Research Article

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# Challenging glyphosate resistance *EPSPS* P106S and TIPS mutations with soybean competition and glyphosate: implications for management

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## Abstract

**BACKGROUND: *Eleusine indica* (L.) Gaertn. (goosegrass) is a major weed in global cropping systems. It has evolved resistance to glyphosate due to single Pro-106-Ser (P106S) or double Thr-102-Ile + Pro-106-Ser (TIPS) *EPSPS* target site mutations. Here, experiments were conducted to evaluate the single effect of soybean competition and its combined effect with a glyphosate field dose (1080 g ae ha$^{-1}$) on the growth and fitness of plants carrying these glyphosate resistance endowing target site mutations.**

**RESULTS: TIPS *E. indica* plants are highly glyphosate-resistant but the double mutation endows a substantial fitness cost. The TIPS fitness penalty increased under the effect of soybean competition resulting in a cost of 95%, 95% and 96% in terms of, respectively, vegetative growth, seed mass and seed number investment. Glyphosate treatment of these glyphosate-resistant TIPS plants showed an increase in growth relative to those without glyphosate. Conversely, for the P106S moderate glyphosate resistance mutation, glyphosate treatment alone reduced survival rate, vegetative growth, aboveground biomass (34%), seed mass (48%) and number (52%) of P106S plants relative to the glyphosate nontreated plants. However, under the combined effects of both soybean competition and the field-recommended glyphosate dose, vegetative growth, aboveground biomass, seed mass and number of P106S and TIPS plants were substantially limited (by ≤99%).**

**CONCLUSION: The ecological environment imposed by intense competition from a soybean crop sets a significant constraint for the landscape-level increase of both the *E. indica* single and double glyphosate resistance mutations in the agroecosystem and highlights the key role of crop competition in limiting the population growth of weeds, whether they are herbicide-resistant or susceptible.**

*Supporting information may be found in the online version of this article.*

**Keywords:** fitness; herbicide resistance; goosegrass; soybean

## 1 INTRODUCTION

Agroecosystems are highly productive environments in which weed species can thrive and must be controlled to protect crop yield. For field crops, herbicides are ubiquitously used and, consequently, herbicide-resistant weeds have evolved. Thus, an agroecosystem landscape has both natural and human-driven selection, especially herbicide selection. Herbicide-resistant weed biotypes show a singular advantage in the presence of the herbicide (resistance benefit) but can exhibit a fitness cost in the absence of the herbicide. As a result, the evolutionary trajectory of herbicide resistance endowing gene mutations is defined by the resulting net effect between the benefit and cost of herbicide resistance within an agricultural landscape.[1,2]

The resistance benefit in an environment under herbicide selection is the exceeding fitness endowed by the resistance mutation over the herbicide-susceptible wild-type (WT). Resistance benefit is a useful trait that accounts, at the population level, for the

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relative fitness of a resistant over the susceptible genotype.[2,3] The magnitude of this resistance benefit, estimated for a particular herbicide and dose, will depend on the specific resistance mutation (i.e. gene and allele), genetic background (i.e. weed species, allelic dominance, ploidy) and environmental conditions (see previous reviews[1,4–8]). For instance, some particular herbicide resistance mechanisms confer a higher fitness benefit (i.e. estimated as plant survival) under warmer *versus* colder temperature conditions.[9,10] A maximum resistance benefit is expected when a resistance mutation minimizes herbicide damage to the extent of endowing a fitness level comparable to that of herbicide non-treated plants.

In general, a genotype displaying a resistance trait to stress is expected to express a cost compared to the susceptible genotype in a stress-free environment (see previous reviews[11–13]). Thus, plants with herbicide resistance mutations may express a fitness cost in a herbicide-free environment in comparison with the WT. However, studies show that herbicide resistance fitness costs are not universal and may depend on specifics of a particular resistance gene mutation, allele, genetic background and ecological environment.[5,11,13–15] For example, the triazine resistance photosystem II *psbA* gene substitution mutation encoding for a serine-264-glycine substitution or cytochrome P450-based enhanced herbicide metabolism have been shown to attract a 20–30% plant fitness cost, contrary to other herbicide resistance traits with negligible fitness penalties.[5,16,17]

If a resistance gene mutation confers a high benefit in the presence of a herbicide and only a negligible cost in the absence of a herbicide, then the resistance mutation can be rapidly fixed at high frequency at the agricultural landscape level. Benefit and cost are opposite sides of plant fitness ($W$) which is a function of the proportion of plants that survive ($S$) from seed dispersal to reproduction and the fecundity ($F$) produced by the surviving adult plants ($W = S \times F$).[18,19]

*Eleusine indica* (L.) Gaertn. (goosegrass), a warm-season $C_4$ annual species is considered to be one of the worst weeds in modern agriculture as a result of its rapid growth, high fecundity, tolerance to drought and heat conditions, and detrimental effects on multiple crops, including corn, soybean, upland rice and cotton, as well as fruit and vegetable orchards.[20] Globally *E. indica* is a weed of soybean (*Glycine max*) crops,[20] and in transgenic glyphosate-tolerant soybean, glyphosate is universally used for *E. indica* control. Consequently, glyphosate resistance evolution has occurred in many populations present in agroecosystems across the globe, since the first reports from Malaysian orchards and oil palm plantations two decades ago.[21–24]

*EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) gene amplification, upregulation and specific point mutations are mechanisms shown to confer target site glyphosate resistance in *E. indica*.[23,25-32] The Pro-106-Ser *EPSPS* point mutation (P106S) has been identified in glyphosate-resistant *E. indica*.[33] In previous studies, we determined that this target site mutation confers a moderate level of glyphosate resistance (4–8-fold) with no evident plant fitness cost.[3,32–34] Notably, a rare and unique double glyphosate resistance *EPSPS* mutation comprising both the Thr-102-Ile and Pro-106-Ser substitutions (hereinafter termed TIPS mutation) has been identified in *E. indica* and shown to endow very high-level glyphosate resistance (113–182-fold) but with a major plant fitness cost (50–85%).[3,29,30,32,34,35] However, estimates of fitness benefit in environments with the joint selection effect of glyphosate treatment and plant competition are lacking and thus our understanding on the rate of enrichment of this glyphosate resistance *EPSPS* mutations (P106S *versus* TIPS) is limited. Likewise, estimates of fitness costs associated with these mutations require a further assessment when in competition with soybean crops.

This study aimed to quantify the single and the combined effects of soybean competition and glyphosate on *E. indica* plants expressing either the *EPSPS* P106S or the TIPS mutation. The results give insight into the evolutionary dynamics of these mutations in an agroecosystem and can contribute to the design of effective management practices for herbicide-resistant populations.

## 2 MATERIAL AND METHODS

### 2.1 Plant material

*Eleusine indica* is a highly self-pollinated and genetically diverse diploid ($2n = 18$) species.[36] Seeds of a field evolved glyphosate-resistant *E. indica* (goosegrass) population were collected in oil palm crops in the Johor region of Malaysia.[24,37] Research on this population revealed resistance alleles of 102-Ile/106-Ser (R), Thr-102/106-Ser (r), and the WT (Thr-102/Pro-106) (WT) at the *EPSPS* locus.[29] However, the R allele corresponds to the double glyphosate resistance *EPSPS* Thr-102-Ile + Pro-106-Ser (TIPS) mutation, and the r allele exhibits the single resistance Pro-106-Ser mutation (P106S). These three genotypes (WT, P106S and TIPS) originally identified within the same population were studied here (Table 1): glyphosate-susceptible plants (homozygous WT) and glyphosate-resistant plants with the homozygous single P106S or double TIPS mutation.

Genotyped homozygous plants were bulked in isolation in glasshouse conditions to produce seeds which resulted in three purified subpopulations containing plants with homozygous WT, P106S or TIPS. First-generation plants of these genotyped subpopulations were used in the experiments. Progenies ($n = 12$) from each genotypic line were randomly marker-analyzed[29] to confirm their genotype and homozygosity before use in the experiments described below.

In order to minimize the fitness cost effects of multiple non-EPSPS herbicide resistance traits identified previously in the field-collected *E. indica* population studied here,[24,29] the homozygous WT, P106S and TIPS genotypes were selected and purified only against the EPSPS locus. As these genotypes were collected from within a single population, each of the EPSPS genotypes

**Table 1.** *EPSPS* mutations, alleles and genotypes identified in the glyphosate-resistant *E. indica* population used in this study

| Mutation | Allele | Genotype | Zygosity |
|---|---|---|---|
| - | Thr-102/Pro-106 (WT) | WT | Homozygous |
| Pro-106-Ser (P106S) | Thr-102/106-Ser (r) | P106S | Homozygous |
| Thr-102-Ile/Pro-106-Ser (TIPS) | 102-Ile/106-Ser (R) | TIPS | Homozygous |

evaluated here has a similar chance of possessing other resistance traits that were not tested by our study. Although the possible effect of other resistance genes (e.g. glufosinate and paraquat), possibly present in the assessed EPSPS genotypes (WT, P106S and TIPS), on plant fitness may not be excluded completed, our EPSPS genotype-based approach is likely to cancel the multiple resistance effect except for the *EPSPS* gene P106S and TIPS mutations.

Two transgenic glyphosate-tolerant soybean cultivars with indeterminate growth habit were used for the treatments under competition: DM4614-IPRO (maturity group 4, Don Mario) and M7110-IPRO (maturity group 6.2, Bayer).

### 2.2 Fitness traits under soybean competition and glyphosate treatment

Fitness traits were estimated in plants grown outdoors (i.e. experimental garden) simulating field conditions during the soybean season. Watering and fertilization were required to provide optimum plant growth (see below). Experiments were replicated in time. Results from a number of these replicated experiments are shown in Supplementary Material (Fig. S1-S3)

The experiments were performed under a completely randomized design, under a factorial arrangement (3 × 2 × 2) with four replicates (a total of 48 experimental units) at the Faculty of Agronomy, University of Buenos Aires (34° 35′ S, 58° 35′ W). Analysis of variance (ANOVA) with the GLM (generalized linear model) procedure was performed in *R* (*glm* function from STATS package v4.2.0)[38] to assess the main effects of genotype (three levels: WT, P106S, TIPS), soybean competition (two levels: with and without) and glyphosate (two levels: with and without), and interactions which all were considered as fixed effects. A standard seeding density of 44 soybean plants $m^{-2}$ and glyphosate recommended field dose of 1080 g ae $ha^{-1}$ were used to assess vegetative (aboveground biomass) and reproductive (seed mass and number) fitness traits of *E. indica* WT, P106S and TIPS genotypes. The function *emmeans* from R/EMMEANS (v1.7.3) was used for mean comparison by Tukey's honestly significant difference (HSD) test ($\alpha = 0.05$).

Nine seeds of each WT, P106S or TIPS genotype and soybean were seeded in large 30-L pots (30 cm width x 30 cm length x 30 cm height) containing an organic substrate comprising equal parts of organic soil, river sand and peat moss. For those experimental units under glyphosate treatment, a higher initial seeding density for the P106S genotype was used (15 seeds) as some plant mortality level (50–20%) was expected to occur.[29] Each *E. indica* genotype was seeded in separate experimental units.

At the four-leaf stage, coinciding with the one-leaf stage of soybean, genotypes were treated with a glyphosate field dose of 1080 g ae $ha^{-1}$[23] (Roundup Control Max, 72% acid equivalent, WSG mono-ammonium salt; Monsanto Argentina, Buenos Aires, Argentina) using an automated cabinet sprayer with a flat-fan nozzle at a spray volume of 140 L $ha^{-1}$ pressurized at 200 kPa with a 2.0 km $h^{-1}$ speed. Regardless of the treatment, random plant thinning was carried out after 14 days of glyphosate application. Seedlings were thinned to two *E. indica* and four soybean (DM4614-IPRO cultivar) plants per experimental unit [Fig. 1(A)]. Two *E. indica* plants and four soybean plants per pot equal 22 plants $m^{-2}$ and 44 plants $m^{-2}$, respectively.

Separately, 120 plants per genotype were treated with glyphosate (1080 g ae $ha^{-1}$) and survival was recorded three weeks after treatment. Plants were recorded as alive if they were actively growing after treatment, and as dead if there was little new growth and no new tiller formation. Plants were regularly watered and were fertilized at the four-leaf stage of soybean [kg $ha^{-1}$: 53 nitrogen (N), 48 phosphorus (P), 79 potassium (K), 12 magnesium (Mg), 0.83 iron (Fe), 0.06 boron (B), 0.08 zinc (Zn) and 0.08 manganese (Mn)].

With measurements taken at 09:00 h, stomatal conductance ($g_s$) was estimated in WT, P106S and TIPS plants grown in the absence and presence of soybean competition under no glyphosate treatment (Porometer type AP4; Delta-T Devices, Cambridge, UK) at 24, 45 and 56 days after germination (DAG) by measuring $g_s$ at the youngest fully expanded leaf in both plants per pot (WT, P106S or TIPS), twice on both abaxial and adaxial leaf surfaces. Photosynthetic active radiation (PAR) at the time of soybean anthesis was measured at different hours (every 2 h between 06:00 h and 20:00 h) to characterize the light environment at the soil level imposed by soybean canopies. PAR data were fitted to a nonlinear regression using the Gauss function [$Y = Y_0$ + (A/(w*sqrt(PI/2))) *exp-2*((x-xc)ˆ2/w2].

Several fitness traits were estimated in WT, P106S and TIPS genotypes to understand the patterns of resource allocation to vegetative and reproductive tissues. At plant maturity (94 DAG), aboveground biomass was harvested, oven-dried at 80 °C for 72 h and weighed. Inflorescences with mature seeds were daily collected from first maturation until final evaluation (60-day period). Immature inflorescences also were collected and regarded as aboveground biomass only at the final evaluation. Inflorescences produced per individual were threshed to separate the seed from chaff and rachis material and the total seed mass was quantified. The number of seeds ($S_n$) produced per plant was estimated as:

$$S_n = \frac{TS_w\,50}{S_w} \quad (1)$$

where $TS_w$ denotes the total seed weight produced per plant and $S_w$ represents the weight of 50 seeds per plant ($n = 4$). The effect of WT, P106S and TIPS on soybean reproduction in the absence and presence of glyphosate was estimated. At soybean maturity, seed pods were collected in the corresponding treatments, oven-dried and weighed.

Stomatal conductance, aboveground biomass, seed mass and seed number were evaluated under different statistical distributions consistent with data characteristics, to obtain the best-adjusted model and, consequently, better inference probability. The normal distribution, with the link identity function, was tested for all responses with the assumptions of normality and homogeneity of variance. Normality and homogeneity of variance were evaluated by Shapiro–Wilk and Levene tests, respectively. Data that did not meet the normality requirements (i.e. plant aboveground vegetative biomass) were transformed by the Box–Cox method[39] for latter comparison with gamma distribution with inverse link function for stomatal conductance, aboveground biomass and seed mass; the Poisson distribution with the log connection function was used for seed number estimates. Models were analyzed graphically as a selection criterion, based on the residual and qq-plot graphs, and in addition, Cook's distance was used to identify possible influential points.[40,41]

### 2.3 Estimation of resistance fitness benefit

The fitness benefit of glyphosate-resistant P106S and TIPS genotypes was estimated in the absence and presence of soybean

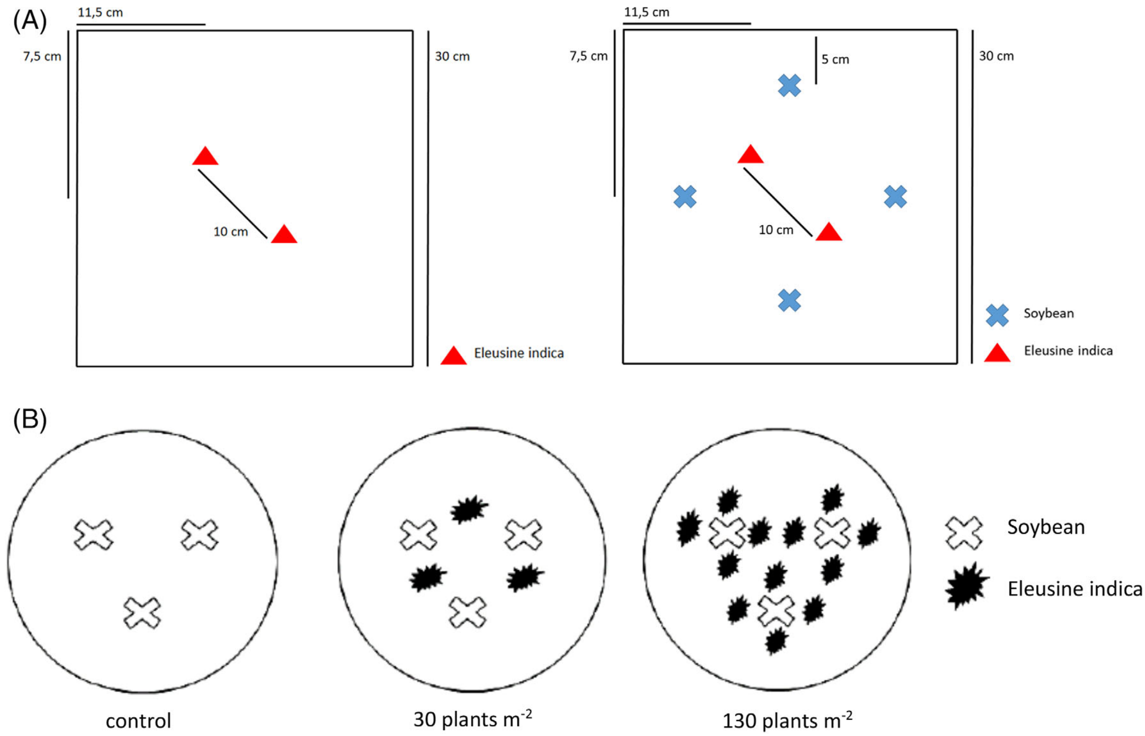


**Figure 1.** Overview of experimental designs. (A) Soybean competitive effects (44 plants $m^{-2}$) on vegetative and reproductive growth of glyphosate-susceptible (WT) and -resistant (P106S, TIPS) *E. indica* genotypes (22 plants $m^{-2}$). (B) *Eleusine indica* genotypic (WT, P106S or TIPS) competitive effects on soybean yield. *Eleusine indica* densities were 30 or 130 plants $m^{-2}$ whereas soybean density was 33 plants $m^{-2}$. Experimental units were fertilized and irrigated and conducted under outdoors growing conditions in an experimental garden.

competition. Operationally, the resistance fitness benefit was estimated as relative (%) to the fittest genotype, being the genotype with the highest *W* after combining *S* and *F* under the selection conditions:

$$W_a = \frac{S_a F_a}{S_f . F_f} \quad (2)$$

where the fitness of genotype *a* ($W_a$) equals the linear combination of survival ($S_a$) and fecundity ($F_a$) under glyphosate treatment relative to the survival ($S_f$) and fecundity ($F_f$) of the fittest genotype *f*.

### 2.4 Estimation of fitness cost

The magnitude (%) of fitness cost (FC) associated with glyphosate resistance also was estimated in the absence and presence of soybean competition as:

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

where *W* denotes the quantitative estimation of a fitness trait (e.g. reproductive biomass) from resistant ($W_R$) P106S or TIPS and susceptible ($W_S$) WT genotypes in a glyphosate-free environment. A resistance fitness cost ranges from 0.99 (extremely high cost) to 0 (zero or negligible cost).

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

### 2.5 Competitive effects of WT, P106 and TIPS genotypes on soybean yield

In order to evaluate the effect of WT, P106S and TIPS on soybean reproduction in the absence and presence of glyphosate, an independent three-level factorial (3 × 3 × 2) experiment of similar design ($n = 4$) and protocol as described above was conducted. *Eleusine indica* plants from each genotype were spatially arranged in each experimental unit at the three densities (0, 30 and 130 plants $m^{-2}$) [Fig. 1(B)]. Density effects were assessed in the absence (control) and presence of glyphosate (1080 g $ha^{-1}$), on soybean (M7110-IPRO cultivar) yield using a crop density of 33 plants $m^{-2}$ (three plants centred). A higher initial seeding density of P106S plants under glyphosate treatment was used as some plant mortality level (20–50%) was expected. After glyphosate treatment, thinning of P106S plants was performed to give equal densities to TIPS plants. *Eleusine indica* and soybean seeds were seeded in large round 30-L pots (35 cm diameter), filled with soil (oxisol) with seeding fertilization of 16, 56 and 32 kg $ha^{-1}$ of N-P-K. Soil chemical properties were: pH 6.7; organic matter 10 g $dm^{-3}$; P-(res.) 18 mg $dm^{-3}$; K, Ca, Mg, H + Al: 0.9%, 54%, 14%, 11%, respectively.

At soybean maturity (124 days after seeding), soybean pods were collected and threshed for grain yield estimation (13% moisture). A three-way ANOVA (GLM) was performed to determine the main fixed effects of genotype (WT, P106S, TIPS), density (0, 3 or 12 plants) and glyphosate on soybean yield. Treatment means were separated by Fisher test ($P \leq 0{,}05$) using InfoStat software.[42]

# 3 RESULTS

Factorial ANOVA revealed an interaction effect of genotype, soybean competition and glyphosate treatment on evaluated fitness

  

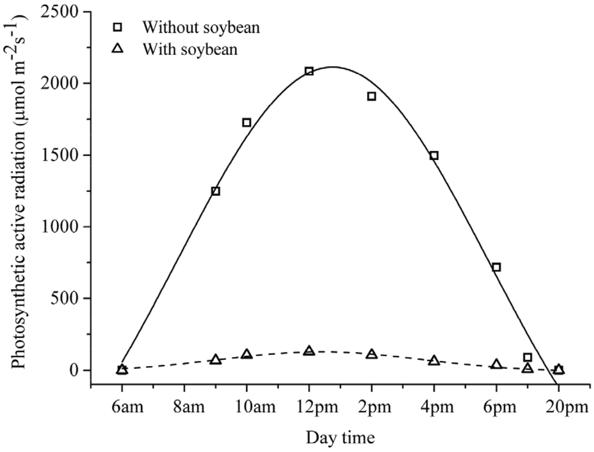


**Figure 2.** Photosynthetic active radiation (µmol $m^{-2}$ $s^{-1}$) (PAR) at the soil surface, measured at the time of soybean anthesis in experimental units without (□) and with (Δ) a soybean crop canopy. Adjusted regression model without soybean ($R^2 = 0.98$): PAR = −1299.2 + (42 495.9/(9.9*sqrt(PI/2)))*exp(−2*((*x* − 12.7)/9.9)^2. Adjusted regression model with soybean crop ($R^2 = 0.96$): PAR = −10.4 + (1125.7/(6.5*sqrt(PI/2)))*exp(−2*((*x* − 12.3)/6.5)^2.

traits ($P < 0.0001$). In the following sections, we decouple the single and combined effects of soybean competition and glyphosate on plant traits accounting for the fitness benefit and cost associated with each EPSPS WT, P106S and TIPS genotype.

Beneath the soybean canopy very little light reached the soil surface (98% PAR reduction). Thus, there was intense competition for light for the WT, P106S and TIPS plants growing within the soybean (Fig. 2).

No effect of growing time (24, 45 *versus* 56 DAG) and leaf surface (adaxial *versus* abaxial) on stomatal conductance was observed ($P > 0.05$). Thus, stomatal conductance was pooled across growing times and leaf surfaces and shown as mean estimates for each genotype ($n = 48$) (Fig. 4).

### 3.1 Effect of soybean competition on WT, P106S and TIPS fitness traits

All three *E. indica* genotypes, WT, P106S and TIPS, growing in competition with soybean but without glyphosate treatment had significantly reduced aboveground vegetative biomass, seed mass and seed number [Fig. 3(A)]. TIPS plants were mostly suppressed from the soybean competition, with 95% less biomass allocated to vegetative and reproductive tissues, relative to the WT (50–65%) or P106S (55–69%) (Table 2). TIPS plants also showed lower stomatal conductance when growing with a soybean crop compared to WT and P106S, but there were no differences among the three genotypes without soybean competition (Fig. 4). P106S plants also showed a reduction in stomatal conductance when competing with soybean, but no difference was observed when compared to WT (Fig. 4). Plants with the TIPS mutation showed a greater fitness cost compared to WT when growing without soybean competition, whereas P106S showed no overall reduction in fitness traits compared to WT, except for a marginal but significant reduction in seed number [Fig. 3(A)].

### 3.2 Effect of glyphosate on WT, P106S and TIPS fitness traits

No WT plants survived glyphosate, whereas all TIPS plants survived. The P106S plants showed 51% mortality, when treated with the recommended glyphosate field dose (1080 g $ha^{-1}$).

The effect of glyphosate on fitness traits of P106S and TIPS is shown in Table 2. The glyphosate effect was calculated by the relative comparison of estimated fitness traits in the glyphosate treated [Fig. 3(B)] *versus* glyphosate nontreated condition (control environment) [Fig. 3(A)], without soybean competition (Soybean–). Biomass, seed biomass and seed number of P106S plants were reduced by

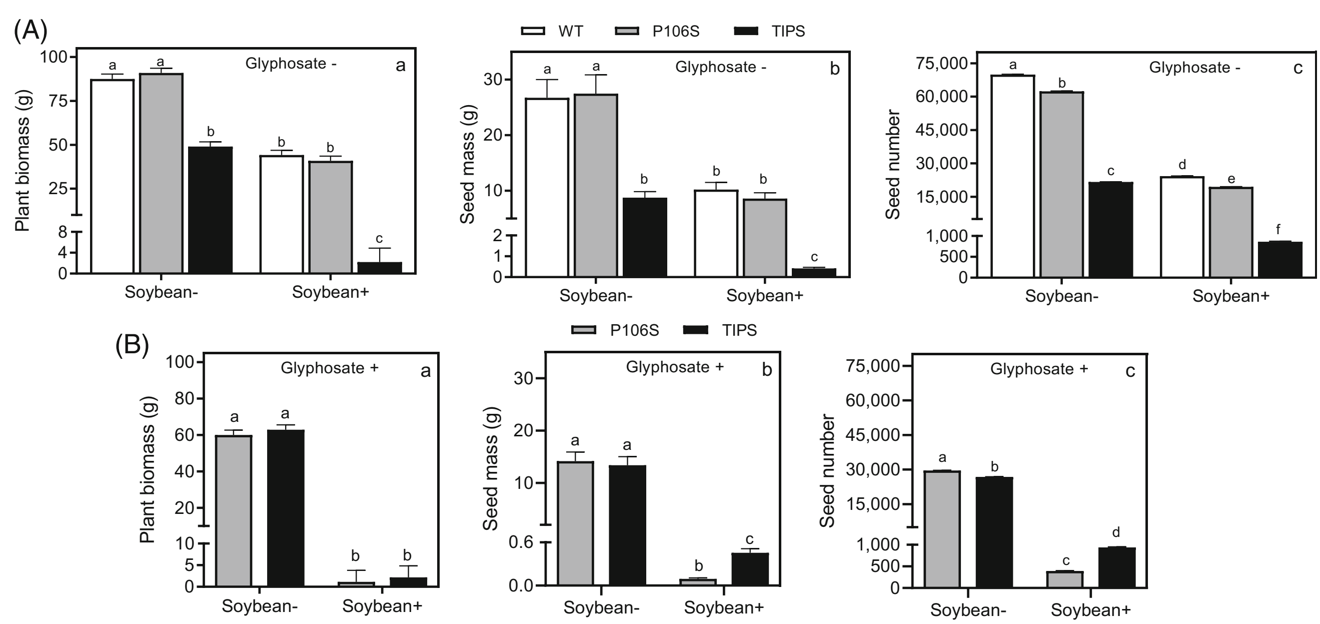


**Figure 3.** Aboveground biomass (a), seed mass (b) and seed number (c) displayed by glyphosate-susceptible (WT) and homozygous resistant (P106S, TIPS) *E. indica* plants when growing in the absence (soybean–) and presence (soybean+) of soybean competition in a glyphosate-free environment (glyphosate–) (A) or under glyphosate selection (1080 g $ha^{-1}$) (glyphosate+) (B). Vertical bars are mean ($n = 4$) estimates per individual with SE. Different letters denote significance genotypic differences by Tukey's HSD test ($\alpha = 0.05$).

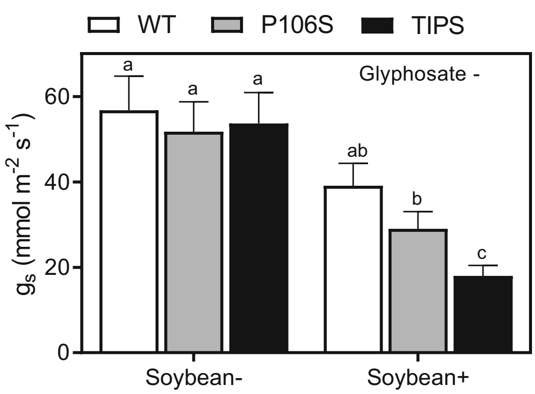


**Figure 4.** Stomatal conductance (mmol $m^{-2}$ $s^{-1}$) estimated in glyphosate-susceptible (WT) and homozygous resistant (P106S, TIPS) single *E. indica* plants when growing in the absence (soybean–) and presence (soybean+) of soybean competition in a glyphosate free environment (glyphosate–). Vertical bars are mean ($n = 48$) estimates with SE. Different letters indicate significance genotypic effects by Tukey's HSD test ($\alpha = 0.05$).

34%, 48% and 52%, respectively, relative to the nontreated controls (Table 2). However, for the TIPS plants there was an increase in biomass under glyphosate treatment compared to the glyphosate nontreated controls (Table 2). Notably, TIPS plants showed 28% higher vegetative growth, 53% increase in seed mass production and 24% higher seed numbers compared to the glyphosate nontreated controls (Table 2).

### 3.3 Combined effect of soybean competition and glyphosate on WT, P106S and TIPS fitness traits

When growing in competition with soybean and with glyphosate treatment, both glyphosate-resistant P106S and TIPS genotypes showed a substantial reduction in aboveground biomass, seed mass and seed number. This finding resulted from the straightforward comparison of the genotypic fitness traits evaluated with *versus* without soybean competition under glyphosate treatment [Fig. 3(B)]. Although the soybean effect on reducing the vegetative growth was similar for both resistant genotypes (40-fold reduction), competition from soybean was noticeably more detrimental to P106S than it was for TIPS plants. The P106S plants showed a 75- and 140-fold reduction in seed mass and number of seeds produced compared to TIPS that showed a 27- and 28-fold reduction, respectively [Fig. 3(B)].

Quantification of the combined effect of soybean competition and glyphosate on fitness traits was possible after comparison of mean fitness estimates of both P106S and TIPS, relative to the no soybean competition and no glyphosate control. The combined effect of soybean competition and glyphosate treatment led to a substantial reduction in vegetative and reproductive growth that ranged from 95% to 99% for both glyphosate-resistant P106S or TIPS (Table 2).

### 3.4 Effect of soybean competition on the estimated fitness cost and resistance benefit of P106S and TIPS

Whereas a fitness cost is quantified in a herbicide-free environment, the resistance benefit is estimated under herbicide selection.[3,34] The assessment of fitness traits helped to estimate the effect of soybean competition on the fitness cost and resistance benefit associated with the glyphosate-resistant P106S *versus* TIPS mutations (Tables 3 and 4).

### 3.5 Soybean effect on fitness cost

Without soybean competition, TIPS plants showed a very high fitness cost, whereas no glyphosate fitness cost was evident in P106S plants (Table 3). TIPS plants showed fitness costs of 44%, 67% and 73% in terms of vegetative growth, seed mass and seed number production, respectively (Table 3). As reported previously, the TIPS double mutation endows a substantial fitness cost.[34] This TIPS fitness cost increased significantly under competition with soybean, resulting in a cost of 95%, 95% and 96% in terms of vegetative growth, seed mass and seed number investment, respectively (Table 3). By contrast, when under intense soybean competition the P106S plants showed a low but significant glyphosate resistance cost of 7%, 16% and 19% associated with vegetative growth, seed mass and seed number production, respectively (Table 3).

**Table 2.** Single and combined effects of soybean competition (soybean+) and glyphosate (glyphosate+) on fitness traits of homozygous EPSPS WT, P106S and TIPS *E. indica* genotypes. Effects are expressed as relative to the fitness traits attained under (no soybean competition no glyphosate treatment; soybean– glyphosate–)

| Genotype | Control (Soybean– Glyphosate–) | Soybean+ | Glyphosate + | Soybean+ glyphosate+ |
|---|---|---|---|---|
| | Vegetative biomass* (g) | Relative effect for each genotype† (% of control) | | |
| WT | 88 | −50.0 | −100 | −100 |
| P106S | 91 | −55.0 | −34.0 | −99.0 |
| TIPS | 49 | −95.5 | +28.5 | −95.6 |
| | Seed mass* (g) | | | |
| WT | 26.7 | −62.0 | −100 | −100 |
| P106S | 27.5 | −69.0 | −48.4 | −99.6 |
| TIPS | 8.8 | −95.0 | +53.0 | −95.0 |
| | Seed number* | | | |
| WT | 69 907 | −65.2 | −100 | −100 |
| P106S | 62 395 | −69.0 | −52.5 | −99.4 |
| TIPS | 21 698 | −96.0 | +23.8 | −95.7 |

*Fitness traits expressed per single plant.
† Minus (–) and plus (+) symbols denote negative and positive relative effects on fitness traits, respectively.

**Table 3.** Estimates of fitness costs associated with glyphosate-resistant *E. indica* homozygous P106S and TIPS genotypes when grown in the absence and presence of soybean competition in a glyphosate-free environment

| Genotypes | Aboveground biomass* | |
|---|---|---|
| | Without soybean | With soybean |
| P106S | 1.03† | 0.07 |
| TIPS | 0.44 | 0.95 |
| Genotypes | Seed mass* | |
| | Without soybean | With soybean |
| P106S | 1.02† | 0.16 |
| TIPS | 0.67 | 0.95 |
| Genotypes | Seed number* | |
| | Without soybean | With soybean |
| P106S | 0.10 | 0.19 |
| TIPS | 0.73 | 0.96 |

Fitness costs were evaluated in an environment with (44 soybean plants $m^{-2}$) and without soybean competition in the absence of glyphosate treatment.
Fitness costs were quantified according to: FC = 1 – ($W_R$/$W_S$), where $W_R$ denotes the quantitative estimation of a fitness trait in the glyphosate-resistant (P106 or TIPS) genotypes, and $W_s$, a fitness trait of the susceptible (WT) genotype.
*Values >1 denote a fitness advantage of the P106S genotype over WT, estimated as relative fitness (RF) = $W_R$/$W_S$.
†Fitness cost values approaching 0.99 indicate extremely high costs and nearby 0 denote low or negligible costs.

### 3.6 Soybean effect on resistance benefit

Quantification of plant fitness with glyphosate treatment revealed that TIPS plants exhibited the highest resistance benefit (i.e. highest relative fitness of 1.0) when growing without soybean competition (Table 4). In the same environment, a reduction of 40% in the resistance benefit was observed in P106S plants (Table 4). However, competition from the soybean crop severely decreased the resistance benefit of both P106S and TIPS by 99% and 96%, respectively (Table 4).

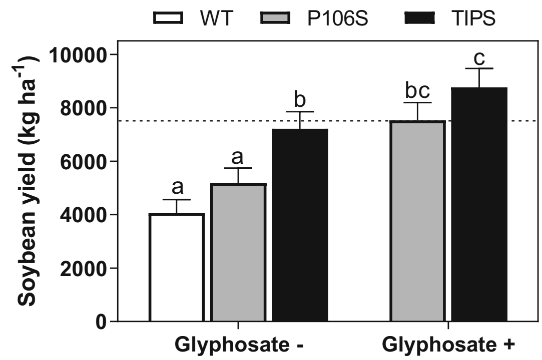


**Figure 5.** Effect of *E. indica* WT, P106S and TIPS on soybean yield with (glyphosate+) and without (glyphosate–) glyphosate treatment (1080 g $ha^{-1}$). Competition effect on soybean yield was evaluated under pooled *E. indica* densities of 30 and 130 plants $m^{-2}$. Vertical bars denote means ($n$ = 6–10) with SE. Different letters indicate significant differences among genotypes according to Tukey's HSD test ($\alpha$ = 5%). Dotted line denotes soybean reference yield (7530 kg $ha^{-1}$) in the absence of competition from *E. indica* genotypes (control treatment).

### 3.7 Competitive effect of WT, P106S and TIPS on soybean crop

Without *E. indica* competition, the glyphosate-tolerant soybean yield was 7530 kg $ha^{-1}$, with or without glyphosate treatment (Fig. 5). The density effect of *E. indica* plants on soybean yield was significant ($P$ = 0.0035), but yield under three and 12 plants did not differ ($P$ > 0.005), and thus density data were pooled. Thus, a two-way ANOVA was performed to assess the main effects of genotype ($P$ = 0.01) and glyphosate ($P$ < 0.0001) on soybean yield under competition with *E. indica*, and both effects were significant.

When grown without glyphosate treatment, the *E. indica* WT and P106S plant competition likewise reduced soybean yield (4500 kg $ha^{-1}$) and this reduction was significantly greater than the competitive effect of TIPS plants (6600 kg $ha^{-1}$). For the glyphosate-tolerant soybean, glyphosate treatment at the recommended field dose (1080 g $ha^{-1}$) had no impact on soybean yield (7530 kg $ha^{-1}$), and was notably higher when growing with either P106S or TIPS compared to that under no glyphosate treatment (Fig. 5).

**Table 4.** Estimates of glyphosate resistance benefits associated with glyphosate-resistant *E. indica* homozygous P106S and TIPS genotypes in response to glyphosate selection

| No competition | | | |
|---|---|---|---|
| Genotype | Survival (*S*)* | Fecundity (*F*)† | Relative fitness (*W*)‡ |
| P106S-rr | 0.51 | 29 639 | 0.600 |
| TIPS-RR^ | 1.00 | 26 862 | 1.000 |
| Soybean competition | | | |
| Genotype | Survival (*S*)* | Fecundity (*F*)† | Relative fitness (*W*)‡ |
| P106S-rr | 0.51 | 392 | 0.008 |
| TIPS-RR | 1.00 | 939 | 0.035 |

Example: Relative fitness ($W$) of P106S–rr under soybean competition = ($S_{P106S-rr} \times F_{P106S-rr}$ / $S_{TIPS-RR} \times F_{TIPS-RR}$); $S$, survival; $F$, fecundity.
^Fittest genotype = TIPS-RR.
*Mean survival of 120 plants exposed at the recommended glyphosate dose of 1080 g ae $ha^{-1}$.
†Fecundity is the average number of seeds produced per single plant.
‡The glyphosate resistance benefit for a particular genotype is calculated as the fitness ($W$) that results from the linear combination of survival ($S$) and fecundity ($F$) under glyphosate treatment relative to the survival ($S$) and fecundity ($F$) of the fittest genotype (TIPS-RR) which was normalized to $W_{TIPS-RR}$ = 1. See Eqn (2) in text.

  

## 4 DISCUSSION

We assessed the fitness effects of the *EPSPS* P106S mutation (low-to-moderate glyphosate resistance with negligible fitness cost) and TIPS mutation (high-level resistance but high fitness cost) in *E. indica* growing in competition with soybean with and without glyphosate selection. Our results show practical implications for the management of glyphosate-resistant *E. indica* and provide an understanding of the trajectories and equilibrium frequencies of glyphosate resistance alleles in *E. indica* in soybean cropping systems.

### 4.1 Soybean competition amplifies the TIPS fitness cost and reveals a P106S fitness cost

TIPS glyphosate-resistant *E. indica* plants grown in optimal conditions displayed a 50% fitness penalty.[34] Furthermore, this constitutive expression of the TIPS fitness cost has been found to increase up to 85% in a light-depleted environment driven by competition with rice.[17,34] The results of the present study confirm the inherent TIPS fitness cost[32,34] and reveals that intense soybean competition amplifies the *E. indica* TIPS fitness cost up to a 95% reduction in vegetative and reproductive growth. Soybean canopy architecture may lead to higher light extinction coefficients compared to other crops such as rice.[43,44] In our experiments, light (PAR) intercepted by the soybean canopy reached 98% imposing a light-deprived environment for *E. indica* growth.

Lower stomatal conductance also was observed in TIPS plants when competing with soybean, a physiological response representing an imbalance in $CO_2$ uptake and water loss with an impact on photosynthesis rate and water use under plant competition.[45,46] A disrupted shikimate pathway owing to impaired *EPSPS* $V_{max}$ and an over-allocation of carbon-rich precursors in the shikimate pathway and other polar metabolites have been identified in plants with the TIPS mutation.[29,34] And these biochemical changes have been proposed as the physiological mechanism responsible for the fitness cost associated with the TIPS mutation.[17] It is likely, then, that in a light-deprived environment caused by shading from a crop canopy, carbon for plant growth and reproduction functions will be more limited in TIPS plants *versus* WT and hence increasing the magnitude of the fitness cost.[17] The fitness penalty associated with the TIPS mutation represents a major adaptive constraint in light-limited and glyphosate nontreated environments.

The EPSPS P106S mutation is the most documented target-site glyphosate resistance mechanism in weed species and no detectable fitness cost of this mutation has been identified in the literature thus far.[3,17,33,34,47] The results of the present study likewise show negligible fitness costs associated with plants carrying the P106S mutation when growing without soybean competition. Previous studies from our laboratory,[3,34] with and without rice competition, concluded that plants exhibiting the P106S mutation showed a negligible fitness difference compared to WT. However, the results presented here show a 19% fitness cost (i.e. seed number) associated with the P106S which is only evident under competition with soybean (Table 3). This result highlights that the expression of fitness costs can be environment-dependent (see review[48]) and thus full identification of fitness costs requires the assembly of the environmental conditions triggering those costs.[19]

It has been argued that any weed management practice aiming to realistically exploit fitness costs associated with herbicide resistance alleles should create the right ecological conditions in the field to expose and exacerbate those predicted fitness costs.[48] Maximizing crop competitiveness (e.g. crop type, crop cultivar, crop density, row orientation) not only will maximize crop productivity,[49,50] but also have the potential to exacerbate the expression of fitness costs, especially those driven by light limitation.[5,51]

### 4.2 Glyphosate unveils differential fitness across EPSPS P106S and TIPS mutations in E. indica

Published studies have shown the ability of the *EPSPS* TIPS mutation to withstand much higher glyphosate doses than the P106S mutation, at both the isolated enzyme and whole-plant level.[3,29] These results have helped define the known target-site *EPSPS* mutations as endowing low to moderate (P106S) *versus* high (TIPS) glyphosate resistance.[3,26] In line with growth stimulation (i.e. hormesis) reported in plants treated with low glyphosate doses,[52] we can speculate on a similar effect observed for the highly glyphosate-resistant TIPS plants, although this needs to be validated.

### 4.3 Combined effect of soybean competition and glyphosate: a constraint limiting glyphosate resistance P106S mutation

The combination of glyphosate treatment (i.e. field dose of 1080 g $ha^{-1}$) and soybean competition greatly limited the growth of P106S mutation plants, compared to soybean competition alone (Table 2). Thus, a glyphosate resistance mutation that confers a low-to-moderate level of resistance (P106S) and competition from soybean greatly negatively impacted the fitness of P106S plants (99%) ultimately reducing seed production from 62 400 (control) to 620. However, for the TIPS mutation which confers a high level of resistance, glyphosate treatment did not further reduce the fitness of TIPS plants which already had shown a significant reduction (96%) from soybean competition. In this case, the strong fitness cost of the TIPS mutation meant that soybean competition alone could greatly reduce the growth and fecundity of TIPS plants. The differential response of P106S and TIPS plants to the combined effect of both soybean competition and glyphosate treatment reflects the inherent weak P106S *versus* the strong TIPS mutation. Thus, both crop competition and glyphosate exploit the weaker resistance mechanism. These results lead to the prediction that higher frequencies of the TIPS allele rather than the P106S allele probably will be present in agroecosystems where *E. indica* is under glyphosate selection and soybean competition.[3,48]

## 5 CONCLUSIONS

The naturally evolved glyphosate resistance endowing single P106S and the double TIPS mutations in the major weed *E. indica* showed significant differences in their responses to the single and combined effects of soybean competition and glyphosate. Although the competitive interaction with the soybean reduced the fitness of plants carrying either of these mutations, the TIPS plants exhibited extremely limited ability to grow and reproduce under crop competition. Conversely, when under glyphosate treatment, P106S plants displayed limited survival, growth and fecundity than plants carrying the TIPS mutation, which showed enhanced plant fitness when glyphosate treated (hormesis). However, P106S plant fitness was substantially limited by the combined effects of both soybean competition and the field-recommended glyphosate dose. These ecological environments set a significant constraint for the spread of both glyphosate resistance mutations in the agroecosystem and highlight the key role of crop competition in limiting the population growth of herbicide-resistant or -susceptible weed ecotypes.

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## DATA AVAILABILITY STATEMENT

Research data are not shared.

## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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