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Reduced absorption and impaired translocation endows glyphosate resistance in *Amaranthus palmeri* harvested in GR soybean from Argentina

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Abstract

Amaranthus palmeri S. Watson is probably the worst glyphosate-resistant (GR) weed worldwide. The EPSPS (5-enolpyruvylshikimate-3-phosphate-synthase) gene amplification has been reported as the major target-site-resistance (TSR) mechanism conferring resistance to glyphosate in this species. In this study, TSR and non-target-site-resistance (NTSR) mechanisms to glyphosate were characterized in a putative resistant *A. palmeri* population (GRP), harvested in a GR-soybean crop from Argentina. Glyphosate resistance was confirmed for the GRP population by dose-response assays. No evidence of TSR mechanisms as well as glyphosate metabolism was found in this population. Moreover, a susceptible population (GSP) that absorbed about 10% more herbicide than the GRP population was evaluated at different periods after treatment. The GSP population translocated about 20% more glyphosate to the remainder of the shoots and roots at 96 h after treatment than the control, while the GRP population retained 62% of herbicide in the treated leaves. This is the first case of glyphosate resistance in *A. palmeri* involving exclusively NTSR mechanisms.

Keywords: EPSPS gene amplification; glyphosate resistance crops; non-target-site-resistance; *Palmer amaranth*; yuyo colorado.

1. Introduction

Several attributes confer to *Amaranthus* species the capacity of becoming major global weeds which are very difficult to control.[1] Among those traits that must be highlighted include the C4 photosynthetic pathway, high growth rate, reproduction capacity, genetic variability, and stress tolerance.[2] The occurrence of *Amaranthus* species becomes even more concerning due to the evolution of multiple herbicide-resistant biotypes.[3] Among them, *Amaranthus palmeri* S. Watson is unique because it is a dioecious species. Compared with other common *Amaranthus* species, *A. palmeri* is the most competitive, sized (height and weight), and prolific weed.[4,5] Under ideal conditions, a single *A. palmeri* plant can shed more than 600 thousand seeds and surpass the 2 m in height.[6]

Amaranthus palmeri is native to the Sonoran Desert in North America, where it could be found from Southern California to Northern Mexico.[7] In about 20 years, it has extended its range from Ontario (Canada) to Brazil and Argentina.[1] Its prone to evolve resistance to herbicides, particularly to glyphosate, which in part explains this rapid spreading together with the commercialization of glyphosate-resistant (GR) crops.[8,9] This species is the most troublesome weed in row crops, especially for cotton and soybean producers on much of the American continent.[9] In Argentina, this species was first reported in 1966,[9] but it was not found again as an alien species in the country until 2004. From this year, *A. palmeri* started to be detected in summer cropping systems in southern parts of Cordoba and San Luis provinces, and from 2012 it was also found in maize, mani, soybean and sorghum in the country.[9]

Resistance to glyphosate governed by target-site-resistance (TSR) mechanisms in *A. palmeri* has received special attention. Since the first report of GR *A. palmeri* in 2006,[10] most of populations, collected mainly from across the United States, have shown target-site mediated resistance to this herbicide.[11,12] The EPSPS (5-enolypyruvylshikimate-3-phosphate synthase) gene amplification has been the most common TSR mechanism to glyphosate described in this species, [11,12] though a point-mutation Pro-106-Ser has also been found in some populations from Mexico.[13] Most recently it has been proposed that amplified EPSPS gene copies of GR *A. palmeri* are present in the form of extrachromosal circular DNA molecules which are transferred to the next generation by tethering to mitotic and meiotic chromosomes.[14]

Non-target-site resistance (NTSR) mechanisms to glyphosate seem not to be relevant in *A. palmeri*. However, some glyphosate-resistant populations of this species, collected in GR-cotton from Mexico in 2015, showed NTSR and TSR mechanisms.[13] The characterization of NTSR mechanisms demonstrated that the restricted absorption and impaired translocation of

glyphosate contributed to the resistance in those *A. palmeri* populations.[13] In other weed species the most widespread NTSR mechanism to glyphosate was also the impaired translocation of glyphosate by sequestering the herbicide into the vacuoles.[15-17] Moreover, a novel NTSR mechanism was described in *Ambrosia trifida*, the rapid cell death in response to the glyphosate application.[18] Finally, metabolism of glyphosate has been studied as a potential NTSR mechanism conferring resistance to this herbicide[20,21].

The diversity of resistance mechanisms to glyphosate highlights the dangers of extrapolating knowledge obtained from one resistant population to others. Considering that for *A. palmeri* almost all reported cases of glyphosate resistance were governed by TSR,[11,12,15] this should encourage to re-investigate the long-overlooked NTSR mechanisms, because they may contribute to resistance in selected populations. In this work, molecular experiments were carried out to confirm if two populations of *Amaranthus* from Cordoba, Argentina, one putative glyphosate resistant (GRP) collected in a GR-soybean field that survived glyphosate applications, and one susceptible (GSP) without a history of glyphosate applications, belonged to *A. palmeri.* In addition, resistance levels to glyphosate and the different NTSR and TSR mechanisms that could be present in the GRP population were also characterized.

2. Materials and Methods

2.1 Plant material

Matured seeds of a putative glyphosate resistant *A. palmeri* population (GRP) used in this research were harvested from GR-soybean fields in Cordoba province (Argentina) in 2016. Seeds of a glyphosate susceptible population (GSP) were also collected in 2016 from an area near the Campus of the University of Cordoba (Argentina), without a history of glyphosate applications. GRP and GSP seeds were sown in pots containing peat wetted at field capacity and maintained under controlled condition (28/18 °C day/night, photoperiod of 16-h, light density of 850 µmol m^{-2} s^{-1}, and 80% relative humidity) in a grower chamber. Seedlings with both cotyledons were transplanted into 250 mL pots containing sand/peat (1:2 v/v), and brought to a greenhouse with temperature and photoperiod similar to that in the growth chamber.

2.2 Species identification

Because *A. palmeri* and *A. hybridus* are difficult to distinguish by Argentinean farmers, since both species have the common name of *yuyo colorado*,[9] genetic analyses were needed to confirm and distinguish *A. palmeri* from other related species found in Argentina. Foliar tissues from 10 plants of each putative *A. palmeri* populations were taken for genomic DNA (gDNA)

isolation using the DNeasy Plant mini kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. The species identification was done by PCR using the specific primers AW90/AW155 developed by Wright et al.[22] Length of amplicons was verified by gel electrophoresis. Individuals of *A. hybridus* and *A. viridis* were also included for distinction using the respective specific primers [AW473/AW483 (1623 bp) and AW477/AW493 (1215 bp), respectively].[22]

2.3 Shikimic acid accumulation fast screening

Ten plants per population were used for a fast screening using shikimic acid accumulation as a parameter to separate plants from resistant to susceptible within a population, as well as to know the homogeneity or heterogeneity in and between *A. palmeri* populations. For individual, three foliar disks (4 mm in diameter) were placed into 2 mL tubes, and then, 1 µL of glyphosate at a concentration of 1000 µM was added to each tube.[23] Four replications per individual were obtained and the assay was repeated three times. The plant which presented a low shikimic acid accumulation implied the high resistance level (GRP), while a high shikimic acid accumulation implied high susceptibility (GSP). They were separated and transplanted into different pots (30 x 60 cm) and, after 3-4 months, new seeds (F_1) were collected for all future experiments.

2.4 Dose-response assays

The F_1 seeds of GRP and GSP populations were germinated as described above in plant material section. Young plants of *A. palmeri* with 4-true leaves were treated with the following increasing doses of glyphosate: 0, 31.25, 62.50, 125, 250, 500, 1000, 2000, and 4000 g ae (acid equivalent) ha^{-1}. The trade formulation of glyphosate used was Roundup Energy® SL (450 g ae L^{-1} as isopropylamine salt, Monsanto). The herbicide treatments were performed using a spray chamber (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with a Tee Jet 8002EVS nozzle pressurized with 200 kPa to deliver 200 L ha^{-1}, 50 cm above the plant level. The experiment was conducted using 10 plants from each population per glyphosate dose. Percentages of plant mortality (LD) and reduction of fresh weight (GR) were determined 28 days after treatment (DAT).

2.5 EPSPS enzyme activity assays

According to Dayan et al.[23], five g of leaf tissue in fine power from each population were transferred to tubes with 100 mL of cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10 % glycerol, 50 mM KCl and 0.5 mM benzamidine), 70 µL of β-mercaptoethanol and 1 % polyvinylpolypyrrolidone (PVPP). After an agitation process and subsequent centrifugation,

$(NH_4)_2SO_4$ was added in a proportion of 45% (w/v) to the supernatant. The mixture was stirred and then centrifuged twice to precipitate the total soluble protein (TPS). All pellets were dissolved in 3 mL of extraction buffer and dialyzed in 2 L of dialysis buffer (30 mm, 1000-MWC dialysis tubing at 4 °C on a stir plate) over 12 hours. The TPS concentrations in the raw extract were determined using the colorimetric method of Bradford.[24]

The specific EPSPS activities of the GRP and GSP *A. palmeri* populations were determined using the EnzCheck phosphate assay Kit (Invitrogen, Carlsbad, CA). The glyphosate concentrations tested to estimate de inhibition of EPSPS activity by 50% (I_{50}) ranged from 0.1 to 1000 µM. The experiments were conducted with five replications of each population per glyphosate concentration and repeated three times. The EPSPS activity was expressed as a percentage of the phosphate (µmol) released µg of TSP^{-1} min^{-1} in comparison to the controls (EPSPS basal).

2.6 EPSPS gene sequencing

Two samples of leaf tissue, one for total RNA extraction and the other for DNA extraction, were collected from ten individuals from each *A. palmeri* population and stored at -80 °C. The total RNA was extracted using the Tri Reagent solution (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer's instructions. The cDNA synthesis was carried out from 1 µg of the total RNA in all samples using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A fragment of the EPSPS gene, including the Thr102 and Pro106 positions, was amplified by using the EPSF1 (5'-ATGTTGGACGCTCTCAGAACTCTTGGT-3') and EPSR1 (5'-GTCATAAGTTTCAATGGCGGTGG-3') primers and PCR conditions described by Gaines et al.[11]. The EPSPS fragments were inserted in the pGEM®-T Easy Vector System (Promega Biotech Iberica, SL, Madrid, Spain) to clone them into competent cells of *E. coli* DH5a (Promega). Sanger sequencing of positive clones was carried out by STABVIDA (Caparica, Portugal).

2.7 EPSPS Gene copy number and amplification

The leaf tissue samples taken for DNA extraction in the previous section were used to determine the EPSPS gene copy number and expression. The gDNA was isolated using the same media as for the identification of species. EPSPS gene copy number (from gDNA) and gene amplification (from cDNA used for EPSPS gene sequencing) assays were performed using the EPSPS and acetolactato synthase (ALS) primers developed by Gaines et al.[11] Reactions were performed using a qRT-PCR Bio-Rad CFX connect thermal cycler and the following amplification profile: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and 95 °C for 15 s. PCR reactions were set up in 20 µL of SYBR Green PCR Master Mix (BIO-RAD),

following the manufacturer's instructions. Controls containing water were included to check for contamination in the qPCR reactions. The *ALS* gene was used as a reference gene to normalize qRT-PCR results. The relative amplification levels were calculated from the threshold cycle (Ct) values and the primer efficiencies by the Pfaffl method.[25] The EPSPS gene copy number in the *A. palmeri* gDNA was determined as described by Gaines et al.[11] Results were expressed as relative EPSPS gene copy number in relation to the *ALS* gene by the Pfaffl method.[25] Triplicate technical replications were used to calculate the mean and standard error of the increase in EPSPS gene amplification or copy number relative to ALS. Standard curves were performed for each primer pair to confirm appropriate efficiency of amplification (E=100±10%).

2.8 ^{14}C-glyphosate absorption and translocation

^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., Saint Louis, MO, USA) and the trade glyphosate formulation were mixed to prepare a solution with 0.834 KBq µL^{-1} of specific activity. The final concentration of the glyphosate solution was 300 g ae ha^{-1} in 200 L ha^{-1}. Twenty-three *A. palmeri* plants per population with 4-true leaves (three plants were reserved for the visualization of ^{14}C-glyphosate) received 1 µL drop (0.834 KBq plant^{-1}) onto the adaxial surface of the second leaf. The plants and subsequent samples [rinse solution, treated leaf (TL), remaining shoot tissue (ST), and roots system (RS)] were handled at 24, 48, 72 and 96 h after treatment (HAT) (five plants per population at each time evaluated) according to Domínguez-Valenzuela et al.[13] The experiment had a completely randomized design, it was repeated twice and the results of absorption and translocation of **^{14}C**-glyphosate were expressed in percentages of the total herbicide recovered and absorbed, respectively.[26]

The distribution of the ^{14}C (in form of ^{14}C-glyphosate or ^{14}C-metabolites) within *A. palmeri* plants was visualized by using a phosphor imager (Cyclone, Perkin-Elmer Packard Bioscience BV) at 96 HAT. The three whole plants of each population reserved were handled as described by Rojano-Delgado et al.[26]

2.9 Glyphosate metabolism

Amaranthus palmeri plants with six true leaves were treated with 300 g ae ha^{-1} glyphosate as in the dose-response assays. The same numbers of plants, without glyphosate treatment, were used as control. Treated and untreated plants were cut and divided into aboveground part (aerial part) and roots at 48 and 96 HAT, washed with distilled water, rapidly frozen in N_2 liquid and stored at –40 °C before being used. Glyphosate and the metabolites aminomethyl phosphonate (AMPA), formaldehyde, glyoxylate and sarcosine were quantified according to Rojano-Delgado et al.[26] Calibration equations were obtained using known

concentrations of standards of glyphosate and the metabolites (Sigma–Aldrich, St. Louis, MI). Five plants per population were used in a completely randomized design and the experiment was repeated three times.

2.10 Data analysis

The parameters GR_{50}, LD_{50} and I_{50} were determined using a three-parameter log-logistic equation $Y= d/1+(x/g)^b$, where *Y* is the response by 50% in relation to control; *x* is the herbicide rate; *d* is the upper limit; *g* is the GR_{50}, LD_{50} or I_{50}; and *b* is curve slope in *g*. Non-linear regression analyses were conducted in the R program using the *drc* package.[27] The R/S ratios of GR_{50}, LD_{50} or I_{50} were calculated to indicate the indices of resistance (RI).

The data of ^{14}C-glyphosate absorption and translocation, glyphosate metabolism, shikimic acid accumulation at 1000 µM glyphosate, and basal enzyme activity were analyzed using the software Statistix, version 9.0 (Analytical Software, USA). Percentage data were transformed into arcsine before the ANOVA, and the model assumptions of normal distribution of errors and homogeneous variance were inspected graphically. Values of $P<0.05$ from the ANOVA were considered significant and the means separated using the Tukey HSD test ($\alpha = 0.05$).

3. Results

3.1 Species identification

Using specific primers for the EPSPS intron 1 of *A. palmeri*, the resulting gel images revealed a band of 697 kb in length, confirming that all plants did belong to this species, both for GRP and GSP populations (**Figure 1**).

3.2 Accumulation of shikimic acid as a biomarker for glyphosate resistance

The highest shikimic acid accumulation was observed in plants from the GSP population, ranging from 0.4 to 0.55 mg mL^{-1}, while for the GRP population values ranged from 0.17 to 0.32 mg mL^{-1} (**Figure 2**).

3.3 Assays of dose-response to glyphosate

Resistance to glyphosate was confirmed for the GRP *A. palmeri* population. The GR_{50} and LD_{50} values estimated for the GSP population were 15.9 and 32.4 g ae ha^{-1}, respectively. According to these values, the GRP population was 34.6 (based on GR_{50}) and 59.7 (based on LD_{50}) times more resistant in comparison to the GSP population (**Figure 3**).

3.4 EPSPS enzyme activity

Both basal and enzyme activities of the EPSPS showed no differences between *A. palmeri* populations. The basal activities were 0.062 and 0.063 μmol Pi μg^{-1} TSP min^{-1} for the GRP and GSP populations, respectively. The I_{50} values of each population were 5.6 and 5.2 μM, respectively (**Figure 4**).

3.5 EPSPS gene sequencing, copy number and gene amplification

The sequenced fragment of the EPSPS of 462 bp did not reveal mutations neither in the Pro106 position nor the Thr102 for any *A. palmeri* population (**Figure 5a**). The EPSPS copy number relative to ALS as well as the gene amplification also showed no differences between GSP and GRP populations. The averages of copy number and gene amplification were 0.97 and 1.41, respectively, for the GSP population, and 1.03 and 1.32 for the GRP population (**Figure 5b**).

3.6 Absorption, translocation and distribution of ^{14}C-glyphosate

The absorption of ^{14}C-glyphosate differed between *A. palmeri* populations, which increased steadily from 12 to 96 HAT in both populations. The percentage of absorbed herbicide at 12 HAT was 18 and 25% for the GRP and GSP, respectively, and up to 70 and 80% at 96 HAT (**Figure 6a**). Similar amounts of ^{14}C-glyphosate were found in the treated leaves in both GRP and GSP plants at 12 HAT (91.4 and 86.4%, respectively); however, GSP plants moved on average 20% more ^{14}C-glyphosate to the remainder of shoot and roots than the GRP plants at 96 HAT. In this evaluated period, in the GRP plants was found 17.2 and 20.8% of the herbicide in shoot and roots, respectively, while in the GSP plants 24.0 and 31.2% (**Table 1**). Phosphor images, obtained at 96 HAT, corroborated these results showing that GRP plants retained the most of ^{14}C-glyphosate within the treated leaf, while GSP plants moved more herbicide to the rest of plant (**Figure 6b**).

3.7 Glyphosate metabolism

Glyphosate metabolites such as AMPA or glyoxylate were not detected in both populations, reinforcing that *A. palmeri* plants translocated only the herbicide. The amounts of glyphosate found in the roots were close to those quantified in the assays of absorption and translocation with ^{14}C-glyphosate. In the GRP plants were found 58.8 and 171.6 nmols glyphosate g^{-1} fresh weight at 48 and 96 HAT, respectively, corresponded to 12.2 and 21.8% of total glyphosate quantified; while for plants GSP, 14.7 and 29.1% (627.2 and 258.4 nmols glyphosate g^{-1} fresh weight, respectively) (**Table 2**).

4. Discussion

4.1 Species identification and resistance confirmation

Species identification following genetic analysis confirmed that both populations, GPS and GRP, belong to *A. palmeri*. The great phenotypic plasticity of *A. palmeri* can give an erroneous identification of this species, because it is also an obligate outcrosser that can hybridize with other *Amaranthus* species.[28] The two main species found in soybean production areas of Cordoba province, Argentina, are *A. palmeri* and *A. hybridus*,[9] therefore, ensuring species identification of *Amaranthus* genus is important since Argentinian farmers have difficulty identifying them morphologically.[9,22]

Once the GPS and GRP populations were distinguished as being *A. palmeri,* the glyphosate resistance was confirmed in the putative GRP population by its low shikimic acid accumulation, an unequivocal biochemical indicator of glyphosate resistance,[29] as well as its higher GR_{50} and LD_{50} values in comparison to the GSP population. Comparing these values with those registered in other glyphosate-resistant *A. palmeri* populations from New Mexico in USA[30], and other countries such as Brazil[1] or Mexico[13], they were similar. The low shikimate accumulation in the GRP population was congruent with the lower impact on growth reduction and plant mortality as glyphosate doses increased in comparison to the GSP population. The low GR_{50} and LD_{50} values estimated for the GSP population resulted from the high inhibition of EPSPS enzyme that produced a high and rapid accumulation of shikimic acid.[29]

4.2 TRS mechanisms characterization

The increase in the enzymatic activity of the EPSPS is indicating that TSR mechanisms are contributing to the resistance to glyphosate. However, the EPSPS basal activity or its inhibition by glyphosate were similar between the GRP and GSP *A. palmeri* populations. The selection of glyphosate resistance in *Amaranthus* species is apparently well described. EPSPS gene amplification have been reported as the major TSR mechanism reported in *A. palmeri,*[1131], as well as in other *Amaranthus* species,[32-34] noting that most of these resistance cases were documented in the USA. The lack of associated fitness cost makes the EPSPS gene duplication an important and widespread glyphosate resistance mechanism,[31] which presumably had a common origin of selection and spread rapidly across the USA.[35] Interestingly, our qPCR results showed the unlikelihood of a greater EPSPS gene copy number and/or its amplification in the GRP population. The distant geographic origin (Argentina) of the GSP and GRP populations compared to the populations of *A. palmeri* from the USA showed that the EPSPS gene amplification cannot be considered as the main mechanism of glyphosate resistance in this species in a generalized way. In addition, EPSPS gene sequencing did not reveal any amino acid substitution at positions Thr102 and Pro106, point mutation sites that

can promote changes reducing the binding of glyphosate with EPSPS, i.e., these substitutions can endow resistance to this herbicide as confirmed for *A. palmeri* from Chihuahua, Mexico,[13] *A. tuberculatus* from Missisippi, USA,[36] as well as other weed species.[15] Consequently, results indicated that TSR mechanisms were not involved in the resistance to glyphosate of the *A. palmeri* GRP. However, it cannot be ruled out that other South American populations of *Amaranthus* species may select for glyphosate resistance by TSR mechanisms.

4.3 NTSR mechanisms characterization

NTSR are important evolutionary mechanisms of herbicide resistance.[16] However, relatively few cases in which the cause of glyphosate resistance is at least a NTSR mechanism have been described. The main NTSR mechanism reported as endowing glyphosate resistance is the alteration of translocation patterns of the herbicide, thus in resistant plants, less glyphosate is translocated to meristematic growing points, and it is retained in the treated leaves.[37] In this research, both quantitative and qualitative results revealed altered patterns of ^{14}C-glyphosate absorption and translocation as the NTSR mechanisms in the GRP *A. palmeri* population from Argentina. Accordingly, the low accumulation of shikimate in GR plants evidenced the occurrence of NTSR mechanisms, since this pattern is due to the reduced foliar absorption or the modified subcellular distribution,[29] reducing the glyphosate amounts that reach the EPSPS. Therefore, it is congruent to infer that low absorption and impaired translocation of glyphosate were the primary and major mechanisms of resistance in the *A. palmeri* GRP population. These results are in consistent with those previously observed in this species,[13] other *Amaranthus* species,[36] or other weed species.[21,29,38-40] The low glyphosate absorption observed in the GRP was likely due to differences in the external leaf surfaces between populations,[40-42] while impaired translocation resulted from the greater retention of herbicide near to the treated area.[21,39] The strongest evidence has shown that the sequestration of glyphosate into the vacuole is the main NTSR mechanism responsible for altering the translocation patterns of this herbicide,[17,43,44] which is regulated by tonoplast-active transporters.[44]

Enhanced metabolism as NTSR mechanism has been reported in plants at most herbicide action sites,[16] but never for glyphosate. In this study, glyphosate was not metabolized in treated leaves of both the GR and GS *A. palmeri* plants. Glyphosate metabolism is not frequent in plants, and so far, it seems not to play an important role as an NTSR mechanism for glyphosate resistance[20]. Therefore, these results allows to conclude that glyphosate metabolism did not contribute to resistance of the GRP population, but also confirmed that *A. palmeri* plants translocated only the parent herbicide, demonstrating that

GRP plants selected for similar mechanisms of resistance to glyphosate like other weeds reinforcing the remarkable repeated evolution of herbicide resistance.[45]

Molecular characterization confirmed that both GSP and GRP populations were *A. palmeri*, and the glyphosate resistance of the second population was confirmed. This research is the first study unraveling the resistance mechanisms in *A. palmeri* from Argentina, revealing the non-involvement of TSR mechanisms, i.e., neither mutations nor EPSPS gene amplification were found in this population. By contrast, the GRP population exhibited a low absorption and impaired translocation of glyphosate as the main resistance mechanisms. This is the first case worldwide of glyphosate resistance in *A. palmeri* based only on NTSR mechanisms. Future experiments are required to unravel the physiological and biochemical basis of the reduced absorption and translocation found in this research, including gene amplification and regulation that could drive the evolution of NTSR to glyphosate in this and other weed species.

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480

481 **FIGURE LEGENDS**

482 **Figure 1.** Gel images of PCR to distinguish between *Amaranthus* species by sequencing the
483 intron 1 of the 5-enolpyruvylshikimate-3-phosphate synthase gene with specific
484 primers. First lane is a 1 kb ladder ranging from 10 to 0.3 kb.

485 **Figure 2.** Shikimic acid accumulation (mg mL^{-1} HCl) at 1000 µM glyphosate in ten GRP and GSP
486 plants of *Amaranthus palmeri* populations from Cordoba, Argentina.

487 **Figure 3**. Dose-response curves relative to percentages of fresh weight reduction (A) and plant
488 survival (B) in two populations (GRP and GSP) of *Amaranthus palmeri* from Cordoba,
489 Argentina; treated with different glyphosate doses evaluated at 28 d after treatment.
490 The log–logistic equations to estimate the GR_{50} values are: GSP $y= 100.0/[1+(dose/GR_{50})^{0.90}]$, and GRP $y= 100.3/[1+(dose/GR^{50})^{1.73}]$. The log–logistic
492 equations to estimates the LD_{50} values are: GSP $y= 99.9/[1+(dose/GR_{50})^{0.63}]$, and GRP $y=$
493 $98.8/[1+(dose/GR^{50})^{1.89}]$. Vertical bars represent the standard error of the mean (n =
494 10).

495 **Figure 4.** 5-enolypyruvylshikimate-3-phosphate synthase enzyme activity in leaf extracts from
496 two populations (GRP and GSP) of *Amaranthus palmeri* from Cordoba, Argentina. The
497 log–logistic equations to estimate the I_{50} values are: GSP $y=$
498 $99.6/[1+(concentration/I_{50})^{0.82}]$, and GRP $y= 99.0/[1+(concentration/I_{50})^{1.06}]$. Vertical
499 bars represent the standard error of the mean (n = 3).

500 **Figure 5.** A) Partial alignment of predicted amino acids of 5-enolpyruvylshikimate-3-
501 phosphatesynthase (EPSPS) genes of two populations (GRP and GSP) of *Amaranthus*
502 *palmeri* from Cordoba, Argentina. Blue boxes include positions 102 and 106
503 corresponding to point mutation sites confirmed to confer glyphosate resistance. B)
504 EPSPS copy numbers relative to the acetolactate synthase gene and EPSPS
505 amplification levels. Vertical bars represent the standard error of the mean (n = 10).

506 **Figure 6.** ^{14}C-glyphosate absorption and translocation in plants of two populations (GRP and
507 GSP) of *Amaranthus palmeri* from Cordoba, Argentina. A) ^{14}C-glyphosate absorption
508 from 12 to 96 h after treatment. Vertical bars represent the standard error of the
509 mean (n= 5). B) Digital (left plants) and autoradiograph (right plants) images that show
510 the distribution of ^{14}C within *A. palmeri* plants at 96 h after treatment. The highest
511 concentration of ^{14}C is highlighted in red.

512

513 **Table 1.** ^{14}C-glyphosate translocation (%) in two populations (GRP and GSP) of *Amaranthus*
514 *palmeri* from Cordoba, Argentina at different hour after treatment (HAT)

Population	HAT	^{14}C distribution (% of absorbed)[a]		
		Treated leaf	Remainder of shoot	Root
GRP	12	91.4 ± 3.5 [ns]	4.4 ± 0.2 [ns]	4.2 ± 0.2 b
GSP		86.4 ± 3.3 [ns]	6.4 ± 0.4 [ns]	7.2 ± 0.6 a
GRP	24	87.4 ± 3.0 a	6.0 ± 0.7 b	6.6 ± 0.6 b
GSP		76.8 ± 4.6 b	11.4 ± 1.2 a	11.8 ± 1.2 a
GRP	48	80.4 ± 3.7 a	8.6 ± 0.4 b	11.0 ± 0.8 b
GSP		69.0 ± 3.7 b	15.8 ± 1.9 a	15.2 ± 1.6 a
GRP	72	73.8 ± 3.2 a	11.2 ± 0.8 b	15.0 ± 2.8 b
GSP		60.6 ± 4.8 b	18.4 ± 1.8 a	21.0 ± 2.4 a
GRP	96	62.0 ± 6.5 a	17.2 ± 2.7 b	20.8 ± 3.3 c
GSP		41.8 ± 5.3 b	24.0 ± 2.3 a	31.2 ± 3.9 a

515 [a] Means with different letter per plant section to a certain evaluation period are statistically
516 different at $P<0.05$ according to the Tukey test. ns= no significant. ± Standard error of the
517 mean (n = 5).

519

520 **Table 2.** Glyphosate metabolism expressed as nmols of glyphosate/metabolites per gram of
521 fresh weight in two populations (GRP and GSP) of *Amaranthus palmeri* at 48 and 96 h
522 after treatment (HAT) with glyphosate at 300 g ae ha^{-1}

Population	HAT	Leaf area		Roots	
		Glyphosate	Metabolites	Glyphosate	Metabolites
GRP	48	421.7 ± 10.6	ND	58.8 ± 8.3	ND
	96	614.9 ± 9.1	ND	171.6 ± 9.4	ND
GSP	48	538.6 ± 9.5	ND	93.2 ± 13.3	ND
	96	627.2 ± 15.2	ND	258.4 ± 15.8	ND

523 ND (not detected)

524

525 **TOC/Abstract Graphics**

526

527

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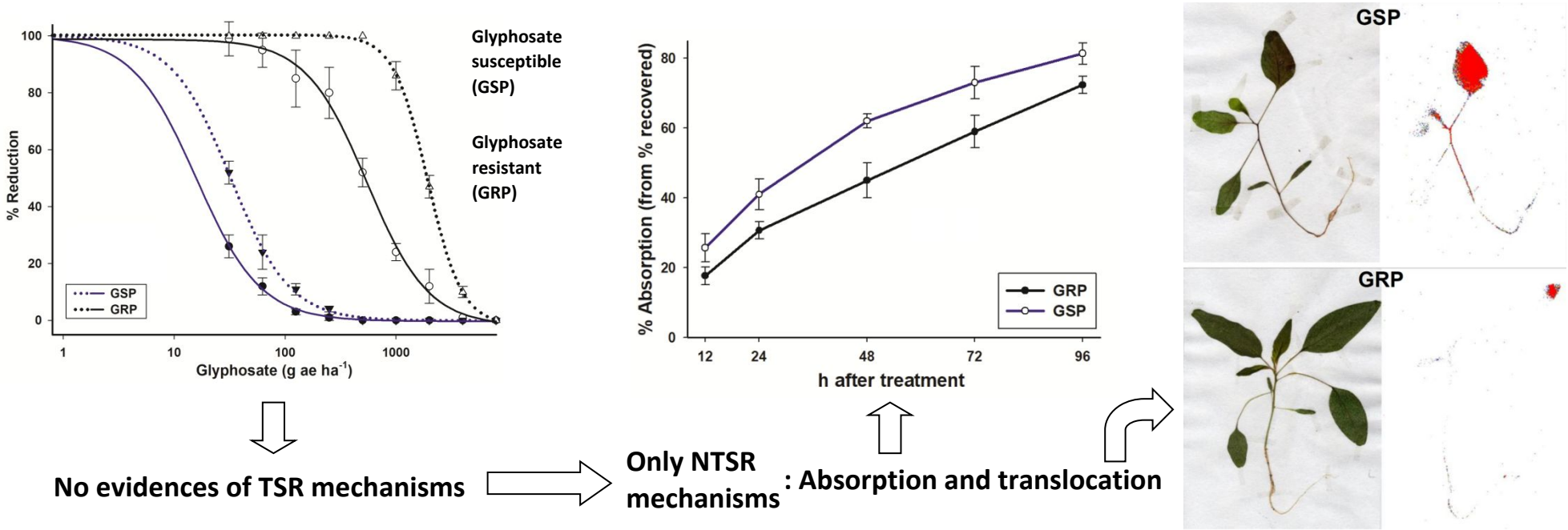
529



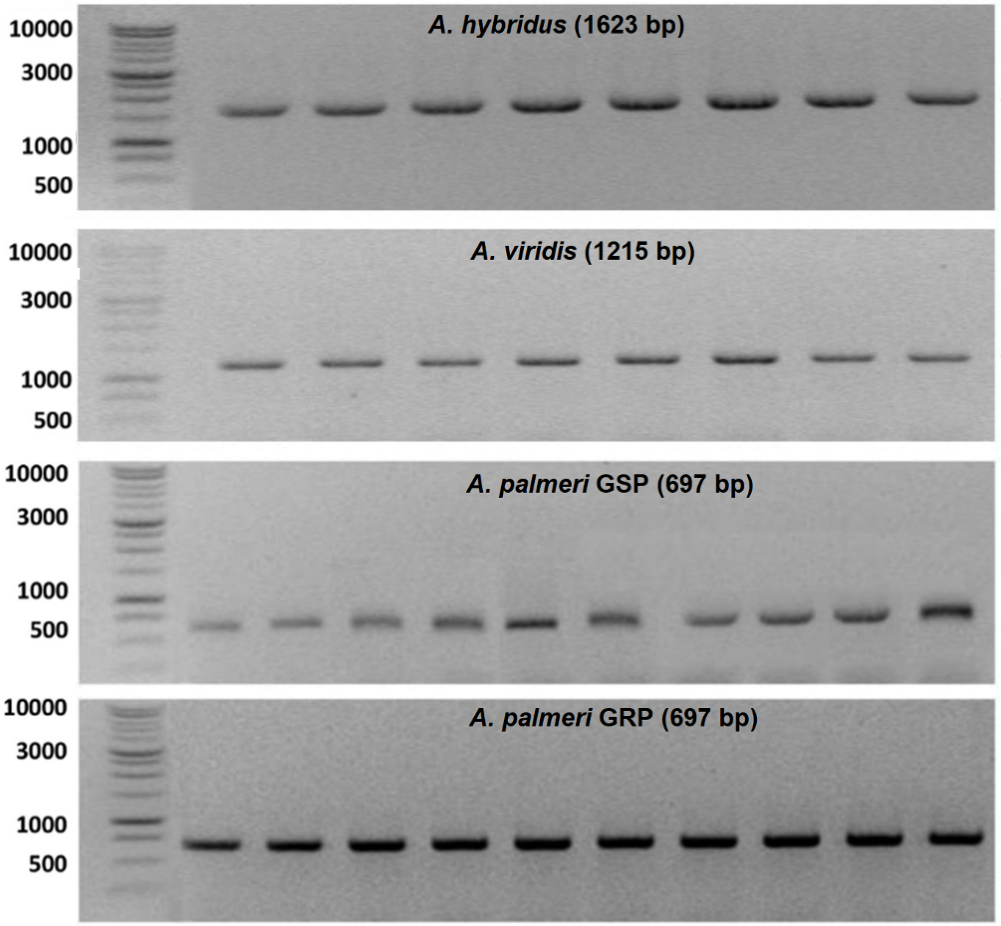


Figure 1. Gel images of PCR to distinguish between Amaranthus species by sequencing the intron 1 of the 5-enolpyruvylshikimate-3-phosphate synthase gene with specific primers. First lane is a 1 kb ladder ranging from 10 to 0.3 kb.

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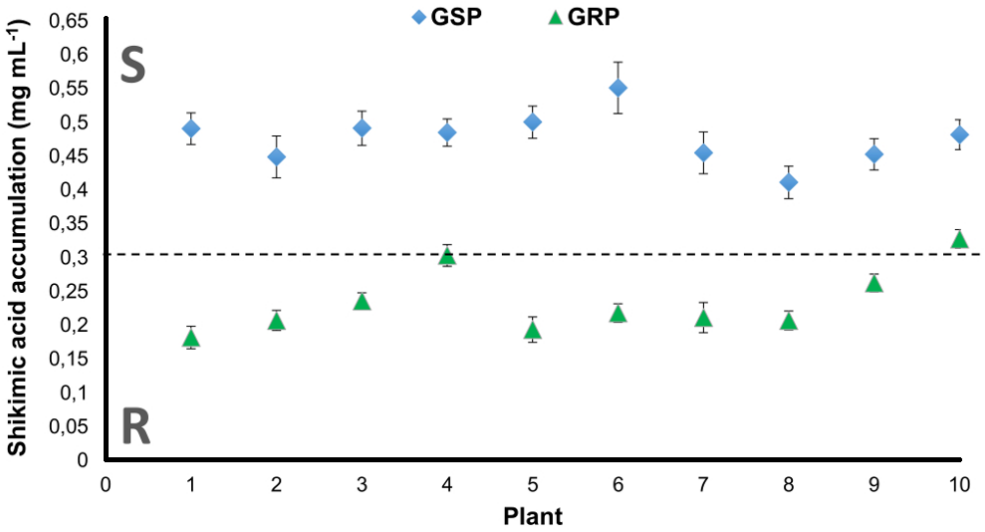
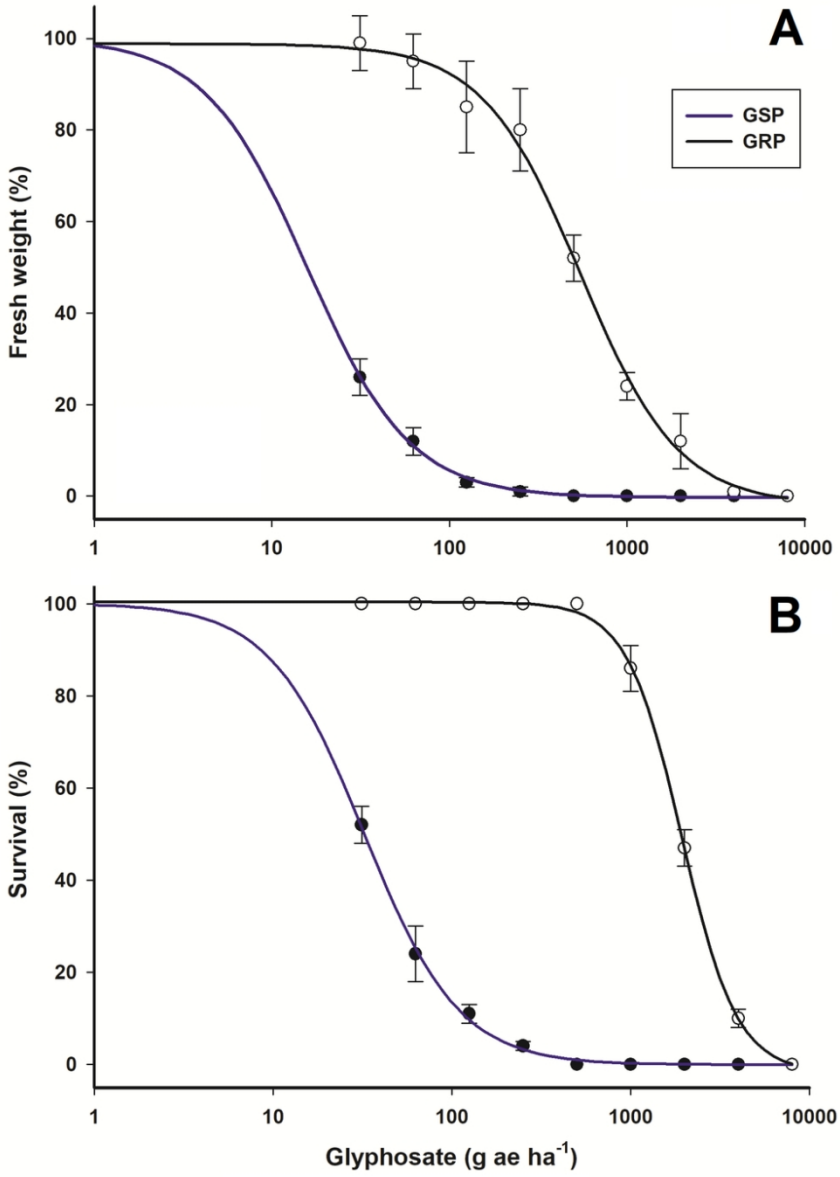


Figure 2. Shikimic acid accumulation (mg mL-1 HCl) at 1000 µM glyphosate in ten GRP and GSP plants of Amaranthus palmeri populations from Cordoba, Argentina.

85x45mm (300 x 300 DPI)



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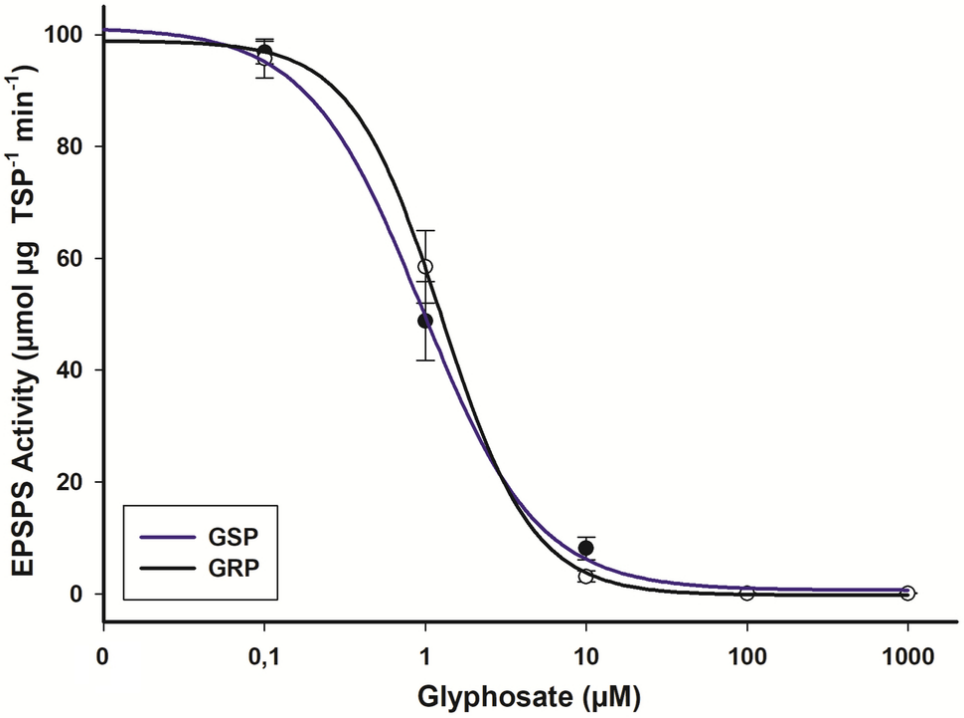
85x119mm (300 x 300 DPI)



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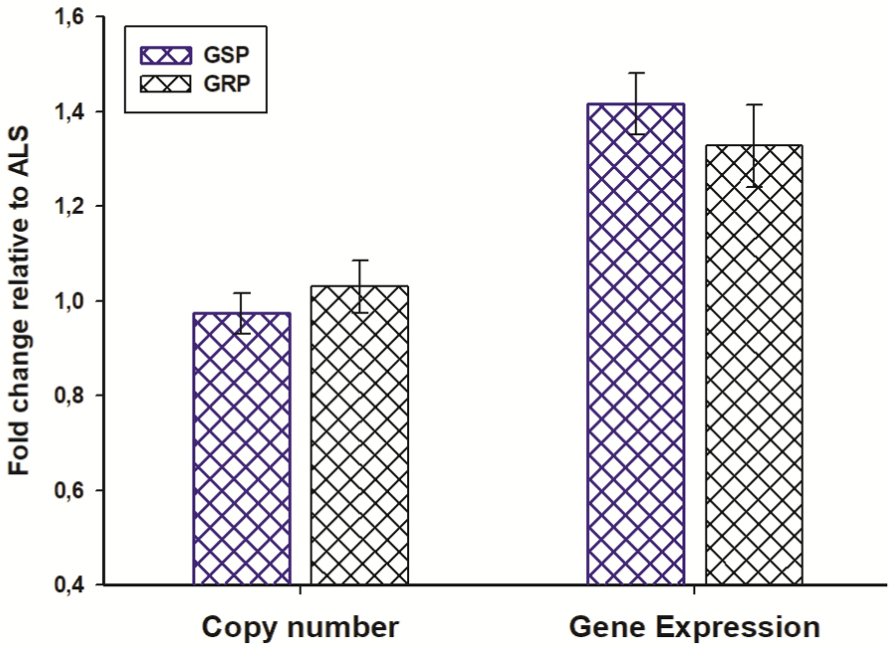
85x62mm (300 x 300 DPI)

A

Amino acid position	101	**102**	103	104	105	**106**	107
Nucleotide consensus	gga	**aca**	gcg	atg	cgc	**cca**	ttg
A. palmeri S (FJ861242.1)	G	**T**	A	M	R	**P**	L
A. palmeri R (FJ861243.1)	G	**T**	A	M	R	**P**	L
A. palmeri GSP	G	**T**	A	M	R	**P**	L
A. palmeri GRP	G	**T**	A	M	R	**P**	L

B



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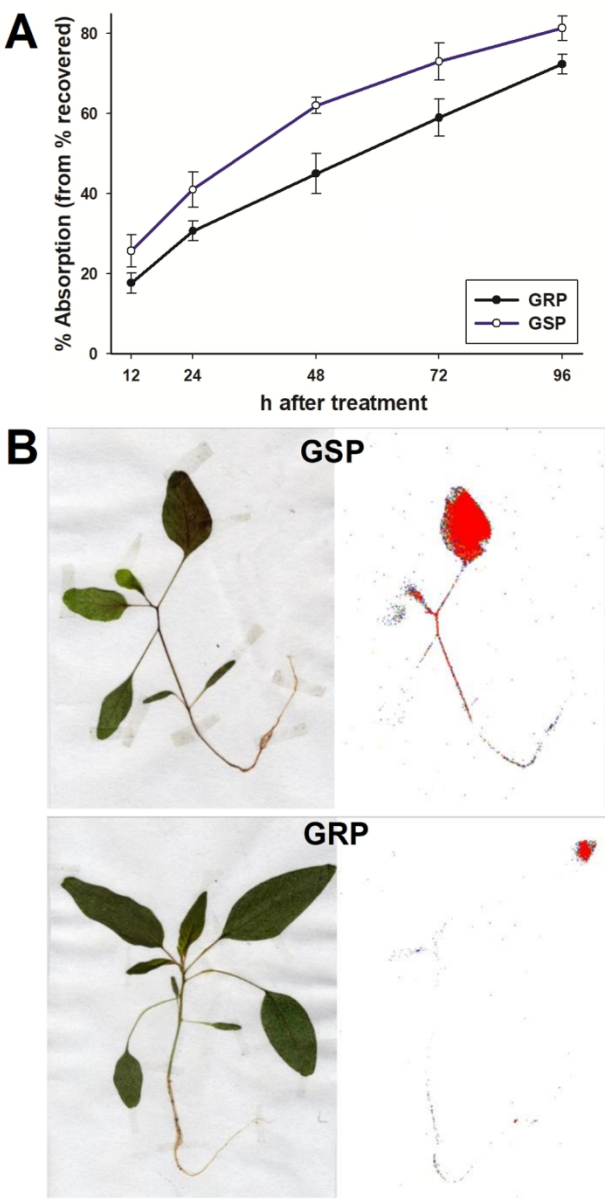

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85x167mm (300 x 300 DPI)