

# No fitness cost of glyphosate resistance endowed by massive *EPSPS* gene amplification in *Amaranthus palmeri*

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Received: 3 September 2013 / Accepted: 9 December 2013 / Published online: 3 January 2014  
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**Abstract** Amplification of the *EPSPS* gene has been previously identified as the glyphosate resistance mechanism in many populations of *Amaranthus palmeri*, a major weed pest in US agriculture. Here, we evaluate the effects of *EPSPS* gene amplification on both the level of glyphosate resistance and fitness cost of resistance. *A. palmeri* individuals resistant to glyphosate by expressing a wide range of *EPSPS* gene copy numbers were evaluated under competitive conditions in the presence or absence of glyphosate. Survival rates to glyphosate and fitness traits of plants under intra-specific competition were assessed. Plants with higher amplification of the *EPSPS* gene (53-fold) showed high levels of glyphosate resistance, whereas less amplification of the *EPSPS* gene (21-fold) endowed a lower level of glyphosate resistance. Without glyphosate but under competitive conditions, plants exhibiting up to 76-fold *EPSPS* gene amplification exhibited similar height, and biomass allocation to vegetative and reproductive organs, compared to glyphosate susceptible *A. palmeri* plants with no amplification of the *EPSPS* gene. Both the additive effects of *EPSPS* gene amplification on the level of glyphosate resistance and the lack of associated fitness costs are key factors contributing to *EPSPS* gene amplification as a widespread and important glyphosate resistance mechanism likely to become much more evident in weed plant species.

**Keywords** Evolution · Fitness traits · Gene over-expression · Herbicide resistance · Target-site resistance · Weeds

## Abbreviations

ALS	Acetolactate synthase
DAT	Days after treatment
EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase
LD <sub>50</sub>	Lethal dose fifty
PCR	Polymerase chain reaction
R	Glyphosate resistant
S	Glyphosate susceptible

## Introduction

The extensive cultivation of crops engineered to be glyphosate resistant has meant excessive reliance on glyphosate for weed control across vast agricultural areas in the Americas and this has led to evolution of glyphosate-resistant weed populations. Glyphosate is herbicidal because it inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimic acid pathway that links carbohydrate metabolism to the synthesis of aromatic compounds (Steinrücken and Amrhein 1980). However, a number of adaptive traits have evolved which endow resistance to glyphosate (Powles and Yu 2010). Glyphosate-resistant plants may exhibit (1) point mutations of the *EPSPS* gene (Baerson et al. 2002; Wakelin and Preston 2006), (2) restricted glyphosate leaf absorption (Michitte et al. 2007; Vila-Aiub et al. 2012) and/or, more commonly, (3) reduced glyphosate translocation (Lorraine-Colwill et al. 2003) due to enhanced vacuolar sequestration (Ge et al. 2010).

A novel glyphosate resistance mechanism is evident in *Amaranthus palmeri* (Palmer amaranth), an economically

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important weed rapidly increasing across large areas of US crop land (Gaines et al. 2010; Ward et al. 2013). Resistance to glyphosate in this species can be conferred by amplification of the *EPSPS* gene which translates into 5-fold to more than 160-fold more *EPSPS* gene copies located throughout the genome (Gaines et al. 2010). A higher number of *EPSPS* gene copies correlate positively with higher *EPSPS* transcription, EPSPS content and activity (Gaines et al. 2010). The EPSPS enzyme of glyphosate-resistant *A. palmeri* plants is equally sensitive to glyphosate inhibition and the much greater content of EPSPS protein essentially acts as a “molecular sponge” to soak up glyphosate and allow normal metabolic functions to continue (Powles 2010).

The fitness advantage of *A. palmeri* plants exhibiting higher *EPSPS* gene expression in the presence of glyphosate is obvious (Gaines et al. 2010, 2011). However, the fitness consequences at the plant level of overproducing EPSPS protein in the absence of glyphosate are unknown and require investigation. Fitness costs have been identified in insects and plants with amplified genes associated with insecticide and herbivory resistance (Raymond et al. 1993; Bekaert et al. 2012).

Constitutively, higher *EPSPS* gene expression means concomitant extra synthesis of *EPSPS* mRNA and EPSPS protein, with potential impacts on plant fitness. In addition, fitness costs might occur if any gene functions are disrupted by insertion of the *EPSPS* gene throughout the *A. palmeri* genome (Gaines et al. 2010), a duplication process that may be directed by a mobile genetic element (Gaines et al. 2010, 2013). To determine whether the amplification of the *EPSPS* gene correlates with fitness costs also requires assessing the untested prediction of a positive correlation between increase in the number of *EPSPS* gene copies and the level of glyphosate resistance attained by plants.

Here, we report on the effects of *EPSPS* gene amplification on fitness of *A. palmeri* in the presence and absence of glyphosate. We quantified the effects of a wide range of *EPSPS* gene amplification (1–160 *EPSPS* gene copies) on the glyphosate resistance level and various fitness traits under a gradient of intra-specific plant competition in the absence of glyphosate. Both plant responses under the presence and absence of glyphosate effect have wider implications to understand the evolution of glyphosate resistance (Bergelson and Purrington 1996; Jasieniuk et al. 1996; Vila-Aiub et al. 2009b).

## Materials and methods

### Creation of segregating $F_2$ populations

Given the importance of genetic background control to assess the effects of resistance genes on fitness (Vila-Aiub

et al. 2009b, 2011),  $F_2$  populations segregating for a wide range of *EPSPS* gene amplification levels were generated.

Seeds of a glyphosate-resistant (R) *A. palmeri* were collected from a field site in Macon County, Georgia (Culpepper et al. 2006), whereas seeds of a known glyphosate susceptible (S) *A. palmeri* population were collected from the University of Georgia Ponder Farm Research Station. The R and S seeds were germinated and grown in a greenhouse and the resistance phenotype of each plant was confirmed using an in vivo leaf disk assay (Shaner et al. 2005). Individual R males were placed next to various S females to create an  $F_1$  generation (S/R). Seeds from the S female plants were collected and stored at 4 °C for 2 months, then germinated and grown to the 4-leaf stage. These S/R  $F_1$  plants were sprayed with a low discriminating rate (0.4 kg a.e. ha<sup>-1</sup>) of formulated glyphosate (potassium salt, Roundup Weather-Max, Monsanto) to select for resistant  $F_1$  progeny. One R  $F_1$  male was selected for hand crossing to one R  $F_1$  female to generate a hand pollinated pseudo- $F_2$  through half-sibling mating. Pollination bags were placed over female inflorescences before emergence, and pollen from the resistant male was applied by hand daily for 2 weeks. Seeds from the female plant (referred to as  $F_2$ -hand) were stored at 4 °C. A second pseudo- $F_2$  population was generated by isolating one resistant R  $F_1$  male with one resistant R  $F_1$  female in a greenhouse and permitting open pollination to occur. Seeds from the female plant (referred to as  $F_2$ -open) were stored at 4 °C. Both the  $F_2$ -hand and  $F_2$ -open segregating families were employed in experiments designed to assess the effect of *EPSPS* gene amplification on phenotypic glyphosate resistance level.

Similarly, glyphosate-resistant individuals exhibiting >40 *EPSPS* copy numbers were identified and selected from within the glyphosate R Georgia population (Shaner et al. 2005). Two R plants (male and female) were paired for hand crossing. The R male individual was the same R plant that originated the  $F_1$  family that was subsequently used to originate both the hand and the open-pollinated  $F_2$  families. The progeny from this cross ( $F_1$  originated from R male × R female) was shown to segregate for the number of *EPSPS* copies from 1 to 76 copies and thus employed in experiments designed to evaluate the pleiotropic effect of *EPSPS* gene amplification on various individual fitness traits.

### *EPSPS* gene copy number

*EPSPS* gene copy number was measured using quantitative real-time PCR (Gaines et al. 2010). For each *EPSPS* gene copy number assessment, plants were grown in a glasshouse with controlled temperature (25–30 °C) and natural summer irradiance. At the 5-leaf stage, the youngest leaf of each plant was collected and DNA was extracted with

Nucleon PhytoPure plant DNA extraction kits. Total RNA was removed from the DNA sample by incubation with RNase A for 30 min at 37 °C. DNA quality and integrity were checked by agarose gel electrophoresis. The concentrations of all DNA samples were measured with Nanodrop spectrophotometer after RNase treatment and normalized to 1 ng  $\mu\text{L}^{-1}$ . To quantify the genomic *EPSPS* gene copy number, the acetolactate synthase (*ALS*) gene was used as a reference gene, because *ALS* has been shown to have stable genomic copy number in *A. palmeri*, and gene copy number of 1 relative to *EPSPS* in glyphosate susceptible individuals (Gaines et al. 2010). The sequences of the *ALS* and *EPSPS* gene primers used were reported before (Gaines et al. 2010). Real-time PCR was performed in a 25  $\mu\text{L}$  reaction containing 10 ng genomic DNA, 1 $\times$  Power SYBR green PCR master mix and 0.1  $\mu\text{M}$  forward and reverse primer each. The program was run on Applied Biosystems 7500 fast Real-Time PCR system under the standard conditions: 50 °C for 20 s, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min; melt-curve analysis was conducted by the following steps: 95 °C for 15 s, 60 °C for 1 min, followed by increasing the temperature by 3.5 °C every 10 s to 95 °C for 30 s, then holding at 60 °C for 15 s. Negative controls consisting of template with no primers and primers with no template were included as well as templates with DNA from known S plants. There were three replicates for each individual sample. Results were expressed as fold increase in *EPSPS* copy number relative to *ALS*. Relative quantification of *EPSPS* was calculated as  $\Delta C_t = (C_t, \text{ALS} - C_t, \text{EPSPS})$ . Increase in *EPSPS* copy number was expressed as  $2^{\Delta C_t}$ .

#### *EPSPS* gene amplification and glyphosate resistance level

Twelve S, 11 R, 79  $F_2$ -hand, and 52  $F_2$ -open individuals were used for experiments to determine the relationship between *EPSPS* gene copy number and glyphosate resistance. Seeds of the R, S,  $F_2$ -hand, and  $F_2$ -open populations were germinated and grown in glasshouse conditions as described. Plants were grown to the 4-leaf stage and DNA samples were collected for the measurement of *EPSPS* copy number as described. All individuals were treated with glyphosate at 200 g a.e.  $\text{ha}^{-1}$ , and survival was evaluated 21 days after treatment (DAT). Next, all individuals surviving 200 g glyphosate  $\text{ha}^{-1}$  were treated with glyphosate at 2,000 g a.e.  $\text{ha}^{-1}$ , and survival was evaluated 21 DAT. Survival scores were assigned depending whether each individual could survive the 200 g  $\text{ha}^{-1}$  treatment and/or the 2,000 g  $\text{ha}^{-1}$  treatment. Whereas glyphosate at 200 g  $\text{ha}^{-1}$  represents four times the  $\text{LD}_{50}$  for a glyphosate susceptible *A. palmeri* population (54 g  $\text{ha}^{-1}$ ), 2,000 g  $\text{ha}^{-1}$  is approximately the same as the  $\text{LD}_{50}$  for the glyphosate-resistant population (1,600 g  $\text{ha}^{-1}$ ) collected in Georgia

(Gaines et al. 2011). These chosen glyphosate rates allowed us to test for both low- and high-level glyphosate resistance. The survival scores at either 200 or 2,000 g glyphosate  $\text{ha}^{-1}$  were plotted against *EPSPS* gene copy number to assess the relationship between increasing *EPSPS* copy number and glyphosate resistance level (i.e., plant survival) in these segregating *A. palmeri* populations.

#### *EPSPS* gene amplification and fitness traits

Experiments were designed to assess fitness traits in individuals from a segregating *A. palmeri* population. Seeds were germinated in commercial expanding peat pellets and transplanted and grown in pots (30 cm diameter  $\times$  40 cm height) containing a potting mix (50 % peatmoss and 50 % river sand). Pots were randomly placed in a glasshouse under controlled temperature (25–30 °C) and natural summer irradiance. Plants were spatially arranged so that growth traits were evaluated under increasing intra-specific competition. Three density treatments were applied: control (isolated plants), 85 and 170 plants  $\text{m}^{-2}$  which corresponded to 1, 4 and 8 plants per pot. In each of the replicated experiments, 10 and 2 pots were used for plants subjected to non-competitive and competitive conditions, respectively. At the end of the growing season, plant height was measured from the highest leaf position to the plant base. Biomass allocation to vegetative (leaves and stem) and reproductive (inflorescences) organs was estimated as dry weight per individual.

#### Statistical analysis

##### *EPSPS* gene amplification and glyphosate resistance level

The ability of plants to survive glyphosate according to the number of *EPSPS* gene copies was estimated using maximum likelihood logistic regression analysis (Statgraphics Centurion XV, StatPoint, Inc., 2005). The individual probability of survival to glyphosate (either 200 or 2,000 g  $\text{ha}^{-1}$ ) (alive = 1 and dead = 0) was modeled as a function of *EPSPS* gene copy numbers using the logistic function:

$$y = \frac{e^{\alpha + \beta x}}{1 + e^{\alpha + \beta x}} \quad (1)$$

where  $y$  is the probability of survival to glyphosate treatment,  $\alpha + \beta$  are unknown parameters to be estimated and  $x$  is the *EPSPS* gene copy number. The significance of the model was tested by analysis of deviance ( $F$  test).

##### *EPSPS* gene amplification and fitness traits

One-way analysis of variance (ANOVA) was conducted to assess the overall effect of plant competition on fitness

traits (vegetative and reproductive biomass and height) and *EPSPS* gene copy number of the segregating  $F_2$  *A. palmeri* plants. Data from replicated experiments were pooled (see “Results”) and mean fitness traits were estimated.

To evaluate the pleiotropic effects of *EPSPS* gene amplification on fitness traits, correlation analysis was performed to identify the degree of relationship between individual plant traits with amplified *EPSPS* gene copy numbers. We used CORR Procedure (SAS 9.2, Citrix Systems, Inc., Florida, USA) to calculate Pearson’s correlation coefficient ( $r$ ) for each pairwise combination of traits (height, vegetative and reproductive biomass) and *EPSPS* copy numbers found in each single plant growing at each density treatment.

## Results

### *EPSPS* gene amplification and glyphosate resistance level

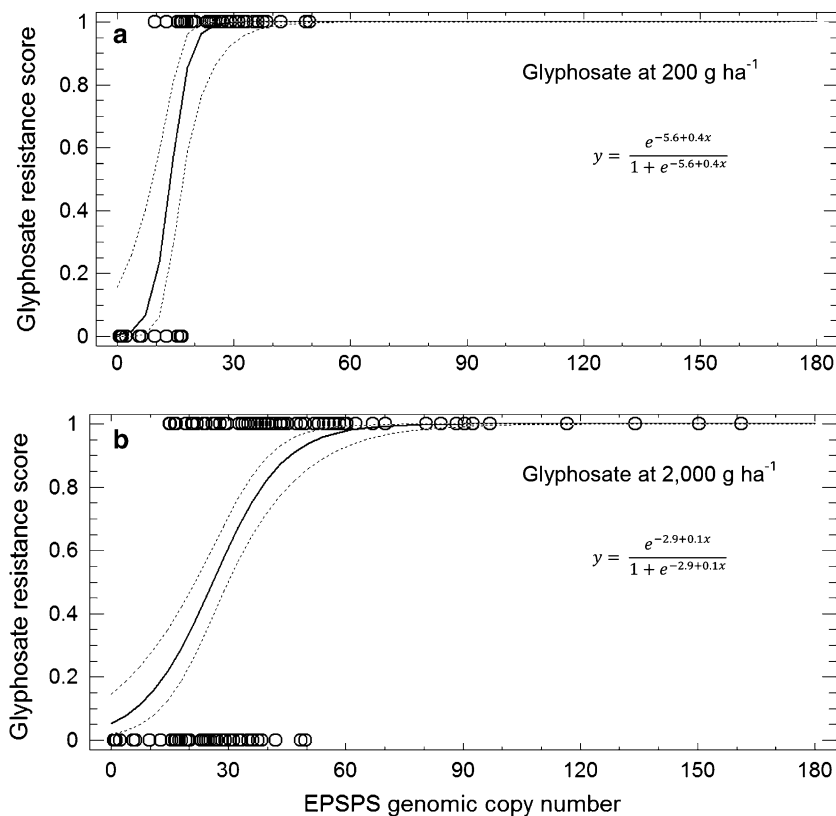
Survival following glyphosate treatment was evaluated in *R. A. palmeri* plants exhibiting a wide range of *EPSPS* genomic copy numbers (1–160 fold increase). Analyses revealed a significant relationship ( $P < 0.0001$ ) between plant survival to glyphosate and quantitative amplification of the *EPSPS* gene. The likelihood of survival from glyphosate treatment increased in plants with higher

number of *EPSPS* copies (Fig. 1). According to the fitted logistic model (Eq. 1) and under glyphosate treatment with  $200 \text{ g ha}^{-1}$ , *A. palmeri* individuals with 14 *EPSPS* gene copy numbers (95 % CI 9–17) showed 50 % chance of surviving glyphosate (Fig. 1a). This survival probability increased to 95 % when plants exhibited 21 *EPSPS* gene copies (95 % CI 18–32) (Fig. 1a). However, when *A. palmeri* plants were exposed to glyphosate at high dose ( $2,000 \text{ g ha}^{-1}$ ), more *EPSPS* copies were required to survive the treatment (Fig. 1b). Twenty-six (95 % CI 22–30) and 53 (95 % CI 46–65) copies of the *EPSPS* gene were required to endow 50 and 95 % chances of survival to glyphosate at  $2,000 \text{ g ha}^{-1}$ , respectively (Fig. 1b).

### *EPSPS* gene amplification and fitness traits

Assessment of various fitness traits was performed to assess the cost of *EPSPS* genomic copy numbers in *A. palmeri* plants under a resource competition gradient. Results from both experiments were similar ( $P > 0.05$ ) and, therefore, pooled to perform the analyses. A total of 67 plants exhibiting from 1 to 76-fold *EPSPS* gene copies were subjected to different intensities of plant competition. As expected, intra-specific competition gradient caused significant reduction in vegetative and reproductive growth ( $P < 0.001$ ) (Table 1). Plants growing at low and high

**Fig. 1** Estimation of glyphosate resistance as probability of surviving *A. palmeri* plants with increasing *EPSPS* genomic copy number. Each point (circles) was obtained after evaluation of individual plants in response to glyphosate treatment at  $200 \text{ g ha}^{-1}$  followed by  $2,000 \text{ g ha}^{-1}$ . **a** Observed values  $>0$  are plants that survived glyphosate at  $200 \text{ g ha}^{-1}$  but were susceptible at  $2,000 \text{ g ha}^{-1}$ . **b** Observed values  $>0$  represent plants that survived glyphosate at both  $200$  and  $2,000 \text{ g ha}^{-1}$ . Observed values  $=0$  represent plants that died after glyphosate at  $200 \text{ g ha}^{-1}$  (a) or either  $200$  or  $2,000 \text{ g ha}^{-1}$  (b). Data were fitted to a logistic function and solid lines represent predicted glyphosate resistance values with 95 % confidence intervals (dotted lines). Sample size ( $n$ ) for (a) is 61 and (b) is 154



**Table 1** Mean aboveground vegetative and reproductive biomass, height and *EPSPS* gene copy number per plant estimated under increasing plant density

Density (plants/m <sup>2</sup> )	Vegetative biomass (g/plant)	Inflorescence biomass (g/plant)	Height (cm)	<i>EPSPS</i> gene (copy number/plant)
0	10.0 <sup>a</sup> (1.2)	3.9 <sup>a</sup> (0.4)	73 (5)	23 (4.8)
85	5.9 <sup>b</sup> (0.8)	1.0 <sup>ab</sup> (0.3)	76 (3)	15 (3.5)
170	3.5 <sup>b</sup> (0.4)	0.5 <sup>b</sup> (0.1)	78 (3)	21 (3.7)
<i>P</i>	<0.0001	0.0024	0.5147	0.44

Values in parenthesis denote standard error of the mean. *P* values after ANOVA analysis. Different superscript letters indicate significant differences among mean values within columns according to Tukey's HSD ( $\alpha = 0.05$ ). Sample size was pooled from two replicated experiments ( $n = 66$ )

density exhibited 40 and 60 % less vegetative biomass and 70 and 85 % less reproductive biomass, respectively, than plants growing without competition. Plants under a competition gradient tended to be 10 % taller compared to isolated plants, but differences were not significant ( $P = 0.5$ ) (Table 1).

The number of *EPSPS* gene copies exhibited by individual plants was unaffected ( $P = 0.44$ ) by the intensity of competition (Table 1). As a result, we evaluated the quantitative changes of the fitness traits (vegetative and reproductive biomass and plant height) in plants displaying a wide range of *EPSPS* gene copy numbers. The number of *EPSPS* gene copies caused no effect on *A. palmeri* growth, regardless of whether plants were growing without competition (Fig. 2a, d, g) or competing at either low (Fig. 2b, e, h) or high (Fig. 2c, f, i) intra-specific densities (Fig. 2). Combining all competition gradients, plant height ( $P = 0.47$ ) and the biomass accumulated in shoots and leaves (i.e., vegetative biomass) ( $P = 0.54$ ) were independent of the *EPSPS* gene copy number exhibited by individual plants.

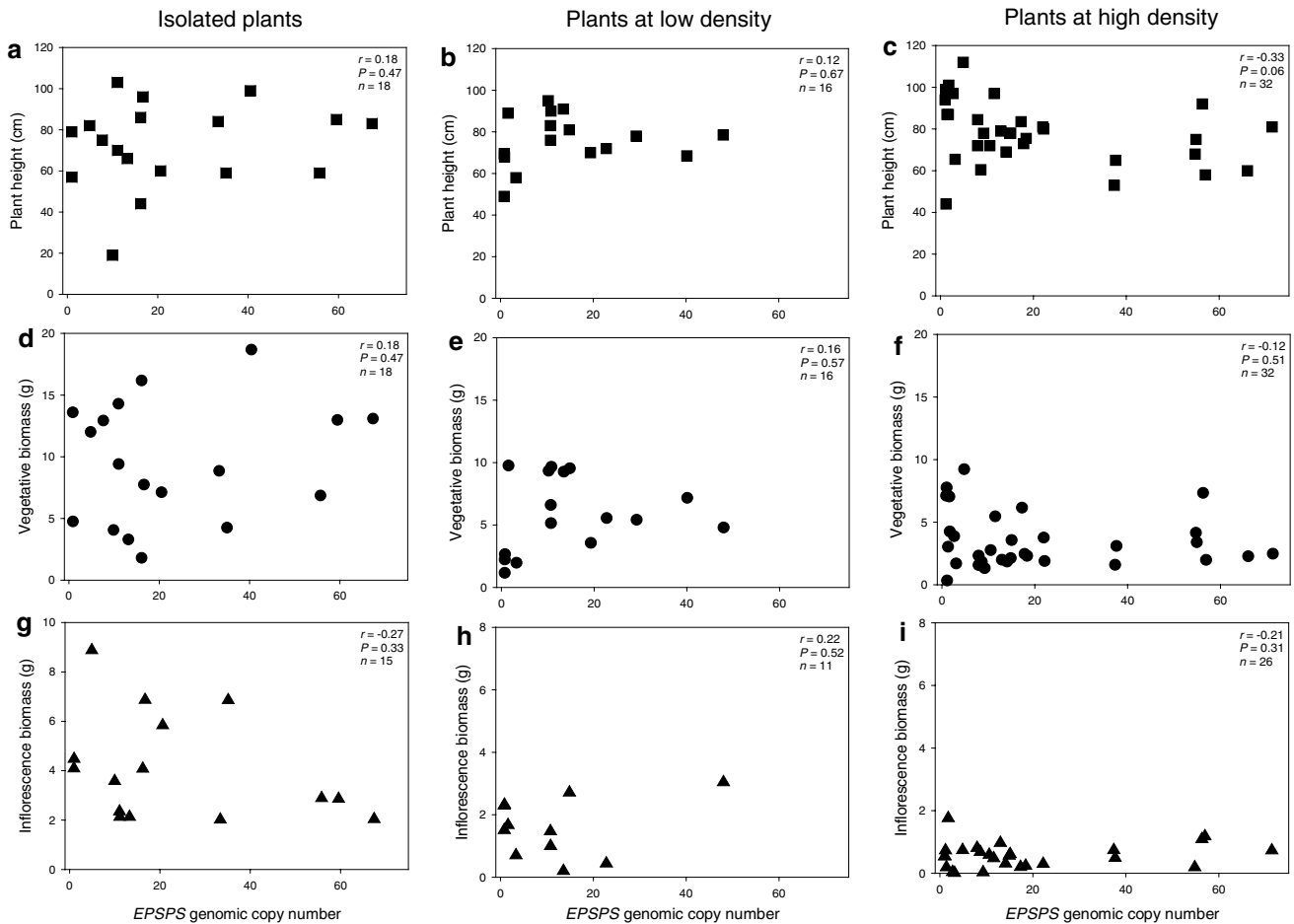
Similarly, no significant correlation ( $P = 0.62$ ) was found between biomass inflorescence and amplification of the *EPSPS* gene regardless of the competition gradient (Fig. 2). Plants with several-fold increase in *EPSPS* copy number allocated the same biomass to inflorescences as plants with no *EPSPS* amplification in each of the plant density treatments (Fig. 2g, h, i). A similar ratio of female and male plants was represented when assessing reproductive biomass in both experiments. As both floral sexes equally contribute to population fitness in the outcrossing *A. palmeri*, no discrimination between female and male inflorescences was carried out during the correlation analysis. To avoid any potential bias in sex-based fitness cost associated with *EPSPS* gene amplification, inflorescence sex (either male or female) was randomly assigned to plants with different *EPSPS* gene copy numbers. No significant correlation ( $P > 0.05$ ) was found between *EPSPS* amplification and either female or male inflorescence biomass regardless of the competition gradient.

## Discussion

Central to evolutionary biology is the principle stating that for herbicide resistance to evolve, a trait must (1) endow a survival and reproductive advantage under herbicide selection; (2) this advantage should exceed any ecological disadvantage or fitness cost (measured in the absence of herbicide treatment); and (3) selection of the trait arisen from within the standing or de novo population gene pool must proceed (Fisher 1958). Rapid herbicide resistance evolution is then expected when the herbicide selected trait endows a significant resistance level with no or negligible fitness costs (for example Yu et al. 2010; Li et al. 2013). The results of the present study not only confirm that amplification of the *EPSPS* gene in *A. palmeri* endows glyphosate resistance, but also reveal that a higher number of *EPSPS* genomic copies enable plant survival at higher glyphosate rates with no cost to the growth and reproduction variables measured in this study.

### *EPSPS* gene amplification and glyphosate resistance level

Glyphosate resistance in *A. palmeri* is endowed by amplification of the *EPSPS* gene which enables overexpression of the *EPSPS* enzyme (Gaines et al. 2010). This resistance mechanism results in the synthesis of *EPSPS* protein in excess that remains uninhibited by glyphosate (Powles 2010). This glyphosate uninhibited *EPSPS* protein enables plant metabolism to continue functioning and thus confers survival after glyphosate treatment. In a previous study, it has been shown that increments in the number of *EPSPS* gene copies had an additive effect on *EPSPS* protein activity, with additional *EPSPS* gene copies conferring a corresponding increase in *EPSPS* activity (Gaines et al. 2010). Here, we extend this additive effect of higher *EPSPS* genomic copies on the ability to survive higher glyphosate rates (Fig. 1). This indicates that the glyphosate dose required to inhibit *EPSPS* activity increases with increasing copy number, as there is more synthesis of *EPSPS* protein that remains uninhibited by glyphosate.



**Fig. 2** Changes in plant height, vegetative (stem and leaves) and reproductive biomass (inflorescences) of plants exhibiting a wide range of *EPSPS* genomic copy numbers. Plants were grown under non-competitive (i.e., isolated plants) (a, d, g) and competitive condi-

tions [low (b, e, h) and high (c, f, i) plant densities]. *P* values after correlation analysis, Pearson's correlation coefficients (*r*) for each pairwise combination of traits and *EPSPS* gene copy numbers. *n* Number of plants in each correlation analysis

The additive effect of additional *EPSPS* copies on glyphosate resistance level may imply a significant evolutionary advantage compared to other glyphosate resistance mechanisms. For a given genetic background and environmental conditions, including the herbicide dose, a particular resistance gene that is selected and fixed often endows an equal protection to all individuals sharing the same resistance. This is translated into a generally “constant” resistance status at the population level, largely dependent on the efficiency of the resistance gene in minimizing herbicide damage. For instance, a target-site mutation in the *EPSPS* gene (Pro-106-Thr) has been shown to endow 2–3-fold glyphosate resistance in a *Lolium rigidum* population which exhibits 50 % survival when exposed to glyphosate at 400 g ha<sup>-1</sup> (Wakelin and Preston 2006).

On the contrary, where the presence of an amplified gene is selected and fixed, there is resistance polymorphism among individuals within a population (Guillemaud et al. 1999). With the amplification of the *EPSPS* gene across the

entire *A. palmeri* genome, the resistance status of a population is a function of the amplification range determining the number of *EPSPS* gene copies present in each plant. Thus, *EPSPS* gene amplification precludes the estimation of an accurate and overall glyphosate resistance level and selection intensity until the amplification range of the *EPSPS* gene at the population level is known. As shown in the present study, *A. palmeri* plants with increasing levels of *EPSPS* gene amplification show increasing levels of glyphosate resistance. As the extent of amplification of the *A. palmeri* *EPSPS* gene has been shown to vary among accessions and plants over time (Gaines et al. 2010, 2011), this polymorphism may represent an advantage in an environment where glyphosate doses fluctuate over time. It may be reasonable to speculate that *EPSPS* gene amplification will contribute to the segregation of individuals exhibiting the minimum number of *EPSPS* gene copies to survive and grow under various glyphosate selective doses. This hypothesis highlights the importance of assessing the

inheritance and stability of the *EPSPS* gene amplification over time in *A. palmeri*.

#### *EPSPS* gene amplification and fitness traits

A common paradigm in evolutionary biology is the expectation that herbicide resistance genes have a fitness cost (Herms and Mattson 1992; Bergelson and Purrington 1996; Vila-Aiub et al. 2009b). This is supported by the observed polymorphism at alleles for herbicide resistance in herbicide unselected populations present in natural and agricultural systems. It is clear that fitness costs pose limits to resistance evolution, provided that the costs outweigh the advantage of the resistance trait. However, it is now known that herbicide fitness costs in plants are not universal and largely depend on the particular resistance mechanism (Vila-Aiub et al. 2005, 2011), specific resistance allele (Menchari et al. 2008), dominance of cost (Roux et al. 2004), pleiotropic effects on the kinetics of herbicide target proteins (Purrington and Bergelson 1999; Ashigh and Tardif 2007; Yu et al. 2010; Li et al. 2013), genetic background (Paris et al. 2008) and environment (Purrington and Bergelson 1999; Gassmann 2005; Tardif et al. 2006; Ashigh and Tardif 2009, 2011; Vila-Aiub et al. 2009a). Furthermore, and in clear contradiction with the fitness cost paradigm, a fitness advantage associated with a particular herbicide resistance allele of the acetyl CoA carboxylase (*ACCase*) gene has been observed (Wang et al. 2010).

The evidence presented here shows that the constitutive amplification of several *EPSPS* gene copies in *A. palmeri* (up to *c.* 80) does not attract evident pleiotropic effects on plant growth and reproductive variables. Our assessment of how *EPSPS* gene amplification influences *A. palmeri* reproduction only involved in the allocation of biomass to male and female inflorescences. However, given the strong relationship usually found between vegetative and seed biomass in vascular plants (Weiner 2004; Weiner et al. 2009) and the fact that similar vegetative biomass was produced by plants exhibiting a wide range of *EPSPS* gene amplification regardless of intensity of competition (Fig. 2), we speculate that no pleiotropic effects on *A. palmeri* fecundity are evident. A recent study has reported that transgenic over-expression of a native *EPSPS* gene developed to confer glyphosate resistance in rice has not only been shown to be associated with no fitness costs but also stimulation of growth and fecundity (Wang et al. 2013).

The lack of apparent fitness costs associated with amplification of the *EPSPS* gene contradicts the observed energy costs associated with amplified resistance genes in other organisms such as bacteria (Stoebel et al. 2008), yeast (Wagner 2005), and insects (Guillemaud et al. 1999; Field and Foster 2002). In *Escherichia coli* mutants with non-functional lactose operon repressors (*lacI*<sup>-</sup>), there is a

significant energy and fitness cost involved in the process of unnecessary gene transcription and protein translation responsible for lactose metabolism in a lactose-free environment (Stoebel et al. 2008).

Invocation of energy drain associated with the extra use of nucleotides, RNA polymerase, tRNA occupying free ribosomes, and excess amino acid production-related toxicity is often employed to account for fitness costs associated with gene amplification and higher gene expression (Wagner 2005; Stoebel et al. 2008). Given the results of this study, if any of the above-mentioned potential causes for fitness costs associated with *EPSPS* gene amplification are occurring, we may conclude that they are not severe enough to translate into impaired ecological fitness under intra-specific competition.

Multigenerational studies involving the assessment of changes in *EPSPS* gene amplification levels in *A. palmeri* plants under no glyphosate treatment are required to confirm the results reported here (Vila-Aiub et al. 2011).

#### Resistance evolution via *EPSPS* gene amplification and pleiotropy

At the early stage of the evolutionary process, herbicides may select for genetic resistance systems that endow an ecological advantage but are not necessarily the most efficient (Uyenoyama 1986). Microevolution and refinement of the original rudimentary mechanism endowing herbicide resistance and its full integration into the genome are possible over time (Uyenoyama 1986). Continuous changes in glyphosate selective conditions in both spatial and temporal scales involving treated and non-treated areas are contributing factors for the improvement of the herbicide resistance expression under both herbicide and natural selection. Therefore, it is argued that regulatory mutations responsible for incremental, progressive and continuous amplification of the *EPSPS* gene may have been selected in ulterior selective stages to the original selection event of the structural resistance trait (Uyenoyama 1986). This is especially possible in common agronomic conditions where glyphosate dose may be increased over time to achieve better weed control. Similarly, acquisition of modifier genes that compensate and reduce pleiotropic costs for the expression of unnecessary *EPSPS* gene expression is possible (Fisher 1928; Cohan et al. 1994; Lenormand et al. 1998). For instance, fitness compensation through the amplification of a gene unrelated to the original antibiotic resistance gene has been observed (Nilsson et al. 2006).

Glyphosate-resistant populations of *A. palmeri* are becoming very widespread in the USA (Heap 2013). Resistance can be due to *EPSPS* gene amplification (Gaines et al. 2010) as well as, of course, other resistance traits (Powles and Yu 2010). For the *EPSPS* gene amplification

resistance mechanism, it is evident that the additive effects of *EPSPS* gene amplification on resistance and the lack of associated fitness costs are key factors likely to mean that resistance due to *EPSPS* gene amplification will become a widespread glyphosate resistance mechanism.

**Acknowledgments** We thank Dr. Pedro E. Gundel (IFEVA-CONICET) for his valuable help in the statistical analyses. This work was supported by a grant from the Grains Research and Development Corporation (GRDC), Australia.

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