

## Potential Transference of CP4 EPSPS to Weed Species from Genetically Modified *Gossypium hirsutum* in Northern Mexico

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† Cándido Márquez-Hernández, our colleague passed away on May 9, 2016 in a tragic car accident. He will be sorely missed by us. This paper was published in his memory

### Abstract

The family of enzymes 5-enolpiruvil shikimato-3-phosphate synthase (EPSPS) is found in plants and microorganisms. The substrates of this enzyme are phosphoenolpyruvate (PEP) and 3-phospho-shikimate and their products are phosphate and 5-enolpyruvylshikimate-3-phosphate that is the biological target of the herbicide glyphosate, which is used in genetically modified crops. The interaction between cultivated genetically modified plants (GMP) and wild plant species could be a transference source of transgenes. Presence of transgenes could be cause and adverse environmental impact on non-target organisms. *Gossypium hirsutum* genotype Bollgard II<sup>®</sup> is a GMP with tolerance to herbicide glyphosate and it has been cultivated during 20 years in Mexico and the possibility to gene flow primary in congeners of the Malvaceae family is possible. The objective of this study was to quantify and identify weed species associated to genetically modified cotton fields and to detect the present of glyphosate-insensitive EPSP synthases (CP4 EPSPS) in these species. The results showed that plants of the families Amaranthaceae, Asteraceae, Boraginaceae, Chenopodiaceae, Convolvulaceae, Fabaceae, Malvaceae, Poaceae, Portulacaceae, Solanaceae and Zygophyllaceae are present in the study site. Twenty-five weed species belonging to these botanical families were collected and identified in the site. From these, two species of the Malvaceae family with potential risk of gene flow plants, *Anoda cristata* and *Sida hederacea* were identified in the site; however, the CP4 EPSPS protein was not detected in none of the collected weed species and only the GM genotype Bollgard II<sup>®</sup> was positive to the CP4 EPSPS protein in the study site.

**Keywords:** genetically modified plants; transgenic proteins; biodiversity; cross contamination; gene flow

### Introduction

The weeds are plants considered unwelcome mainly in human-controlled sites; are at this time present in extensive range environments around the world (Snir *et al.*, 2015).

However, from the view point of the taxonomy, the expression weed has no botanic meaning because of a plant that is a weed in one perspective is not a weed when developing in a condition where it is in fact desired, and where one species of plant is an appreciated crop plant, another species in the same group might be a severe weed

(Janick, 1979). The presence of weeds is one of the main problems that limit production in the cultivated species. The weeds have high adaptation to disturbed areas by agricultural activities and if they are not controlled in a timely and efficiently, reduce the yield of crops significantly.

Fiber cotton dominates the natural textile industry worldwide, refined cottonseed is used to produce high quality cottonseed oil as meal for human and animal. *Gossypium* is the cotton genus; it is part of the Malvaceae family that grows in more than 50 countries (Mehboob-ur-Rahman et al., 2012). Cotton is predominantly self-pollinating plant, i.e., fertilize themselves with their own pollen, and sometimes their pollen can be transfereed by insects (Cunningham, 2014). Around to 15 pest insect species infest cotton, with abundant losses and the pest insects control is an arduous and time-consuming task. The pest and weed-management use very toxic pesticides and herbicides result a public health discomfort (Bakhsh et al., 2015).

Biotechnological manipulation of several plants (genetically modified plants - GMP), improved the survived and yield of cultivate crops around the world, made it resistant to environmental stress, insects, herbicides and some diseases. This method removes the potentially unwelcome effects of linked alleles, which could be accidentally introduced into a crop gene pool in a traditional breeding program follows the Mendel's principles of genetic, as well is faster than the conventional breeding (Chapman and Burke, 2006; Juturu et al., 2015). However, the transgenes that confereed the ability to better growth and survive, could be taken for others organism, including the weeds plants (Sammons and Gaines, 2014). Potential hybridization intraspecific or interspecific could be occurs between variants or wild type, or with sexually compatible species, correspondently. The potential environmental risk is considered high between untransformed parent lines or in closely related weeds plants, moreover all the possibilities might happen (Ryffel, 2014).

Instead the first cotton genetically modified plants developed in 1996, several traits have been introduced. Insect, virus, bacteria, fungi, drought, chilling, heat, salt, and herbicide tolerance; as well as, manipulation of oil contain and fiber features have been reported (Center Environmental Risk Assessment, 2010; Bakhsh et al., 2015; Holst-Jensen et al., 2012; Emani, 2016). Developing strains glyphosate tolerant, made the culture of cotton friendly with environment. Several cotton producer countries have been adopted genetically modified varieties tolerant to herbicides (Manalil et al., 2017). Genetically modified cotton (*Gossypium* spp.) cover around the 70% of cultivated cotton in the United States of America (Chapman and Burke, 2006). Mexico has approved 18 GMP cotton, 17 as food and feed use. Nevertheless, Mexico is considered as primary diversity center for the genus *Gossypium* (Wegier et al., 2011). Therefore the potential propagation unwanted of the tolerance transgenes and keep the diversity are a national concerns, as well as essential topic for global food security (Quist and Chapela, 2001).

Gene flow of GMP crops to wild species have been reported (Rieseberg et al., 2003; Stewart et al., 2003; Warwick et al., 2003), beside with the possible persistence

of the transgenes in nature (Warwick et al., 2008). In cotton the pollen-mediated gene flow in greenhouse, confirmed that the resistant phenotype to herbicides and insect were transfer, and it be determined by pollinator species, distances between plants and the receptor plant (Yan et al., 2015, Cunningham et al., 2014). Freire (2002) suggest that genetically modified cotton can have pollination with wild cotton and native plants. The principal way to introduce new genes at the native population of plants is through the movement of seed or propagules to outside of the cultivate areas; a potential crossover with other sexually compatible families is possible (Center Environmental Risk Assessment, 2010).

Particular interest has the propagation of the gen EPSPS that codified for the protein 5-enolpyruvyl shikimate-3-phosphate synthase, originally isolated from one strain of *Agrobacterium* sp. (CP4 EPSPS), that was successfully incorporated to cotton and other crops (Harrison et al., 1996). After several field trials the genetically modified cotton strain glyphosate-tolerant has a huge potential to control weeds in cotton fields (Bakhsh et al., 2015). Indeed, the genetically modified gen is now used as selectable marker for detecting the gene flow between cultivated and native plants (Watrud et al., 2004; Weigier et al., 2011; Ryffel, 2014).

Genetically modified cotton has been sown in several part of the Mexican territory since 1996 which subsidizes purchase for the government. In 2003, 25 % of the nation was cover with GMP cotton. In 1996 the genetically modified cotton with tolerance to the herbicide glyphosate was approved in Mexico and all the farmers were be able to sown it (Vassant and Dinesh, 2016).

Reports for the cotton associated with weeds species described 126 species in Mexico; in Sonora, Mexico were described 104 weed species (Villaseñor and Espinosa, 1998) meanwhile, in San Luis Rio Colorado, Sonora, Mexico, a city located close to the border with US, 27 weed species has been recorded (Herrera-Andrade et al., 2010; Hernández et al., 2010). The objective of this study was to quantify and identify weed species associated to genetically modified cotton fields and to detect the present of glyphosate-insensitive EPSP synthases (CP4 EPSPS) in the weed species.

## Materials and Methods

### Study area

Delimited collecting area was plotted in the municipality of San Luis Rio Colorado, Sonora (32° 16' 25.54" N; 114° 57' 52.35" W) at the Border to Mexico-USA (Fig. 1). In the agricultural cycle of 2011, two hectares were cultivated with transgenic cotton genotype Bollgard II with resistance to insects and tolerance to herbicides. One sample of each weed plant present in the experimental plot, were collected and transported to the Agricultural Biology Laboratory at the Universidad Juarez del Estado de Durango. Also, one sample of the genetically modified cotton genotype Bollgard II was collected. The botanical identification was carried out using specialized codes (Villarreal-Quintanilla, 1983; Lot and Chiang, 1986; Villaseñor and Espinosa, 1998; Elpel, 2000).

### Detection test

For each collected and identified weed plant, leaves were tested for the presence of CP4 EPSPS using the QuickStix™ Combo Kit for Bollgard II R/ Roundup Ready R Leaf and Seed (EnviroLogix, Portland, ME, USA), as well the cotton genotype Bollgard II was used as positive control. Approximately 1 cm of leaf was placed into an Eppendorf tube containing 0.5 mL of extraction buffer. The tissues were macerated until it was completely triturated. The supernatant were transfer to a clean tube and one reactive strip was introduced in order to detect the presence of the transgene protein CP4 EPSPS in the correspondently line of the strip. Negative samples only show one line in about 4 mm below the top pads. Positive samples to CP4 EPSPS develop one additional band few millimeters down to the control line. This methodology was also used by Huang *et al.* (2007) and Yue *et al.* (2008).

## Results and Discussion

### Collected weed plants

Eleven botanical families were found in the 25 species collected of weed plants in San Luis Rio Colorado, Sonora experimental plot (Table 1) there is very close to the previous reports describing a richness of 27 species (Herrera-Andrade *et al.*, 2010; Hernández *et al.*, 2010). According with Booth and Swanson (2002), Perdomo *et al.* (2004) and Manalil *et al.* (2017) this difference could be due for disturbances in the land, the spatial and temporal variability in the communities and by the different strategies of cultivation. In Sonora, Mexico, Villaseñor and Espinoza (1998) reported a high number of weed species associated with the cotton agroecosystem while Martínez-Díaz and Jiménez-León (2009) reported 82 species of weeds in agricultural fields and orchards in La Costa de Hermosillo,

western Sonora; only 32 species were non-natives. Also, Tamayo (2010) reported 56 weed species associated with the cotton fields in Sonora, Mexico, which include *Malva parviflora* from the Malvaceae family. The floristic abundance in the different ecosystems including farmlands, is associated with the cultivation practices and environmental conditions of the habitats; these attributes are important elements that define the existence of species in a site (Dauber *et al.*, 2003), possibly because of that, the differences found in weed species at the study site were different with those reported in Sonora, Mexico. The Malvaceae family found in the experimental plots in San Luis Rio Colorado, Sonora, Mexico was represented by *Anoda cristata* and *Sida hederacea* that are the species more vulnerable to presenting gene flow with genetically modified cotton (Center Environmental Risk Assessment, 2010).

### Identifying of the CP4 EPSPS protein

From the 25 weeds species identifies in the experimental plot in San Luis Rio Colorado, Sonora, Mexico; none one gave positive to the presence of CP4 EPSPS protein, using the certificated test to QuickStix™ Combo Kit for Bollgard II R/ Roundup Ready R Leaf and Seed and the cotton genotype Bollgard II was the unique that presented the positive result. This finding suggest that the potential gene flow between the transgenic cotton sown for around 18 years in the region and the weed plants associated has not yet occurred, or that protein is not expressed for these plants. Similar results reported Márquez-Hernández *et al.* (2015) in a study done to detect the presence of the CP4 EPSPS protein in weed flora in genetically modified cotton fields in Coahuila and Durango, Mexico, who found four weed species of the Malvaceae family, *Malvastrum coromandelianu*, *Sphaeralcea angustifolia*, *Anoda cristata* and *Sida hederacea* in both, Coahuila and Durango, Mexico,

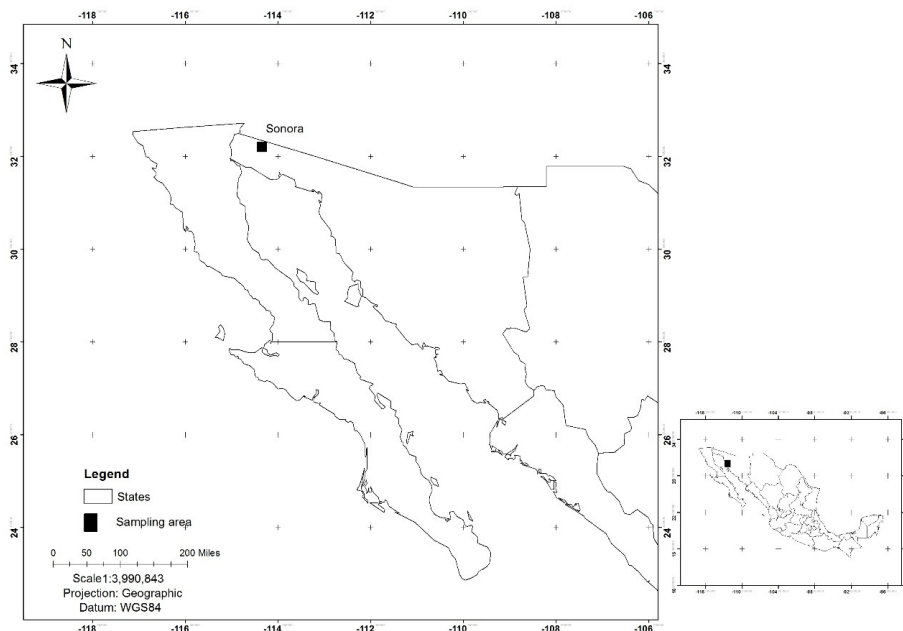


Fig. 1. Location of cultivated plots of genetically modified cotton in San Luis Rio Colorado, Sonora, Mexico

Table 1. Presence of the CP4 EPSPS protein in weed species associated to genetically modified cotton in San Luis Rio Colorado, Sonora, Mexico

Species number	Family	Scientific name	Control line	CP4 EPSPS
1	Amaranthaceae	<i>Amaranthus hybridus</i>	*	
2	Asteraceae	<i>Helianthus annuus</i>	*	
3	Asteraceae	<i>Pluchea sericea</i>	*	
4	Asteraceae	<i>Sonchus oleraceus</i>	*	
5	Asteraceae	<i>Verbesina encelioides</i>	*	
6	Asteraceae	<i>Xanthium strumarium</i>	*	
7	Boraginaceae	<i>Heliotropium curassavicum</i>	*	
8	Chenopodiaceae	<i>Chenopodium album</i>	*	
9	Chenopodiaceae	<i>Chenopodium murale</i>	*	
10	Convolvulaceae	<i>Convolvulus arvensis</i>	*	
11	Convolvulaceae	<i>Ipomoea purpurea</i>	*	
12	Fabaceae	<i>Hoffmanseggia glauca</i>	*	
13	Malvaceae	<i>Anoda cristata</i>	*	
14	Malvaceae	<i>Sida hederacea</i>	*	
15	Poaceae	<i>Chloris submutica</i>	*	
16	Poaceae	<i>Cynodon dactylon</i>	*	
17	Poaceae	<i>Echinochloa colona</i>	*	
18	Poaceae	<i>Echinochloa crus galli</i>	*	
19	Poaceae	<i>Eragrostis mexicana</i>	*	
20	Poaceae	<i>Phalaris minor</i>	*	
21	Poaceae	<i>Setaria verticillata</i>	*	
22	Poaceae	<i>Sorghum halepense</i>	*	
23	Portulacaceae	<i>Portulaca oleracea</i>	*	
24	Solanaceae	<i>Solanum elaeagnifolium</i>	*	
25	Zygophyllaceae	<i>Tribulus terrestris</i>	*	
26	Malvaceae	<i>Gossypium hirsutum</i>	*	*

but the CP4 EPSPS protein was not detected in none of these weed plants located in the agroecosystem of genetically modified cotton in these study sites.

Several hypotheses allow explicating this result could be the deficient transport of pollen by wind or insects (Gómez, 2002; Guzmán *et al.*, 2008). Other causes could be the incompatibility among the sexual form of this genetically modified seed and the native weed plants or their chromosomal complement is not productive. But also, the lack of synchrony among the susceptible species, the genetically modified cotton and their fertility periods is considered as barrier (Center Environmental Risk Assessment, 2010). Generally, the current evidence of genetic flow is the presence of fertile progeny carried the genetically modified varieties (Center Environmental Risk Assessment, 2010; Risk Assessment Search Mechanism, 2010; Mallory-Smith *et al.*, 2015), which in this case were not detected because of that introgression of the tolerance to glyphosate was not found in weed species in the genetically modified cotton fields in San Luis Rio Colorado, Sonora, Mexico. Similar results reported Warwick *et al.* (2008); Mallory-Smith and Zapiola (2008) and Center Environmental Risk Assessment (2010), also, Brubaker *et al.* (1999) found that Australian wild cotton species fail to serve as recipients to transgenes from commercial species of *Gossypium hirsutum*, because of the prezygotic barriers that separate the genetically modified tetraploid cotton from their wild (diploids) Australian relatives.

## Conclusions

Eleven botanical families were found in the 25 species collected of weed plants in San Luis Rio Colorado, Sonora experimental plot, which is very close to the previous reports describing a richness of 27 species associated to genetically modified cotton. From these species, *Anoda cristata* and *Sida hederacea* belong to the Malvaceae family are species identified with potential risk of gene flow plants; however the CP4 EPSPS protein no was detected in none collected weed species, including two species of the Malvaceae family. Only the GM genotype Bollgard II was positive to the CP4 EPSPS protein in the study site.

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### Authors' contributions

JEA, JRER contributed to weed species collection; PPR, JJRP, JRER and CMH participated with analysis of laboratory, data and manuscript preparation; AT and CMH wrote and edited the first draft of the manuscript; BMA wrote the final version of the manuscript. All authors read and approved the final manuscript.

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