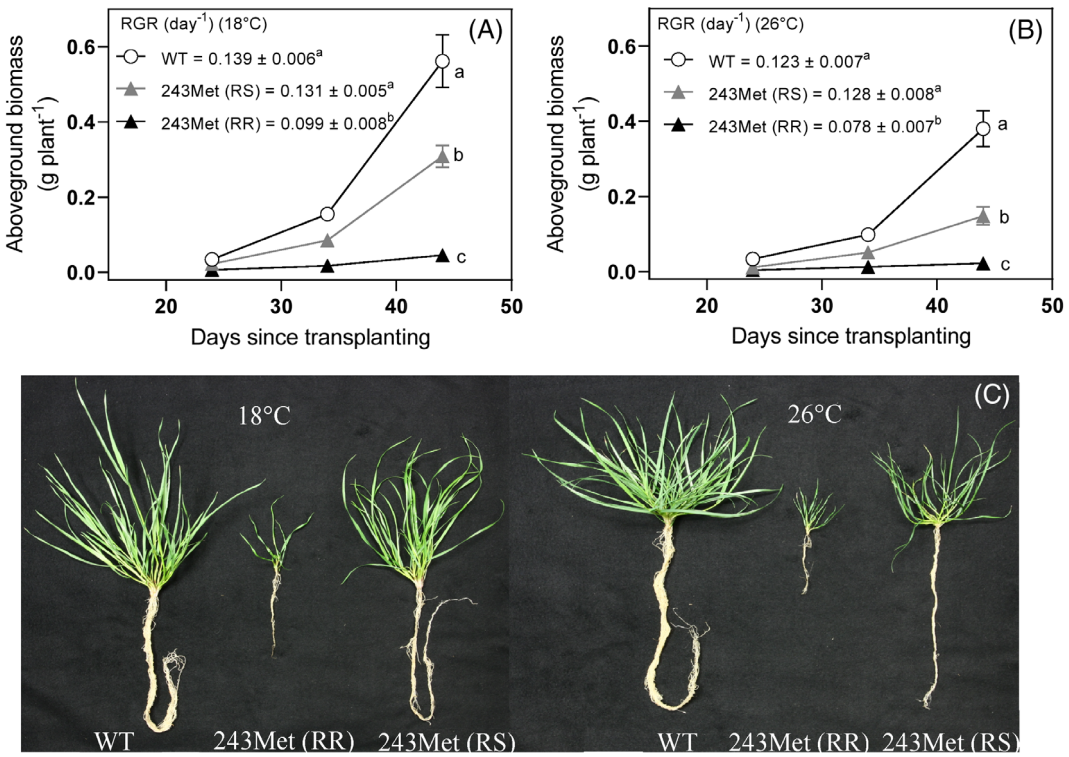

FIGURE 3. Changes in aboveground biomass and relative growth rate (RGR) of *Lolium rigidum* heterozygous (RS), and homozygous (RR) 243Met and wild-type (WT) genotypes estimated during a 3-week growth period under average growing temperature conditions of 18°C (A) versus 26°C (B). Data are means ± SE ($n = 10$). (C) Plant growth of heterozygous (RS) and homozygous (RR) 243Met and WT genotypes 44 days after transplanting under growing temperature conditions of 18°C and 26°C. Different lower case letters indicate significant differences in the RGR of shoots within a 3-week growth period and shoot–leaf biomass among genotypes at 44 days after transplanting according to Tukey's HSD test ($\alpha = 5\%$).

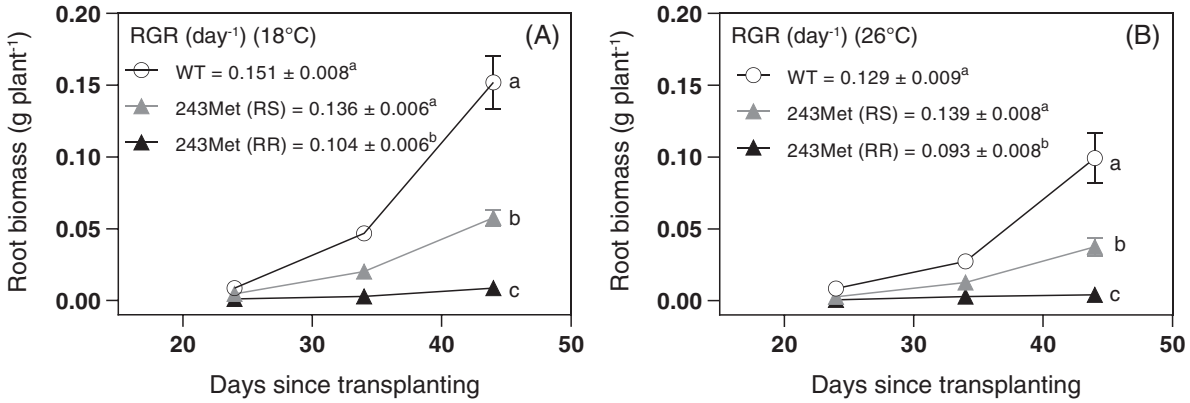

FIGURE 4. Changes in root biomass and relative growth rate (RGR) of *Lolium rigidum* heterozygous (RS), homozygous (RR) 243Met and wild-type (WT) genotypes estimated during a 3-week growth period under average growing temperature conditions of 18°C (A) versus 26°C (B). Data are means ± SE (n = 10). Different lower case letters indicate significant differences in the RGR of roots within a 3-week growth period and root biomass among genotypes at 44 days after transplanting according to Tukey's HSD test (α = 5%).

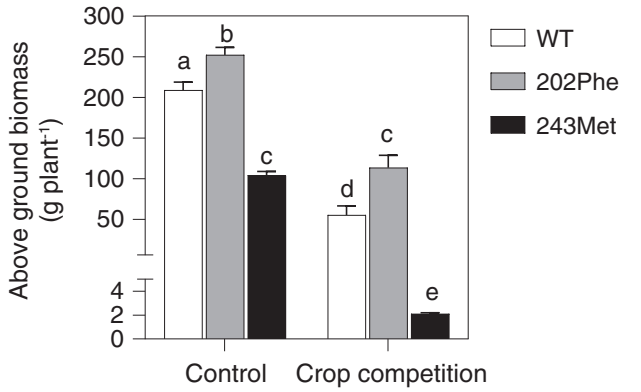

FIGURE 5. Aboveground biomass of *Lolium rigidum* homozygous (RR) genotypes [wild-type (WT), 202Phe and 243Met] under control and competitive conditions with a wheat crop. Bars are mean values ± SE (n = 25). Different lower case letters indicate significant differences among treatments according to Tukey's HSD test (α = 5%).

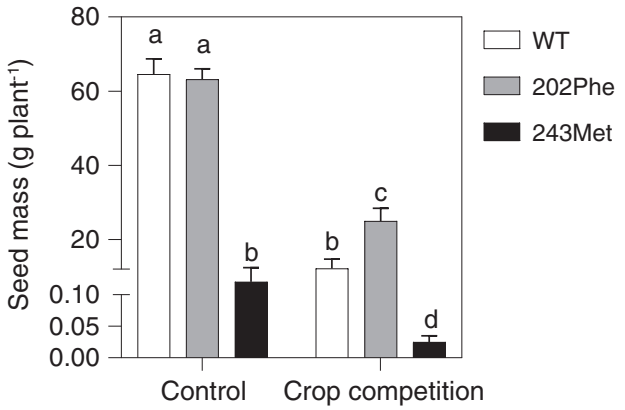

FIGURE 6. Seed mass produced by *Lolium rigidum* genotypes [wild-type (WT), 202Phe and 243Met] under control and competitive conditions with a wheat crop. Bars are mean values ± SE (n = 25), except for 243Met under competition (n = 8). Different lower case letters indicate significant differences among treatments according to Tukey's HSD test (α = 5%).

individual 243Met plants under wheat competition only accounted for, respectively, 0.2% and 0.3% of WT plants growing under similar conditions (Figures 5–7). It is noteworthy that under wheat competition, WT plants showed a range of seed production of eleven thousand and thirty-seven thousand seeds per plant, whereas 243Met plants produced 2–70 seeds per plant (Fig. 7). Interestingly, aboveground biomass, seed mass and seed number produced by 202Phe plants under wheat competition were 1.8- to 2.0-fold higher than WT plants (Figures 5–7).

Based on the assessed plant traits, fitness costs associated with the Val–202–Phe and Arg–243–Met mutations were estimated (Table 1). High fitness costs in terms of vegetative biomass (50%), seed mass (83%) and seed number (75%) were observed in 243Met plants (Table 1). However, the fitness cost imposed by 243Met at the population level (considering the rate of 32% of plants surviving the environmental conditions imposed by the competitive wheat effects), was nearly lethal, with costs above 99% in terms of progeny size (seed mass and number) (Table 1). Estimation of the fitness cost at the individual level (based on all single plants surviving the crop effect) also reached values above 99% (data not shown). Fitness costs associated with 202Phe were not detectable regardless of the presence of the wheat crop. On the contrary, a significant fitness advantage was observed in

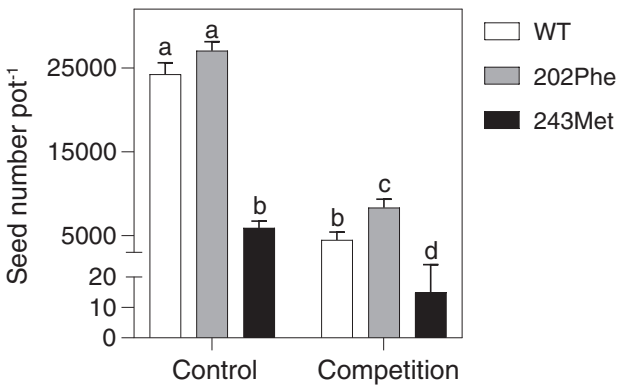

FIGURE 7. Number of seeds produced by *Lolium rigidum* genotypes [wild-type (WT), 202Phe and 243Met] under control and competitive conditions with a wheat crop. Bars are mean ± SE (n = 25), except for 243Met under competition (n = 8). Different lower case letters indicate significant differences among treatments according to Tukey's HSD test (α = 5%).

202Phe compared with WT plants (Table 1). Conversely, the productivity of the wheat crop (aboveground vegetative biomass) varied depending on the effect of the competing target plant

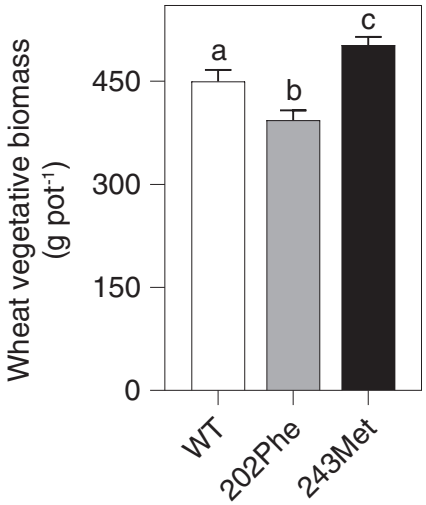

FIGURE 8. Wheat aboveground dry biomass under competition with *Lolium rigidum* homozygous wild-type (WT), 202Phe and 243Met genotypes. Bars are mean estimates ± SE ($n = 25$). Different lower case letters indicate significant differences among treatments according to Tukey's HSD test ($\alpha = 5\%$).

TABLE 1. Fitness cost estimates associated with homozygous 202Phe and 243Met genotypes for α-tubulin resistance mutations Val–202–Phe and Arg–243–Met, respectively, in *Lolium rigidum* relative to wild-type (WT) under control and competitive conditions with a wheat crop in absence of herbicide treatment (Equation 3 in text)

Genotypes	No crop competition	Wheat competition
Aboveground biomass[a]		
202Phe[b]	1.21	2.05
243Met	0.50	0.96
Seed mass[a]		
202Phe[b]	1.02	2.05
243Met	0.83	>0.99
Seed number[a]		
202Phe[b]	1.11	1.86
243Met	0.75	>0.99

[a] Fitness cost values approaching 0.99 or 0 (zero) are indicative of nearly lethal and negligible costs, respectively.
[b] Values higher than 1 denote a fitness advantage of the 202Phe genotype over wild-type, estimated as relative fitness (RF) = W_R/W_S.

genotype ($p < 0.0001$). As expected, the highest wheat productivity was observed in competition with plants of the 243Met genotype, whereas the lowest productivity was achieved when competing with the 202Phe genotype (Figure 8), reflecting clear-cut contrasting competitiveness of the two resistant genotypes.

4 DISCUSSION

Careful control of genetic background and the specific identification of resistance-endowing gene traits are key factors in unequivocally ascribing fitness costs to specific herbicide resistance genes/mechanisms.[4,28] Identification and selection of WT, 202Phe and 243Met genotypes growing within a single *L. rigidum* population[12,20,21] enabled a more accurate assessment of putative fitness costs in this study.

Fitness costs associated with target-site herbicide resistance mutations are likely to be expressed when mutations drive significant changes in the structure and geometry of the herbicide target protein in such a way that catalytic activity of enzymatic proteins or function of structural proteins is somehow impaired. This is the case, for instance, for some particular glyphosate resistance mutations, endowing a high level of glyphosate resistance in bacteria and plants, but also severely compromising enolpyruvylshikimate-3-phosphate synthase substrate affinity and catalytic activity, and thus disturbing cell metabolic pathways, leading to high fitness costs.[6,29,30]

Fitness costs associated with target-site dinitroaniline resistance are expected for any mutations of tubulin genes that have the potential to affect microtubule polymerization, a vital function for normal cell division and the cytoskeleton.[13] Trifluralin can bind to the α-tubulin in *L. rigidum* but less so in plants with resistance endowed by Arg–243–Met/Lys mutations. These amino acid substitutions clash with some important residues (e.g., Arg2 and Gln133) in the trifluralin binding site, causing a spatial shift of the trifluralin-binding domain and creating unstable binding and therefore resistance.[20] However, α-tubulin mutations at the Arg243 residue alter the contact interface with the β-tubulin subunit during certain stages of the microtubule life cycle, resulting in abnormal tubulin polymerization.[20] This abnormal tubulin assembly might be the cause of right-handed helical twisting in young leaves of *L. rigidum* plants homozygous for the Arg–243–Met mutation.[20]

4.1 Deleterious and semi-dominant fitness effects of the α-tubulin Arg–243–Met mutation

The results of this study show the deleterious effects on *L. rigidum* fitness of the dinitroaniline resistance α-tubulin Arg–243–Met mutation in the homozygous state. In an herbicide-free environment, resistant 243Met-RR plants exhibited mortality, severely diminished root and aboveground vegetative growth, and very poor fecundity when compared with WT, which led to fitness costs ranging from 50% to 80% at both the vegetative and reproductive stage. When resistant plants grew and developed in a competitive environment with a wheat crop, the fitness consequence of the Arg–243–Met-RR mutation escalated to a nearly lethal cost (>99.9%). The magnitude of this fitness cost has not been observed for any other target-site resistance mutation in any other field-evolved herbicide-resistant weed species. Likely, the identification of plants with this homozygous resistant genotype was only possible after artificially pairing heterozygous plants with the Arg–243–Met mutation.[20] This result coincides with the lack of detection of the homozygous Arg–243–Met mutation in naturally evolved dinitroaniline-resistant *L. rigidum* populations.[13,20,21,31] Errors in cell division leading to reduced size and a 20% fitness penalty in fecundity have been reported in *Setaria* plants with the α-tubulin Thr–239–Ile resistance mutation in the homozygous state.[23]

By contrast, fitness cost for plants heterozygous (RS) for the Arg–243–Met mutation was not evident in terms of plant mortality rates and RGR estimates for both root and aboveground vegetative biomass, which were similar to WT and significantly higher than 243Met-RR under contrasting temperature growth conditions. However, lack of RGR differences between WT and heterozygous 243Met-RS plants at contrasting temperature growth

conditions did not preclude plants with 243Met-RS exhibiting lower shoot–leaf and root biomass than WT before the RGR measurement (e.g., at 24 DAT), which led to reduced growth of 243Met-RS compared with WT when evaluated at 44 DAT. This indicates the semi-dominant nature of the fitness cost associated with the Arg–243–Met mutation[32] which likely indicates that tubulin polymerization and microtubule assembly are still operational in RS plants where at least half of the expressed α-tubulin is WT.

Lolium rigidum plants heterozygous for the Arg–243–Met mutation have been shown to have reduced dwarfism and right-handed helical growth compared with plants homozygous for the mutation at the earliest plant growth stage, with return to normal growth during early vegetative growth.[20] Previous work from our laboratory has also documented that rice seedlings expressing the Arg–243–Met mutation exhibited abnormal leaf morphology and slow growth.[13] Alpha-tubulin protein is critical for cell elongation and division and various α-tubulin rice mutants have been shown with distorted growth and dwarfism.[33]

Dinitroaniline herbicides disrupt microtubule assembly in not only plants, but also protozoan parasites.[34,35] Several α-tubulin target-site mutations endowing dinitroaniline resistance in the protist *Toxoplasma gondii* have been shown to have detrimental effects on parasite fitness.[22] The resistant parasites exhibit abnormal morphology, altered nuclear division and a 5–13% increase in replication defects.[36]

Some experimental evidence helps predict that evolution of the homozygous Arg–243–Met mutation in *L. rigidum* is unlikely to be selected in cropping systems. Constraints arise from the almost lethal fitness costs of the Arg–243–Met at the homozygous state as shown in this study, the likely incomplete recessive trait of dinitroaniline resistance [i.e., resistance is only evident in homozygous (RR) but not heterozygous (RS) 243Met genotypes],[20,37] and the finding of compound heterozygous *L. rigidum* plants with the Arg–243–Met and other resistance mutations co-occurring in the field via outcrossing.

Under field conditions, homozygous 243Met-RR plants likely arise from obligatory crossing among heterozygous 243Met-RS plants that survived dinitroaniline herbicide applications. As the results suggest, a very low frequency of 243Met-RR plants would remain in fields under no crop competition and no herbicide treatment. However, crop competition would lead to the likely loss of this homozygous genotype as a result of a high level of population mortality and poor growth and fecundity.[38,39]

4.2 Positive fitness effects of the homozygous α-tubulin Val–202–Phe mutation

Whereas the nearly lethal effects conferred by the Arg–243–Met-RR mutation were evident in population fitness, fitness costs in homozygous plants exhibiting the α-tubulin Val–202–Phe resistance mutation were undetectable in the tested environment. Rather, the results of this study showed a surprising twofold fitness advantage in vegetative growth and fecundity of 202Phe plants when compared with WT in a competitive environment. This may not only reflect that the Val–202–Phe mutation is the most common field-evolved α-tubulin mutation reported in *L. rigidum*,[13,20] but also account for the higher loss in wheat productivity when the crop was competing with 202Phe compared with WT.

A fitness advantage associated with a herbicide resistance mutation contradicts evolutionary ecology principles and theory,[40–43] although a few studies have also reported positive fitness effects of target-site resistance mutations[44] and gene overexpression.[45] The mechanistic basis for this fitness advantage requires an understanding of the molecular effects of the Val–202–Phe mutation on microtubule polymerization and functionality. Further research is needed to elucidate whether the reported fitness advantage associated with the Val–202–Phe is a direct effect of compensatory mutations at the α-tubulin gene,[36] or an indirect effect of co-segregation of non-resistance loci and/or selection of evolved fitness-enhancing alleles.[46] A fitness advantage associated with a resistance mutation can arise as sequential evolution of other α-tubulin compensatory mutations that not only reverse the fitness penalty,[36] but also increase fitness. Several single nucleotide polymorphisms have been identified in the α-tubulin genes, some of which are not associated with dinitroaniline resistance in *L. rigidum*.[47] The implications of these single nucleotide polymorphism mutations on the fitness of plants carrying the Val–202–Phe mutation may become clearer after further evaluation. Ultimately, a long-term experiment showing an increase in the Val–202–Phe mutation frequency over generations in the absence of herbicide treatment would validate the fitness advantage associated with this resistance mutation.

Similar to the acetyl-CoA carboxylase Ile–1781–Leu resistance point mutation, a fitness advantage associated with the Val–202–Phe would suggest no apparent constraints for the allele to persist and be fixed in *L. rigidum* and other species, in the absence of herbicide selection.[48–50] Therefore, reducing dinitroaniline herbicide selection pressure is unlikely to decrease the population frequency of the Val–202–Phe mutation, and other weed control strategies (alternate chemical, mechanical, biological and cultural) would be required to delay and mitigate resistance evolution associated with this particular mutation.

5 CONCLUSION

Target-site resistance to dinitroaniline herbicides bestowed by the α-tubulin Arg–243–Met mutation can have an exceptional fitness cost with nearly lethal effects on resistant *L. rigidum* plants. This extraordinary adaptation cost associated with the Arg–243–Met mutation is evident in homozygous resistant plants (i.e., likely semi-dominant fitness cost) with nearly lethal effects on both plant survival and reproduction. This finding helps understand the absence of homozygous 243Met plants in natural *L. rigidum* populations.[13] The fitness advantage observed in *L. rigidum* plants homozygous for the resistance α-tubulin Val–202–Phe mutation merits further investigation.

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AUTHOR CONTRIBUTIONS

Yanhui Wang, Qin Yu and Martin Vila-Aiub designed this work. Yanhui Wang, Heping Han and Jinyi Chen performed the experiments. Yanhui Wang, Qin Yu and Martin Vila-Aiub analyzed the data and wrote the manuscript. Hugh J. Beckie and Stephen B. Powles revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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