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Pollen-mediated gene flow in the cultivation of transgenic cotton under experimental field conditions in Spain

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ABSTRACT

In line with current regulations and in order to ensure the feasible coexistence of genetically modified (GM) cotton (*Gossypium hirsutum* L.) cultivars with conventional cultivars, it is essential to assess the potential for pollen-mediated gene flow (PMGF). Experiments measuring gene flow in cotton were carried out in two locations in the province of Seville, in southern Spain's region of Andalusia, first in Peñaflor in 2007 and then in Lebrija in 2009 and again in 2010. In 2009 and 2010, the experiment consisted of a 4 ha square field of non-GM cotton, in the middle of which a 40 × 40 m central plot of GM cotton was sown. The GM cotton used as pollen source was glyphosate-tolerant cotton and a non-GM counterpart was used as pollen receptor. In 2007, the non-GM field was a 4.8 ha rectangle (120 × 400 m) in the middle of which a 20 × 100 m plot was sown with the GM insect-protected *Bt* Cotton, which displayed tolerance to the herbicide glufosinate due to the presence of the selectable marker gene. Recipient and donor cultivars went into bloom in synchrony and uniformly. The progeny of non-GM plants grown at distances of from 1 m to 50 m along eight directions radiating from the central GM plot (up to 150 m in some directions) was screened for herbicide resistance to measure PMGF frequency. In the Lebrija experiments, putative hybrids recorded after glyphosate screening were confirmed as true hybrids by Polymerase Chain Reaction (PCR), while glufosinate was used for herbicide screening and Enzyme-Linked ImmunoSorbent Assay (ELISA) was used to confirm resistance of the Peñaflor hybrids. In all cases, PMGF declined exponentially as distance from the pollen source increased. Outcrossing differed among locations, with highest values obtained in 2007 in Peñaflor, where the average across the eight directions was 3% (0.9–9.8%) at 1 m from the GM source, and with values of 0.2% (0–0.5%) and 0.06% (0–0.3%), at 10 m and 25 m respectively. In Lebrija, these values, averaged across directions and years, were 0.17% (0–0.64) at 1 m, 0.19% (0–0.98) at 10 m and 0.01% (0–0.16) at 25 m. Although experimental design, cultivars, insect vectors and environmental conditions could be sources of variation, under the circumstances of this assay, PMGF values at 10 m distance were always below the 0.9% threshold based on seeds permissible under European Union (EU) regulation.

1. Introduction

Cotton (*Gossypium hirsutum* L.) is one of the most important non-food crops in the world. Its products are destined to different industries as textiles, cosmetic, feed or chemicals. European Union (EU) cotton production represents only 1% of global cotton production and the two primary cotton-producing Member States are Greece, with 80% of the cotton area, and Spain, with 20%, and even if it represents less than 0.2% of the total value of European agricultural production, it is important in socioeconomic terms for both countries. Cotton is one of the world's four major genetically modified (GM) crops. Several characteristics such as biotic (insects, viruses, bacteria and fungi) resistance, abiotic (drought, chilling, heat, salt), herbicide tolerance, manipulation of oil and fiber traits have been reported to date (Bakhsh et al., 2015). In 2013, total global GM cotton was grown in 20 million hectares, representing 70% of the area planted to cotton in the world (James, 2013). The EU has

Abbreviations: *Bt*, *Bacillus thuringiensis*; ELISA, Enzyme-Linked ImmunoSorbent Assay; EPSPS, 5-EnolPyruvyl Shikimate-3-Phosphate Synthase; GM, Genetically Modified; IPM, Integrated Pest Management; PAT, Phosphinothricin Acetyl Transferase; PCR, Polymerase Chain Reaction; PMGF, Pollen Mediated Gene Flow.

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Table 1
Meteorological data during the experimental cotton field growing seasons in Andalusia, Spain.

Field	Year	Month	Temperature (°C)	Precipitation (mm)	Relative humidity (RH, %)	Prevailing winds	
						Direction (n° days)	Avg. speed (km h^{-1})
Peñaflor	2007	May	19.1 ± 3.0	138.2	60 ± 13	SW (17)	4.1 ± 1.9
		June	23.3 ± 1.9	2.2	51 ± 11	SW (16)	5.1 ± 2.0
		July	27.8 ± 2.1	0.0	38 ± 5	W (15)	3.9 ± 1.0
		August	26.7 ± 2.0	2.6	45 ± 8	W (13)	4.3 ± 1.4
		September	23.7 ± 2.3	32.2	59 ± 10	SW (11)	4.0 ± 1.7
		October	18.3 ± 2.2	18.8	62 ± 9	NE (24)	4.2 ± 1.6
Lebrija	2009	May	19.2 ± 1.9	2.8	65 ± 11	SW (11)	8.6 ± 2.4
		June	23.5 ± 3.4	3.8	65 ± 10	SW (18)	10.1 ± 3.2
		July	24.9 ± 1.7	1.4	60 ± 7	SW (20)	8.2 ± 1.2
		August	25.9 ± 2.0	0.8	61 ± 8	SW (11)	6.8 ± 1.4
		September	22.7 ± 2.5	25.6	66 ± 11	SW (13)	6.8 ± 2.2
		October	20.3 ± 1.5	17.4	71 ± 8	SW (12)	6.7 ± 3.2
Lebrija	2010	May	18.7 ± 2.9	19.2	60 ± 12	SW (13)	10.0 ± 2.9
		June	21.5 ± 2.5	28.2	67 ± 8	SW (23)	9.6 ± 3.5
		July	26.0 ± 1.8	0.2	58 ± 7	SW (17)	7.4 ± 1.6
		August	26.6 ± 1.9	1.4	60 ± 9	SW (16)	6.7 ± 1.3
		September	22.8 ± 2.4	3.2	62 ± 8	SW (12)	6.8 ± 1.8
		October	17.0 ± 1.9	52.0	69 ± 10	SW (13)	8.0 ± 4.1

approved the import of several lines of GM cotton but only for use as food and feed, not for cultivation. Although applications for GM cotton for cultivation have been submitted for evaluation in the EU they were withdrawn by the companies. One of the issues associated with GM cultivation is the transfer of genes by hybridization, which can occur at inter- and intra-specific level and could lead to the escape of transgenes. In Europe there have been no reports of sexually compatible wild relatives of *G. hirsutum* (Tutin and Heywood, 1992). Once GM cotton is released for commercial production, potential gene transfer from GM cotton will be restricted to non-GM neighboring cultivars and to occasional feral populations. Therefore, the farmers could lose the ability to choose between conventional, organic or GM-based crop productions while complying with EU legislation on labelling and/or purity standards, which is moreover a prerequisite to preserving consumers' freedom of choice (Devos et al., 2009). EU regulations on food and feed derived products establish a 0.9% labeling threshold for the adventitious presence of GM material in non-GM food and feed (Regulation EC No. 1829/2003). Thus, problems may arise if no measures are taken prior to the release and commercialization of any GM cotton cultivars.

Cotton is a predominantly self-pollinated crop (Niles and Feaster, 1984), but it has some degree of outcrossing and may be cross-pollinated by certain insects, primarily bees and bumblebees (McGregor, 1959; Moffett et al., 1975). Loden and Richmond (1951) reviewed cross-pollination data in cotton available in the literature until 1950 and found a large variability from 1 to 81%, compromising the purity of breeding stocks and varieties. This variability was also detected in the 60s in the Mississippi Valley (USA), with rates between 10 and 47% (Simpson, 1954; Simpson and Duncan, 1956; Sappenfield, 1963). Poehlman (1959) and Allard (1960) also cited similar cross-pollination rates, 5–25% and 5–50%, respectively, and often above 10%. However, in studies conducted after the 60 s, rates of pollen-mediated gene flow (PMGF) in cotton rarely exceed 10% in plants grown in close proximity to the pollen sources (Meredith and Bridge, 1973; Umbeck et al., 1991; Llewellyn and Fitt, 1996; Xanthopoulos and Kechagia, 2000; Zhang et al., 2005; van Deynze et al., 2005; Llewellyn et al., 2007). In all studies, cross-pollination rates decline rapidly as distance increases from the pollen source (see Andersson and de Vicente, 2010 review). These substantial changes in cross-pollination rates over time may be due to changes in crop management in intensive agricultural systems (Kremen et al., 2002). A more recent study that measured transgene flow from *Bt* to non-*Bt* cotton commercial fields in Arizona, showed that PMGF occurred at rates below 1% at field edges (Heuberger et al., 2010). However, pollen is not the only possible source of adventitious presence and other sources exist as seed-mediated gene flow, volunteer cotton or unwanted mixing.

Data on PMGF between cotton cultivars in Spain is scarce. This information is essential to establish appropriate measures, as isolation distances between GM and non-GM cotton, which will allow for the coexistence of GM and conventional cotton crops. The objective of this study is to estimate PMGF rates under the specific conditions of cotton cultivation in Spain.

2. Materials and methods

2.1. Experimental site location

Three field experiments on PMGF in cotton were carried out in the Guadalquivir River valley in the province of Seville, in southern Spain's region of Andalusia, where is grown 98% of existing cotton in Spain. The first experiment, carried out in 2007, took place in Peñaflor (37°44′10″ North, 5°19′12″ West). Two more experiments were carried out in 2009 and 2010 in Lebrija (36°58′10″N, 6°4′34″W). The climate of the region is typically Mediterranean with mild, wet winters and autumns and long, dry summers. Mean annual rainfall is 598 mm, mostly distributed from October to May and almost absent during the summer cropping season. Climatic data were obtained from weather stations "Lora del Rio" (37°39′37″N, 5°32′19″W, 68 m a.s.l.) in 2007 and "Lebrija I" (36°58′40″N, 6°7′30″ O, 25 m a.s.l.) in 2009 and 2010. Annual growing seasons' average air temperatures, maximum and minimum temperatures, relative humidity, total rainfall and wind speed and direction are shown in Table 1.

2.2. Plant material

The GM cotton cultivar used as pollen donor in 2007 was "WideStrike" Insect-Protected *Bt* Cotton (Phytogen 440W, Dow AgroSciences). This cotton contained the insecticidal genes cry1Ac and cry1F derived from *Bacillus thuringiensis* (*Bt*) and expressed insecticidal proteins Cry1Ac and Cry1F (Bt toxins), which are toxic to specific lepidopteran caterpillar insects, including cotton's major caterpillar pests. This line also displayed tolerance to the herbicide glufosinate ammonium due to the presence of the selectable