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Genetic inheritance of dinitroaniline resistance in an annual ryegrass population

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ABSTRACT

The increasing number of weedy species resistant to dinitroaniline herbicides warrants studies on the evolutionary factors contributing to resistance evolution, including genetic inheritance of resistance traits. In this study, we investigated the genetic control of trifluralin resistance in a well-characterised *Lolium rigidum* Gaud. population from Western Australia. This population was purified to contain plants homozygous for the Val-202-Phe α-tubulin mutation, and used as the resistant (R) parents and crossed with susceptible (S) parents to produce eight reciprocal F1 families. Trifluralin dose response curves of the eight F1 families indicate that trifluralin resistance in this population is inherited as an incomplete recessive nuclear trait. The F1 plants were crossed within each families to establish eight pseudo-F2 (ψ-F2) families. Segregation ratio of resistance and susceptibility in ψ-F2 families were determined using the discriminating trifluralin rates of 120 and 480 g a.i. ha^{-1}. At 480 g a.i. ha^{-1} trifluralin, the segregation ratio in almost all ψ-F2 families (except one) was fit to 1:3 (resistance: susceptibility) one recessive gene control model. However, at 120 g a.i. ha^{-1} trifluralin, the segregation ratios in half of the families did not fit this model, indicating involvement of one or more genes in resistance at the lower rate. These results showed complexity of genetic inheritance of trifluralin resistance in this *L. rigidum* population possessing the Val-202-Phe α-tubulin mutation.

1. Introduction

The anti-microtubule, pre-emergence dinitroaniline herbicides (e.g. trifluralin, pendimethalin, oryzalin, etc.) are globally used to control weeds in grain crops, cotton, soybean, turf grass and many vegetable crops. One of the most commonly used dinitroaniline herbicides, trifluralin, plays a vital role in controlling grass weeds in Australian no-till farming systems [1]. Trifluralin works by binding to microtubules in actively dividing cells of germinating seeds/seedlings. In these mitotic cells, microtubules form a spindle apparatus that correctly positions chromosomes at the cell midplane and then directs separated chromatids to opposite ends of the cell [2]. Trifluralin disrupts the polymerisation of microtubules, and the loss of spindle apparatus arrests the mitosis at prometaphase, resulting in isodiametric cells with abnormal, polymorphic nuclei [3].

Trifluralin resistance in the important grass weed *Lolium rigidum* was first reported in 1995 in South Australia where trifluralin had been applied repeatedly for years [4,5]. Since then, the number of *L. rigidum* populations resistant to trifluralin has been increasing across Australia [6–8], and trifluralin-resistant *L. rigidum* populations from South and Western Australia have recently been characterised [9,10]. Dinitroaniline resistance has also been reported in *Eleusine indica* [11], *Setaria viridis* [12], *Amaranthus palmeri* [13], *Poa annua* L. [14] and *Alopecurus aequalis* [15]. Thus far, trifluralin resistance mechanisms have only been investigated in a few species, revealing target-site resistance (TSR) due to α-tubulin mutations in *E. indica*, *S. viridis* and *L. rigidum* [16–19]. We have reported both TSR α-tubulin mutations and non-target-site resistance (NTSR) endowed by enhanced trifluralin metabolism in *L. rigidum* [20].

Studies revealing the genetic pattern of dinitroaniline resistance were conducted on two autogamous weed species, *S. viridis* [21] and *E. indica* [22], with Leu-136-Phe, Thr-239-Ile and Met-268-Thr α-tubulin mutations respectively [17,18]. In contrast to the semi-dominant or dominant single gene inheritance pattern of resistance to many herbicides, dinitroaniline resistance is controlled by a single, nuclear, recessive tubulin gene in these two species. This recessive mode of inheritance is relatively uncommon in the herbicide resistance literature, with only homozygous mutants being able to survive the normal herbicide dose. This generally results in relatively slow TSR evolution to dinitroaniline herbicides, as compared to dominant or semi-dormant

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resistance traits [23,24].

Thus far, trifluralin resistance inheritance genetics has not been elucidated in cross-pollinated *L. rigidum*. Therefore, this genetic inheritance study was performed with a well-characterised, highly dinitroaniline-resistant *L. rigidum* population [10] which has the known resistant α-tubulin mutation, Val-202-Phe [10,15]. In this study, pre-genotyped homozygous Val-202-Phe mutants were used as the resistant parent to investigate the heredity of trifluralin resistance in *L. rigidum*.

2. Materials and methods

2.1. Plant material

The *L. rigidum* population (M4/16) collected from a 2010 resistance survey in Western Australia [7] displays high level resistance to dinitroaniline herbicides [10]. In 2015, plants surviving 960 g a.i. ha^{-1} trifluralin were sequenced, and most individuals contain the resistance-endowing Val-202-Phe α-tubulin mutation ([10], unpublished data). More than 20 individuals homozygous for this Val-202-Phe mutation were bulk-crossed to generate a sub population. In 2016, the sub population was sprayed with 960 g a.i. ha^{-1} trifluralin, and survivors genotyped for the 202 locus [10]. Plants confirmed to have the homozygous Val-202-Phe mutation were repotted individually and grown to maturity (hereafter referred to as R plants). A herbicide susceptible population SVLR1 (hereafter referred to as S) [25] was also used.

2.2. Generation of F1 and pseudo-F2 families

Genotyped R plants were pair-crossed with S plants to produce the first filial generation (F1 families). The pot pairs (eight pairs in total) were placed in either an isolated glasshouse at 20/15 °C day/night temperature under natural sunlight, or outdoors in a 1.5 m high plastic enclosure to prevent entry of external pollen. Plants grew during the normal growing season from May to November, 2016. F1 seeds were collected separately from the eight S and eight R mother plants in December, 2016. Seeds harvested from the S and R parent of each pair-cross were named as F1P1S-F1P8S and F1P1R-F1P8R, respectively. A trifluralin dose-response test (see section 2.3) was conducted for the F1 families and the parent populations.

To generate pseudo-F2 families (*ψ*-F2), F1 families with low, moderate and high levels of resistance to trifluralin were initially selected. However due to lack of synchronization in flowering time and the availability of seeds, finally four F1 families were chosen (F1P1S, F1P2S, F1P3S and F1P8S) from the eight F1 maternal susceptible plants. About twenty plants from each of the four F1 families were grown to the flowering stage. Four individuals within each F1 family with a matching flowering time were selected to make two pair-crosses *ψ*-F2, resulting in total eight *ψ*-F2 families, named as F2P1-F2P8.

2.3. Trifluralin dose-response of the parent populations and F1 families

This experiment was conducted between March and May, 2017. Seeds were pre-germinated on moistened filter paper and then sown onto the surface of potting mix as described in [10]. The pots with just-germinating seeds from the parent S and F1 families on the soil surface were treated with trifluralin at 0, 30, 60, 120, 240, and 480 g a.i. ha^{-1} (20 seedlings per pot, three pots per rate), while 0, 120, 240, and 480 g a.i. ha^{-1} was used for the parent R population. Trifluralin (Triflur X 480 g L^{-1} Nufarm®) was applied as a commercial formulation in 118 L ha^{-1} water, delivered in two passes at 200 kPa with a speed of 1 m s^{-1}, using a cabinet sprayer equipped with two flat fan nozzles (TeeJet®XR11001 flat fan, Spraying Systems Co, Wheaton, IL, USA). After spraying, seeds were immediately covered with a 1 cm layer of untreated soil and lightly watered. Treated seedlings were kept in the glasshouse at day/night temperatures of around 25/10 °C under natural sunlight. Survival rate was determined three weeks after trifluralin application, and plants were recorded as survivors if they emerged from the soil and produced healthy new growth.

The herbicide rate causing 50% mortality (LD_{50}) of plants was calculated using SigmaPlot® (version 13.0, 2014 Systat Software, Inc.) software capable of non-linear regression analysis. The data were fitted to the four-parameter log-logistic model

$$y = c + (d - c) / [1 + (x/x_0)^{b}]$$

where d is the upper limit representing plant survival at low herbicide rates close to those of untreated controls, c is the lower limit representing plant survival at infinitely high herbicide rates, x_0 is the trifluralin rate conferring 50% plant survival and b is the slope around x_0. A t-test was conducted to compare the LD_{50} of F1 families from the susceptible and resistant mother plant within one paired cross using the software PRISM (version 5.0; GraphPad Software, Inc., San Diego, CA, USA). The LD_{50} was averaged if no significant differences were identified between the two pair-cross families.

The degree of dominance (D) of trifluralin resistance in F1 families was calculated following the formula

$$D = \frac{2X_2 - X_1 - X_3}{X_1 - X_3}$$

where D is the degree of dominance, and X_1, X_2 and X_3 are the log(LD_{50}) of the R, F1 and S populations, respectively. $D = 0$ indicates intermediate resistance, $D = 1$ complete dominance, $0 < D < 1$ incomplete dominance, $-1 < D < 0$ incompletely recessive and $D = -1$ completely recessive [26].

2.4. Assessing trifluralin resistance segregation in ψ-F2 families

This experiment was conducted between March and May, 2018. Two discriminating trifluralin rates (120 and 480 g a.i. ha^{-1}) were used for the *ψ*-F2 test, based on the responses of the parent and F1 populations in the experiment described in section 2.3. Seeds of the eight *ψ*-F2 families, the corresponding F1 families and the parent families were pre-germinated and sown in plastic trays containing potting mix (300 × 400 × 100 mm, 100 seedlings per tray). Fifty seeds from the S, R and F1 families and 100 seeds from each *ψ*-F2 family were sprayed with trifluralin at the two discriminating rates. The glasshouse conditions were the same as described in 2.3. Plant mortality was assessed 21 days after treatment.

The experimental null hypothesis was that the Val-202 locus was the single locus conferring trifluralin resistance in this *L. rigidum* population. Under this premise, the phenotype segregation ratio of *ψ*-F2 families should follow Mendel's Law as 1R:2F1:1S. The segregation analysis in *ψ*-F2 families was performed based on the observed survival ratio (alive: total treated plants) compared to the expected survival ratio assuming the single gene control model. For the *ψ*-F2 families, the expected number of surviving plants under one-gene control model was calibrated according to the following equation [27,28]:

$$\text{Exp. } \psi\text{-F2} = 0.25 \times \text{Obs R} + 0.5 \times \text{Obs F1} + 0.25 \times \text{Obs S}$$

where R, F1 and S are the percentage of observed survivors of the R, F1 (in this case, derived from S mother plants) and S families at each trifluralin dose (120 and 480 g a.i. ha^{-1}). For each dose, the expected number of surviving plants for *ψ*-F2 families was calculated, with the total number of treated plants multiplied by the theoretical one locus segregation ratio for *ψ*-F2 families.

A Chi-square goodness-of-fit test (χ^2) on segregation of trifluralin resistance in *ψ*-F2 families was performed [27,29]. For one locus segregation, the null hypothesis is that the *ψ*-F2 resistance phenotype segregates as 0.25R:0.5F1:0.25S. The significance level is $\alpha = 0.05$, and if the P-value is < 0.05, the null hypothesis is rejected. A heterogeneity test was conducted to examine for variations in trifluralin resistance among *ψ*-F2 families.

2.5. Genotyping of the ψ-F2 family

After the χ^2 test, further genotyping was performed on one ψ-F2 family that did not fit the one-gene control model ($P < 0.05$). For sequencing convenience, a new pair of primers (LrTubulinF1: GGCCTT GGTTCTCTTCTCCTT, 5-R-seq: CAGGCCATGTACTTGCCGTG) were designed for Val-202 locus inspection using genomic DNA. Genomic DNA was extracted from the leaf tissue (about 100 mg) of ψ-F2 family survivors according to Yu et al. [30]. The PCR cycling ran as follows: 94 °C 5 min, 35 cycles of 94 °C 30 s, 60 °C 30 s, and 72 °C 60 s, followed by a final extension step of 7 min at 72 °C. PCR products were directly sequenced after gel purification, and the chromatogram files of all sequences were visually checked using the Chromas software (version 2.5.1; Technelysium Pty Ltd, Australia).

3. Results

3.1. Trifluralin response of parent populations and F1 families

Dose-response experiments were conducted on S and R parents, and 8 reciprocal F1 families to determine the trifluralin resistance inheritance pattern. No significant differences in LD_{50} values within each reciprocal F1 family were identified except for the reciprocal families of F1P1 and F1P7. Therefore, the LD_{50} within the other six paired F1 families were each averaged for calculation of dominance. The degree of dominance in F1 families varied from incompletely recessive to incompletely dominant (Table 1, Fig. 1). Resistance in the three F1 families (F1P3, F1P4 and F1P8) were identified to be incompletely recessive with the degree of dominance between -0.16 and -0.4 (Table 1), and the dose-response curves were close to the S parent (represented in Fig. 1B). Only one family (F1P5) showed a resistance level intermediate between the R and S (Fig. 1C), with a degree of dominance close to 0, at −0.04 (Table 1). In contrast, two F1 families (F1P2 and F1P6) exhibited incomplete dominant resistance (0.12 and 0.25 respectively) with the dose-response curves much closer to the R parent (Table 1, Fig. 1D). Pooled data from all 8 F1 families (Fig. 1A) demonstrated a resistance level intermediate between the S and R parental populations. Dose-response curves of six out of eight reciprocal F1 families (Table 1), as well as pooled F1 families were similar to each other ($P = 0.5$, Fig. 1A).

3.2. Resistance segregation in ψ-F2 families

When treated at 120 g a.i. ha^{-1}, the observed level of mortality for the four ψ-F2 families (F2P1, F2P2, F2P4 and F2P5) was similar to the predicted values assuming a 1R:2F1:1S phenotype segregation ($P > 0.05$) (Table 2), consistent with a single gene effect. In contrast, the other four ψ-F2 families (F2P3, F2P6, F2P7 and F2P8) did not fit the single-gene hypothesis ($P < 0.05$), with more than expected plants surviving 120 g a.i. ha^{-1} trifluralin treatment (Table 2). For example, 41 out of 100 plants in F2P6 were expected to survive at 120 g a.i. ha^{-1} trifluralin, whereas there were 71 survivors observed (Table 2). In contrast, at 480 g a.i. ha^{-1} trifluralin, most (seven of eight) ψ-F2 families matched the predicted values for a single gene effect ($P > 0.05$), except for the F2P6 ($P < 0.01$, Table 3). Again, the reason for the lack of fit in this family was due to higher than expected survivorship. As the segregation ratio of resistance to susceptibility in ψ-F2 families was close to 1:3, and the survival rate of all F1 progenies close to zero (Table 3), this corresponds to monogenic recessive trait. The heterogeneity was significant at 120 g a.i. ha^{-1} ($P < 0.01$, Table 2), indicating a large variation in resistance segregation among the ψ-F2 families at the lower herbicide rate. However, better homogeneity was achieved at 480 g a.i. ha^{-1} trifluralin ($P = 0.16$, Table 3), indicating less variation among the ψ-F2 families at the higher trifluralin rate.

3.3. Genotyping of the F2P6 family

As the phenotype segregation ratio of F2P6 did not fit the one-gene model at the two discriminating rates due to more than predicted survivors (Table 2&3), it was speculated that in addition to the Val-202-

Table 1
Estimated parameters (*d*, *c* and *b*) and effective trifluralin dose to control 50% of *Lolium rigidum* parents and F1 families.

Population[a]	Parameter[b]			LD_{50} (SE)[c]	P-value[d]	Degree of dominance[e]
	d (SE)	*c* (SE)	*b* (SE)			
S parent	99 (2.4)	−1.7 (2.2)	2.2 (0.2)	54 (2.7)	< 0.0001	–
R parent	101 (2)	−2.2 (2.3)	2.5 (0.2)	733 (25)		–
F1P1S	99 (0.9)	−0.3 (1.9)	2.8 (0.2)	150 (3.8)	< 0.05	–
F1P1R	99 (0.5)	−1.1 (0.9)	4.4 (0.1)	175 (2.1)		
F1P2S	104 (3.7)	−27 (33)	2 (0.6)	287 (86)	0.8	0.25
F1P2R	100 (3.0)	−13 (21)	2 (0.5)	262 (61)		
F1P3S	100 (2.7)	2.7 (5.4)	2.7 (0.5)	142 (11)	0.1	−0.16
F1P3R	101 (2.4)	4.9 (6.2)	2.7 (0.5)	180 (14)		
F1P4S	102 (3.9)	−20 (19)	1.7 (0.4)	204 (46)	0.1	−0.2
F1P4R	101 (6.9)	8.2 (7.3)	4.3 (2.2)	101 (13)		
F1P5S	100 (1.1)	−1.6 (2.4)	3.6 (0.3)	186 (5.3)	0.9	−0.05
F1P5R	95 (4.4)	3.1 (7.8)	6 (2.5)	189 (23)		
F1P6S	101 (0.9)	−1.5 (3.3)	3.3 (0.3)	228 (6.8)	0.7	0.12
F1P6R	103 (2.3)	−5.8 (11)	2.6 (0.5)	237 (24)		
F1P7S	102 (2)	−3.4 (3.9)	2.7 (0.3)	138 (7.1)	< 0.05	–
F1P7R	106 (6.6)	1.7 (3.9)	3.5 (0.7)	106 (6.6)		
F1P8S	100 (2)	−1.6 (3)	2.7 (0.3)	114 (5.1)	0.4	−0.4
F1P8R	98 (1.7)	3.1 (2.4)	3.2 (0.4)	121 (4.3)		
F1 pooled	99 (1.1)	−1.7 (2.8)	2.5 (0.2)	167 (6)	–	−0.13

[a] S, trifluralin-susceptible *L. rigidum* population SVLR1; R, trifluralin-resistant *L. rigidum* population M4/16 collected from Western Australia in 2010. F1S1-F1S8, F1R1-F1R8 are progeny resulting from crosses of S × R parents, harvested from the S and R mother plants, respectively.

[b] *d*, the upper limit; *c*, the lower limit; *b*, the slope.

[c] LD_{50} (g a.i. ha^{-1}), the effective dose of trifluralin needed to reach 50% control of a population. SE, standard error.

[d] Significance of a *t*-test comparison of LD_{50} values between the S and R parents, and between the pairs of F1 families resulting from each S × R cross (e.g. F1P1S vs. F1P1R, etc.). A P-value > 0.05 means there was no significant difference between families.

[e] Degree of dominance was calculated using the formula $D = [(2X_2\text{-}X_1\text{-}X_3)/(X_1\text{-}X_3)]$, where X1, X2 and X3 represent the log (LD_{50}) of the R, F1 and S populations, respectively.

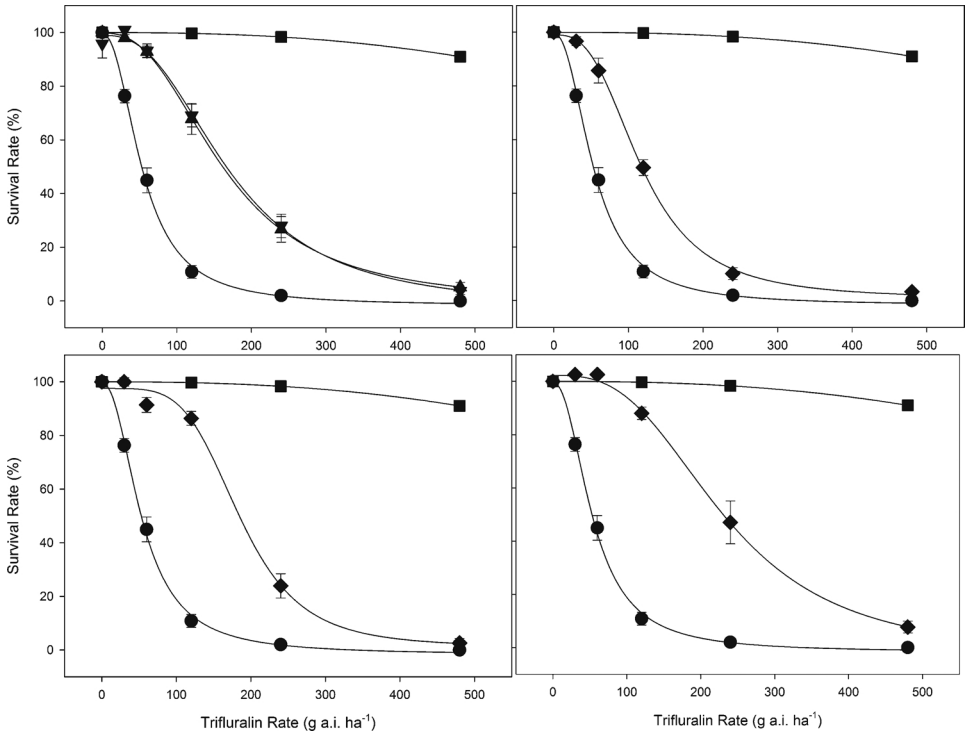

Fig. 1. Trifluralin dose-response of the parental susceptible (S) (circle), parental resistant (R) (square), F1 maternal S (F1S, up triangle), F1 maternal R (F1R, down triangle) and combined F1 (diamond) populations of *L. rigidum*. Data in A are the means of all 8 F1 families, and data in B, C and D are from single F1 families F1P8, F1P5 and F1P6 representing three different types of responses to trifluralin, respectively.

Table 2
Phenotypic resistance segregation observed in eight pseudo-F2 (F2) families and their corresponding F1 families at a trifluralin dose of 120 g a.i. ha^{-1}. Chi-square (χ^2) analysis for expected F2 plant survival assuming trifluralin resistance controlled by one major allele. Survivors were plants that emerged and grew as expected for healthy plants.

Family name	Observed		Total	Expected		χ^2	*P*
	Died	Survived		Died	Survived		
F2 segregation				1R:2F1:1S			
F2P1	46	54	100	51	49	1.00	0.32
F2P2	50	50	100	51	49	0.04	0.84
F2P3	44	56	100	54	46	4.03	0.04
F2P4	49	51	100	54	46	1.01	0.32
F2P5	65	35	100	59	41	1.49	0.22
F2P6	29	71	100	59	41	37.21	< 0.01
F2P7	20	80	100	63	37	79.32	< 0.01
F2P8	36	64	100	63	37	31.27	< 0.01
Total	339	461	800	454	346	67.35	< 0.01
Heterogeneity test						88.01	< 0.01
S	48	2	50				
R	2	48	50				
F1P1S	26	24	50				
F1P1R	27	23	50				
F1P2S	29	21	50				
F1P2R	24	26	50				
F1P3S	34	16	50				
F1P3R	31	19	50				
F1P8S	38	12	50				
F1P8R	26	24	50				
Total F1	235	165	400				

Table 3
Phenotypic resistance segregation observed in eight pseudo-F2 (F2) families and their corresponding F2 families at a trifluralin dose of 480 g a.i. ha^{-1}. Chi-square (χ^2) analysis for expected F2 plant survival assuming trifluralin resistance controlled by one major allele. Survivors were plants that emerged and grew as expected for healthy plants.

Family name	Observed		Total	Expected		χ^2	*P*
	Died	Survived		Died	Survived		
F2 segregation				1R:2F1:1S			
F2P1	80	20	100	79.5	20.5	0.02	0.90
F2P2	82	18	100	79.5	20.5	0.38	0.54
F2P3	84	16	100	82.5	17.5	0.16	0.69
F2P4	84	16	100	82.5	17.5	0.16	0.69
F2P5	77	23	100	81.5	18.5	1.34	0.25
F2P6	70	30	100	81.5	18.5	8.77	< 0.01
F2P7	81	19	100	79	21	0.24	0.62
F2P8	80	20	100	82.5	17.5	0.43	0.51
Total	638	162	800	645.5	154.5	0.45	0.50
Heterogeneity test						10.63	0.16
Parental S	50	0	50				
Parental R	15	35	50				
F1P1S	47	3	50				
F1P1R	50	0	50				
F1P2S	50	0	50				
F1P2R	49	1	50				
F1P3S	49	1	50				
F1P3R	50	0	50				
F1P8S	50	3	50				
F1P8R	48	2	50				
Total F1	393	7	400				

Phe mutation, some other genes may also contribute to plant survival. Sequencing results exhibited that, in the 30 F2P6 plants surviving 480 g a.i. ha^{-1} trifluralin, there were 18 homozygous mutant Phe-202 (RR), 6 heterozygous (RS) and 6 homozygous wild type Val-202 (WT) plants. As the expected number of survivors at 480 g a.i. ha^{-1} is 18.5 and they are all expected to be homozygous Phe-202 mutants, it was the 12 additional surviving plants (RS and WT) that caused skew to the theoretical values.

4. Discussion

With the identification that a Val-202-Phe α-tubulin gene mutation in this *L. rigidum* population endows resistance to trifluralin and other dinitroaniline herbicides (Chen et al. 2018, and unpublished data), we examined genetic inheritance of trifluralin resistance in this population. The genetic inheritance study was carried out using F1 and *ψ*-F2 families derived from pre-genotyped parental plants. Overall, trifluralin

resistance inheritance endowed by the Val-202-Phe mutation in this *L. rigidum* population was identified to be semi-recessive over the dose range tested. At specific rates, the mutant α-tubulin is inherited as a single, recessive nuclear gene at 480 g a.i. ha^{-1} trifluralin. And at low trifluralin dose (120 g a.i. ha^{-1}) other minor genes may play a role. This research emphasizes the necessity to use multiple herbicide rates for the determination of resistance inheritance trait, as used in other genetic studies [28,31–33].

4.1. Dominance of trifluralin resistance inheritance in L. rigidum

Trifluralin dose-response and LD_{50} values showed no significant difference in most (six out of eight) reciprocal F1 family pairs (Table 1). Also, the pooled dose-response curves from the eight F1 family pairs exhibited no obvious maternal inheritance pattern (Fig. 1A). Hence, trifluralin resistance trait in this population is nuclear-encoded. Generally, an incomplete recessive genetic inheritance pattern was identified in F1 families (Fig. 1A). This result partially concurs with the recessive inheritance pattern of dinitroaniline resistance in *E. indica* and *S. viridis*. The variations in genetic dominance of trifluralin resistance observed in F1s (Fig. 1), and between the current and previous studies, are likely due to the herbicide rates used and the cross-pollination nature of *L. rigidum*. Doses of trifluralin were applied here to *L. rigidum* F1 families, whereas single discriminating herbicide rates were used to determine the recessive inheritance trait in *E. indica* [22] and *S. viridis* [18]. The resistance heredity traits may vary with herbicide rates used, necessitating the use of multiple relevant herbicide rates (e.g. discriminating and/or field recommended rates) to evaluate the dominance level of resistance [28,31–33]. In addition, different from highly self-pollinated *E. indica* and *S. viridis* species, *L. rigidum* is an obligate cross-pollinated species with great genetic diversity, and this is reflected in trifluralin dose-response curves of F1 families from different parental pairs.

4.2. Gene number controlling trifluralin resistance inheritance in L. rigidum

Generally, different number of gene loci contribute to trifluralin resistance at different rates in this *L. rigidum* population. At 480 g a.i. ha^{-1} trifluralin, a single, recessive nuclear gene is responsible for resistance (Table 3), which is the α-tubulin gene containing the Val-202-Phe mutation in this *L. rigidum* population. The recessive TSR inheritance pattern in *L. rigidum* resembles that in *E. indica* [22] and *S. viridis* [18], which partially explains the relative slow dinitroaniline evolution in weeds. At the lower trifluralin rate (120 g a.i. ha^{-1}), other minor genes may also be involved in resistance in the four ψ-F2 families (F2P3, F2P6, F2P7 and F2P8) (Table 2). Particularly, in the genotyped ψ-F2 family F2P6 that consistently does not follow a single gene mode at both discriminating rates, other genes in addition to the 202 mutant α-tubulin gene are demonstrated to contribute to resistance, as the 12 heterozygous and wild type plants unexpectedly survived the treatment of 480 g a.i. ha^{-1} trifluralin. These minor resistance-conferring genes are unknown but possibly metabolism-based NTSR genes, as has been identified in other trifluralin resistant *L. rigidum* populations [20,34].

Herbicide resistance is mostly inherited and controlled by an incomplete dominant gene [23]. In *L. rigidum*, for example, resistance to paraquat [32], glyphosate [35,36] and ACCase-inhibitors [37] were demonstrated to be controlled by a single major dominant or semi-dominant nuclear gene. In contrast, recessive herbicide resistance inheritance is rare and only revealed in limited weedy species. Resistance to dinitroaniline herbicides conferred by the nuclear α-tubulin mutations Thr-239-Ile and Leu-136-Phe in *E. indica* and *S. viridis* [17,18], together with the Val-202-Phe in *L. rigidum* here at 480 g a.i. ha^{-1} trifluralin were all established to be inherited recessively. Another case is resistance to auxinic herbicides in *Centaurea solstitialis* conferred by a single nuclear recessive gene [38], with the molecular basis as yet unknown.

4.3. Trifluralin resistance inheritance in L. rigidum

It follows that dinitroaniline herbicides should have less chance for TSR evolution, as homozygosity is required for plant survival under field rates. Particularly, as plant tubulins are encoded by a small nuclear gene family with multiple gene copies [39,40], it is anticipated that a heterozygous tubulin mutation may be insufficient in endowing resistance. However, dinitroaniline herbicide resistance evolution in *L. rigidum* cannot be underestimated, as the obligate cross-pollinated nature and the great genetic diversity helps overcome the recessive nature of resistance. In comparison to self-pollinated weedy species, *L. rigidum* is capable of accumulating various resistance-conferring genes in single plants by cross-pollination, either TSR or NTSR based. For example, individual plants stacking multiple tubulin mutations at 202 + 243, 202 + 239, or 239 + 243 were reported in *L. rigidum* populations [9,19]. At the same time, NTSR may coexist with TSR in this and other dinitroaniline resistant populations, facilitating resistance evolution in *L. rigidum*. For instance, using relatively higher trifluralin rates may be effective in controlling heterozygous TSR mutants, but may not be sufficient for "TSR + NTSR" or "NTSR" individuals. Therefore, simply increasing herbicide application rates, or using alternative herbicides cannot ultimately fix the resistance problem. Mechanical and agronomic weed management tactics (harvest weed seed control, crop competition etc.), additional to judicious and diverse chemical controls should be integrated to achieve better results.

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