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Best practice document for the coexistence of genetically modified cotton with conventional and organic farming

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Executive summary

The Technical Working Group (TWG) for Cotton is the third one of the European Coexistence Bureau (ECoB) and is established for elaboration of the coexistence issues between genetically modified (GM) cotton cultivation and non-GM cotton and honey production in the EU.

The present technical report analysed the possible sources for potential cross-pollination with GM cotton and adventitious admixture of GM cotton material such as seeds and pollen and presents consensually agreed by TWG for Cotton best practices for coexistence. The terms of reference for this review are presented in Section 1. The scope of the Best Practice Document is coexistence in cotton production in the EU. It includes the coexistence between GM cotton cultivation and honey production but excludes coexistence in seed production.

The ECoB TWG for Cotton held two meetings in October 2014 and April 2015 and examined the state-of-the-art from scientific literature, research projects and empirical evidence provided by existing studies for segregation in cotton production looking at the factors determining the cross-pollination rates in cotton as well as other sources of admixture of GM material in conventional cotton harvests and EU-produced honey. The review of this information (coming from a total of 194 references) is presented in a structured manner in Sections 5 and 6 of this document. Finally, the TWG for Cotton reviewed the up todate approaches for the detection and identification of traces of GM cotton material in non-GM cotton harvests and honey (Section 7).

The TWG for Cotton of the ECoB, based on the analysis of the evidence summarised in this document submitted proposals for best management practices, which form the ground the agreed consensus recommendations presented in Section 8, complemented by ex-ante view about their economic impact (Section 9).

Contents

1. Introduction

1.1. Legal background

The European legislative framework for coexsitance in agriculture is created to ensure that cultivation of genetically modified (GM) crops is carried out in a way that allows different agricultural systems to co-exist side by side in a sustainable manner, which in turn promotes freedom of choice throughout the food chain. The coexistence rules support market forces to operate freely in compliance with the Community legislation. The legislative basis in the EU for the coexistence of GM and non-GM crops is established by the relevant legislation for the release of GMOs into the environment, and food and feed legislation for the labelling requirements of GMOs presence. Both pieces of legislation provide a harmonised approach for the assessment of all potential environmental and health risks which might potentially be connected to placing of GMOs on the market.

Directive 2001/18/EC[1] on the deliberate release of GMOs into the environment and Regulation No 1829/2003[2] on GM food and feed ensure strict control of placing on the market GMOs in the EU. All GMOs and food and feedstuffs derived from them have to be clearly labelled to ensure freedom of customer choice. In addition to that, as an exemption of the labelling requirements, the European legislation takes into consideration the presence of technically unavoidable or adventitious traces of GM material. Directive 2008/27/EC[3] which amended Directive 2001/18/EC established the threshold of 0.9% for commodities intended for direct processing, which comprises all crop harvests (excluding the case when they are intended for seed production) below which traces of market-approved GM products do not require labelling. The Regulation (EC) No 1829/2003 establishes the same threshold for food and feed. With Directive 2014/63/EU[4] amending Council Directive 2001/110/EC relating to honey the threshold of 0.9% adventitious admixture of GM pollen over total honey was adopted. These labelling rules are also valid for organic products, including food and feed, according to Regulation (EC) No 834/2007[5].

The adopted threshold for labelling exclusion is aplicable only for adventitious, technically unavoidable admixtures. For farm-scale activities which are performed in open-space environment, it has always been understood that some admixing will occur. To control adventitious GM presence, adequate technical and organisational measures during cultivation, on-farm storage and transportation are required. Therefore the potential admixing below the threshold for which particular coexistence measures are designed, is possible and is technically unavoidable and adventitious. Thus the effectiveness of the coexistence measures used to limit the potential intermixing to below certain threshold defines what is “adventitious or technically unavoidable” in terms of coexistence for open-space farm activities.

As local environmental conditions and farm structures may have a significant impact on the effectiveness and efficiency of coexistence measures their development is under the remit of individual Member States (MS).

Recommendation 2010/C 200/01[6] of the EC provides guidelines for development of national coexistence measures to avoid the unintended presence of GMOs in conventional and organic crops, replacing Commission Recommendation 556/2003[7]. Recommendation 2010/C 200/01 recognizes that the market demand for particular food crops may result in economic damage to operators who would wish to market them as not containing GMOs, even

1 Directive 001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. OJ L 106, 17.4.2001, p. 1–39

2 Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. OJ L 268, 18.10.2003, p. 1–23

3 Directive 2008/27/EC of the European Parliament and of the Council of 11 March 2008 amending Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, as regards the implementing powers conferred on the Commission, OJ L 81, 20.3.2008, p. 45-47

4 Directive 2014/63/EU of the European Parliament and of the Council of 15 May 2014 amending Council Directive 2001/110/EC relating to honey. OJ L 164, 3.6.2014, p. 1–5

5 Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No 2092/91. OJ L 189, 20.7.2007, p. 1–23

6 OJ C 200, 22.7.2010, p. 1-5

7 Commission Recommendation 556/2003 of 23 July 2003 on guidelines for the development of national strategies and best practices to ensure the co-existence of genetically modified crops with conventional and organic farming. OJ L 189, 29.7.2003, p. 36.

if GMO traces are present at a level below 0.9%. Therefore MS may establish different thresholds for adventitious and technically unavoidable admixture of GMOs in non-GM harvests, taking into account the demands of the consumers and their market. The Recommendation also takes into consideration the extreme diversity of European farming systems, natural and economic conditions and clarifies that under certain climatic and/or agronomic conditions MS may exclude GMO cultivation from large areas, if other measures are not sufficient to ensure coexistence.

Directive 2015/412[8] amended Directive 2001/18/EC regarding the possibility for MS to restrict or prohibit the cultivation of GMOs in their territory. This Directive reaffirms the existing approach for development of coexistence measures, established by the Commission Recommendation of 13 July 2010. The Directive 2015/412 places on MS (in which GMOs are cultivated) the responsibility to take appropriate measures in border areas of their territory with the aim of avoiding possible cross-border contamination into neighbouring MS in which the cultivation of these GMOs is prohibited, unless such measures are unnecessary in light of particular geographical conditions.

1.2. The role of the European Coexistence Bureau

The diversity of agricultural practices and legal environments among the MS, has led to adoption of the subsidiarity approach in the EU for the implementation of coexistence regulations. Although the development of coexistence measures is under the remit of individual EU MSs, the European Commission retains several roles in this process. One important role is the technical advice offered to MSs through the European Coexistence Bureau (ECoB).

The mission of the ECoB, created in 2008, is to organise the exchange of technical and scientific information on the best agricultural management practices for coexistence and, on the basis of this process, to develop consensually agreed crop-specific guidelines for technical coexistence measures. The ECoB is managed by and located on the premises of the Joint Research Centre (JRC) of the European Commission.

The work of ECoB is organised into crop-specific Technical Working Groups consisting of experts nominated by EU MSs. Their main task is to develop Best Practice Documents (BPDs). The BPDs of ECoB comprise a methodological tool to assist development of national coexistence measures, based on scientific evidence and practical experience.

Presently the ECoB is comprised of three TWG for: maize, soybean and cotton. The first TWG for maize crop production started its work in 2008. The TWG for maize has developed three BPDs for:

- Coexistence of GM maize crop production with conventional and organic farming (Czarnak-Kłos and Rodriguez-Cerezo, 2010);
- Monitoring efficiency of coexistence measures in maize crop production (Rizov and Rodriguez-Cerezo, 2014); and
- Coexistence of GM maize and honey production (Rizov and Rodriguez-Cerezo, 2013).
- The second TWG, for soybean, was established in 2013 and developed a BPD for Coexistence of genetically modified soybean crops with conventional and organic farming (Rizov and Rodriguez-Cerezo, 2015).

TWG for cotton started work on this BPD in July 2014.

1.3. Scope of the Best Practice Document

This document focuses on the development, based on current scientific knowledge and agricultural practices, of a set of best agricultural management practices that will ensure coexistence of GM cotton with conventional and organic cotton while maintaining economic and agronomic efficiency of the farms. The TWG for Cotton was also asked to examine the issue of coexistence between GM cotton cultivation and honey production in the EU. The scope of the BPD is coexistence in the cultivation of cotton in the EU.

It is assumed that for the purpose of this document, the coexistence measures should be addressed to GM cotton producers. All these measures should be proportionate, technically and economically consistent.

The document considers both the need for compliance with the regulated labelling threshold of 0.9% as well as with lower thresholds of adventitious presence of GM material (0.1%) which may be required by private operators in some markets.

The document exclusively considers GM cotton with a single gene transformation event.

8 Directive 2015/412 of the European Parliament and of the Council of 11 March 2015, amending Directive 2001/18/EC as regards the possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory OJ L 68, 13.3.2015 , p. 1–8

2. Cotton biology

2.1. Evolutionary and directed selection of genus Gossypium for lint production

Cotton genus *Gossypium* belongs to a monophyletic family *Malvaceae* and its origin has been dated to 20 million years ago (LaDuke and Dobley, 1995 and Seelanan et al., 1997). There is a great diversity in cotton species (Fryxel, 1971).

The most striking aspect of the evolution of cotton genus is that due to its wide geographical distribution, mainly in tropical and subtropical regions (Wendel and Cronn, 2003), it has been associated with ancient cultures on different continents, which led to a process of domestication, convergent or parallel, from divergent and geographically isolated wild ancestors (Fryxel, 1979 and Wendel et al., 2010).

Commercially important cotton is the result of a polyploidization event between Old and New World cottons that occurred over one million years ago (Wendel et al., 2012 and Wendel and Cronn, 2003). *Gossypium hirsutum* and *Gossypium barbadense* are the main commercially cultivated tetraploid cotton species (Adams and Wendel, 2004). The present-day cultivated diploid species are *Gossypium herbaceum L.* and *Gossypium arboreum L.* and they cannot be crossed with tetraploid ones.

G. barbadense (Pima, Egyptian or American-Egyptian cotton) is native to South America (Brubaker et al., 1999) and *G. hirsutum L.* (Upland cotton) originated from the northern coast of the Yucatan peninsula in Mexico (Beasley, 1942).

Gossypium hirsutum was initially domesticated at least 5000 years ago and, following millennia of directional selection, domesticated forms now produce long, strong, and fine white fibres along with a dramatically enhanced fibre yield. In addition to this increase in fibre length, strength, and quality, the domestication process brought about other morphological transformations, including decreased plant stature, earlier flowering, and loss of seed dormancy. However, the intensive breeding for desirable fibre properties has resulted in decreased genetic diversity and the loss of potentially desirable characteristics, such as resistance to pests or tolerance to drought. Notably, one-third of the genes (about 5000 genes) of the employed genome are differentially expressed in cotton fibre development as a consequence of cotton domestication (Yoo and Wendel, 2014). These data suggest that the human selection has reprogrammed the transcriptome on a massive scale, and that part of this rewiring entails a reallocation from stress response pathways toward fibre growth, explaining the pest and drought susceptibility. The directed selection during the initial domestication and subsequent crop improvement has resulted in a biased upregulation of components of the transcriptional network (Wang et al., 2012 and Paterson et al., 2012) that are important for agronomically advanced fibre, especially in the early stages of development (Chaudhary et al., 2009 and Rapp et al., 2010). The majority of present cultivars are a mixture of closely related pure lines (Van Deynze, 2005)

The most dominant forms of cotton today are *Gossypium hirsutum* cultivars, which are spread across over 50 countries in both hemispheres (Beasley, 1942). About 90 percent of the annual global harvest and practically 100% of the European cotton harvest is derived from *G. hirsutum*. Gossypium hirsutum has been grown in southern Europe since the 19th century (Davies, 1967). One negative outcome of the wide cultivation of *G. hirsutum* cultivars is the increased incidence of attack by pests. Hence, world-wide cotton cultivation uses more pesticides than any other crop (Truscott, 2010).

2.2. Lint development

Cotton differs from other crops in that the harvestable portion is not the seed but rather the lint fibres that are appendages of the seed. In modern cultivars, 30% of the ovule epidermal cells initiate into fibres from the outermost layer of integument at anthesis (Basra and Malik, 1984 and Tiwari and Wilkins, 1995). Each fibre of cotton is a highly elongated and thickened single cell (trichoma) of maternal origin that initiates from the ovule epidermis and rapidly elongates isodiametrically to 2 - 3 cm in *G. hirsutum* (the most commonly grown cotton cultivar) and to over 6 cm in *G. barbadense* (considered as extra-long staple cotton). In contrast, the diameter of a cotton fibre is relatively thin: *G. hirsutum* fibres have an average diameter of 11 - 22 µm (Ranjan et al., 2012).

The cotton lint fibres undergo rapid and synchronous elongation during seed development and intensive interaction between maternal (fibre) and embryonic tissues in seeds takes place. Fibre development consists of four overlapping stages: (a) extreme elongation via primary wall synthesis; (b) transitional wall thickening and primary wall remodelling; (c) secondary wall thickening via deposition of nearly pure cellulose; and (d) ill-defined "maturation" and cell death processes occur before the boll opens to reveal the fluffy fibre within the cotton fruit (or boll). Typically four carpels (or locules) of one fruit contain about 32 seeds and ~500,000 long cotton fibres (lint fibre: Bowman et al., 2001). A population of thick-walled but short fibres (fuzz fibre or linters) also exists on the outside of the cotton seed.

Mature fibre is a biological composite of cellulose, water, small quantities of proteins, pectins, hemicellulose, mineral substances, wax, and small amounts of organic acids, sugars, and pigments that provide excellent wearability and aesthetics (Basra and Malik, 1984, Ryser, 1985 and Arthur, 1990).

2.3. Flower, pollen and seed morphology

Flower

Gossypium species have a complete hermaphroditic (containing both male and female structures), solitary, terminal, axial and pentamerous flower that begins to form four to five weeks after planting (Macfarlane et al., 2002). Cotton flowers are large (5–9 cm) and are borne on the sympodial branches. The style is 2–5 cm long and terminates in the 0.5-1 cm - long stigma. The stigma of *G. barbadense* extends well above the anthers, unlike *G. hirsutum* (McGregor, 1976), and this may affect the likelihood of cross pollination occurring. However, for both species the stigma is only receptive at the time of dehiscence on flower opening, ensuring that cotton is predominantly inbreeding (Thomson, 1966 and Mungomery and Glassop, 1969). Cotton flowers develop along fruiting branches that extend out from one or more main stems, with flowering progressing sequentially from the bottom to the top of the plant and out to the fruiting branches. The cream colored (Acala cotton) or pale yellow flowers (Pima cotton) open in the morning shortly after dawn, turn pink in the afternoon, and close at night, never to reopen (Liogier, 1994). The stigma is receptive only until early afternoon. So, despite the cotton flower being large and showy to attract insects, the majority of seed set is the result of self-pollination not cross-pollination (Free, 1993). The ovary contains 5–10 ovules in each of 3–5 sections, or locules. The stamina sheath, which encloses most of the style, bears numerous stamens 0.5–1 cm long, each terminating in an anther that normally produces an abundance of viable self-fertile pollen (McGregor, 1976). There are approximately 20,000 pollen grains per flower (Ter Avanesian, 1978).

The cotton flowers have both floral and extra-floral nectaries (Moffett, 1983). Secretion of bracteal (extrafloral) nectar starts 5-6 days before flowering and initially peaks on the day of anthesis (Adjei-Maafo and Wilson, 1983; Wäckers and Bonifay, 2004).

Pollen

The cotton pollen is dispersed through the anthers after the flower opens, remaining viable from approximately 12 to 24 hr in corolla (Cobley, 1956). However, there is a difference in pollen release and flower opening between species. *Gossypium barbadense* pollen is released early just as flowers are opening, whereas Upland pollen (*G. hirsutum*) is not available to pollinators until the flower is much more open. Pollen viability is low in the early morning and peaks at midday (Van Deynze et al., 2011). Only a small proportion of pollen can still perform pollination at 8:00 a.m. the next day. Under field conditions, pollen is usually viable for 4–8 h (Richards et al., 2005).

Pollen grains germinate within 30 min after deposition on the stigma then fertilisation of ovules occurs within 24-48 after pollination (Pundir, 1972). The great majority of seeds are as a result of self-pollination (John and David, 1995).

The high temperatures found in *G. hirsutum* flowers which are exposed to full sun has been shown to lead to reduced pollen grain germination *in vitro* (McGregor, 1976; Burke et al., 2004). Kakani et al. (2005), by studying the cardinal temperatures (lowest, optimum and highest for survival) of 12 cultivars of cotton, showed that the averages for pollen germination and growth are 14 °C (minimum), 31 °C (optimum) and 43 °C (maximum).

Cotton pollen is large, heavy (El Nagger, 2004), and sticky (coated with a viscous material that causes them to adhere to each other), with long spines and is not easily dispersed by wind (Thies, 1953, McGregor, 1976 and Jenkins, 1992). Vaissière and Vinson (1994) demonstrated that only 16% of foraging honey bees that landed on cotton flowers collected pollen. This reduced efficiency in cotton pollen collection was associated primarily with the length of the spines on cotton pollen which physically interfered with the pollen aggregating process used by honey bees. The density of the spines is $4.9x10^{-3}$ spines/μm^2 and of $8.3x10^{-3}$ spines/μm^2 for *G. barbadense* and *G. hirsutum* respectively (Kakani et al., 1999).

A number of authors (Kearney and Harrison, 1932, Saad, 1960, Kakani et al., 1999 and El Nagger, 2004) have demonstrated that *G. barbadense* pollen is larger than *G. hirsutum*, ranging between 66 – 115 µm and 85 – 103 µm respectively.

Seeds

Cotton seeds are large, covered with thick fibres and enclosed in a tough boll that retains most of the seeds on the plant.

Therefore the gene flow mediated by incidental seeds dispersal is likely to be minimal. However, as cotton does not generally reproduce vegetatively (Serdy et al., 1995), spread within the environment occurs by seed dispersal. Dispersal of cotton seeds is a physical process.

The seed coat bears two types of fibres: long lint fibres, valued by the textile industry; and short, fuzzy fibres, known as linters used in various products including foods. After ginning, the cotton seed is still covered in linters and is known as 'fuzzy seed'. After acid treatment to remove the linters (a process known as delinting) the cotton seeds are ovoid in shape, slightly pointed, about 10 mm long x 4 mm wide and weigh about 80 mg (George, 2007), and are dark brown in colour (called 'black seed'). Cotton seed has a hard seed coat covered by a slightly waxy cuticle (Tharp, 1960). Beneath the epidermal cells, which produce the seed hairs, there is a thin outer seed coat with an inner epidermis. This has a different origin from that of the inner seed coat but the two are fused together (Christiansen et al., 1960; Merideth,et al., 1984). Each boll produces about 20 - 25 seeds.

Cotton seeds in general do not posses seed dormancy. Very few lines have dormancy and it breaks by the time seeds undergo grow out tests and other processing schedules. It is widely accepted that dormancy in cotton seeds is induced by low soil temperature and/or soil moisture (OGTR, 2002). Additionally, some forms of cotton may produce 'hard seeds' that, upon drying, become impermeable to water and suffer delayed germination (Christiansen and Moore, 1959). This 'induced dormancy' can be overcome by various treatments.

2.4. Insect impact on cross-pollination

Cotton is a facultative self-pollinator and an opportunistic out-crosser when insect pollinators are present (Oosterhuis and Jernstedt 1999). Since pollen grains of cotton are too heavy to be air-borne, wind does not play any role in cross pollination (Tanda and Goyal, 1979 and Umbeck et al., 1991), so insects are the likely natural agents for any pollen transfer.

Cotton pollen dispersal studies consistently demonstrate that when outcrossing occurs, it is localized around the pollen source and decreases significantly with distance (Thomson, 1966, Galal et al., 1972, Theron and van Staden, 1975, Elfawal et al., 1976, Chauhan et al., 1983, Umbeck et al., 1991 and Llewellyn and Fitt, 1996). This presumably represents the effective foraging range of insect pollinators and their feeding habits. For example most foraging honeybees have been routinely observed in cotton plants to collect nectar (from both in-floral and extra-floral nectary glands) rather than pollen.

In different studies for pollinator insects, 35 bee species were found on the cotton field by Moffet et al. (1980). Wild bees such as bumble bees (*Bombus spp.*) and black bees (*Melissodes spp.*), are known as real pollinators (Vaissiere et al., 1984 and Saeed et al., 2012) and honeybees are secondary pollinators because they generally do not collect pollens or do not carry them far away (Moffet and Shipman, 1978 and Waller et al., 1985). Additionally, wild bees mostly prefer nectar on the flower and carry the pollens that are adhered on their body from one flower to others and cause out-crossing. While honeybees visiting flowers follow specific directions and clean their body from the pollens that are getting them heavy. The study of Saeed et al. (2012) on impact of bumble bees on cotton pollination in greenhouse conditions indicated that they can significantly improve cotton reproductive success, but without providing data whether this is due to cross pollination or improved selfing.

Honeybees will forage the food source nearest to their hive with the maximum reward, especially when pollen and nectar are abundant (Eisikowitch and Loper, 1984) and avoid collecting pollen or nectar from sources where competition from different colonies is high (Gary et al., 1972; Visscher and Seeley, 1982). Furthermore, honeybees will work a specific crop and even specific varieties if they differ in nutritional value (Free, 1993).

The impact of honeybee activity has been evaluated by different techniques, such as: comparing fields with and without managed honeybees (Shishikin, 1952, Vaissiere et al., 1984 and Vaissiere, 1991); yield in relation to the level of bee visitation (Rhodes, 2002); and bagging individual flowers and comparing to unbagged ones (Radoev and Bozhinov, 1961). However, the most common method has been by using cages, either to exclude bees from flowers or to cage them with flowers (Moffett and Stith, 1972, Waller et al., 1985, El-Sarrag et al., 1993 and Rhodes, 2002).

Honeybee pollination has been reported to: decrease boll shedding (McGregor et al., 1955), improve seed germination (Radoev and Bozhirov, 1961 and El-Sarrag et al., 1993), increase seed oil content (El-Sarrag et al., 1993), improve lint quality (McGregor et al., 1955, Kuliev, 1958, Vaissiere et al., 1984 and Rhodes, 2002), as well as increase yield (total boll weight) for different cotton varieties (Kuliev, 1958, Wafa and Ibrahim, 1960, Radoev and Bozhinov, 1961, El-Sarrag et al., 1993 and Rhodes, 2002) . For example, McGregor et al. (1955) assessed the influence of bee activity on boll set, number of seeds and yield, by confining honeybees with Pima and Upland cotton plants under plastic screen cages and comparing with caged plants without bees. While they recorded 24.5% higher yield of cotton seed from Pima cotton caged with bees, there was no yield increase in Upland cotton, although there was earlier fruit set.

The measurable benefits from bee activity probably derive from bees increasing the amount and distribution of pollen on stigmas. Honeybee visits to cotton flowers increase the amount of pollen deposited on the stigmas of the same plant and of foreign pollen introduced from other plants (of the same species) to the stigma. The bees' activity

promotes self-pollination by pushing the anthers against the stigma while collecting nectar from nectaries present on the internal base of the flower (Silva, 2007). This pollinator behavior promotes the mixing of natural pollen in the stigma and creates conditions for competition between pollen from different sources. However, the proportion of foreign versus own pollen is not known. For instance, Kearney (1923) reported that stigmas of bagged cotton flowers were not always completely covered with pollen, but this was rarely the case with open flowers when insect visitation, particularly by honeybees, was high. This was further confirmed by Vaissiere et al. (1984) who reported that in the absence of bees, the number of pollen grains on stigmas was very low (0-7). In the presence of managed honeybees, this figure was often much higher. Vaissiere et al. (1984) also suggested that factors other than bee visitation, such as flower or plant biology or the microclimate around flowers, were important in determining the number of pollen grains transferred onto stigmas within any one day. Furthermore, Vaissière and Vinson (1994) demonstrated that cotton pollen is seldom harvested along with nectar and that honeybee nectar foragers routinely remove accumulated pollen from their haircoat during foraging trips. Loper (1986) and Loper and DeGrandi-Hoffman (1994) also reported that honeybees, before returning to the hive, spend between 15 min and 20-30 min respectively to scrape the pollen grains from their body with their legs: this implies that the honeybees actively reject cotton pollen.

2.5. Crop biology and cultivation

The cotton plant has indeterminate growth habit and the position of the fruits on the plant is indicative of the time of the season at which they were originally set. Cotton possesses extreme sensitivity to adverse environmental conditions, while under favourable moisture and temperature conditions the growth of the cotton is very predictable. Growth follows a well-defined and consistent pattern expressed in days.

Temperature has a major influence on the rate of development and growth of the cotton plant and this determines the geographic range in which cotton can be grown (Freeland et al., 2006, ACCRC, 2001, Reddy et al., 2006, and Robertson et al., 2007).

Although originating in the tropics and subtropics, cotton has come to be cultivated mostly in subtropical and warm-temperate zones — regions which provide more than half of world production presently. This geographical shift of cultivated areas requires adjustment of the species' photoperiod from the naturally short-day plant to a day-neutral plant that could be cultivated as an annual crop in the longer summers (Smith et al., 1999).

Cotton is grown worldwide between latitudes of 45° north and 30° south, in areas that have at least 160 frost-free days. Cotton is a 'heat loving' plant, however, more than 50% of the world crop is grown in temperate zones above 30° N latitude. Additionally, cotton is grown under similar climatic and soil constraints. The majority of cotton is grown in areas that receive between 50 and 150 cm of rainfall per year.

Maximum productivity of cotton is achieved in regions of high temperatures, high light intensity, good soil moisture, and soil fertility (Hartman and Flocker, 1981). Cotton is planted when the minimum soil temperature at 10 cm depth is 14°C for at least three successive days. For germination it requires a daily minimum temperature of 16°C and 21°C to 27°C for proper crop growth. Growth and development of cotton plants below 12°C is minimal and a long, hot growing season is crucial for achieving good yields (Constable and Shaw, 1988). During the fruiting phase, a day temperature ranging from 27°C to 32°C and cool nights are needed. The sowing season of cotton varies between east and south of Europe starting at the beginning of April and continuing through mid May, provided that fields are not wet. The optimum daytime temperature range for *G. hirsutum* is 30-35ºC, with a loss of fruit above 35ºC, and with a 50% yield reduction at 25ºC (Reddy et al., 1992).

After planting *G. hirsutum* needs 180-200 frost-free days for normal development, with an average of 150 days of suitable temperatures (i.e. 1200 heat units above 15.5ºC accumulated) (Duke, 1983). For *G. barbadense*, 200-250 days are needed (Unruh and Silvertooth, 1997). Although the values differ between varieties, from the planting of cotton to 60% boll opening about 2050 day degrees (heat units) are the required minimum (Ritchie et al., 2007 and OGTR, 2008).

Cotton development is also sensitive to water deficit during flowering and boll development (Constable and Hearn, 1981 and Turner et al., 1986). Recent research has shown that the developing pollen (Burke et al., 2002) and pollen tube growth (Snider et al., 2011) are highly sensitive to this environmental stress.

The extra-long staple of *Gossypium barbadense* is very demanding in terms of climatic conditions and irrigation, and only grows in a few countries (e.g. in Egypt and the United States), but not in Europe.

The developmental phases for cotton can be divided into six main growth stages: (1) from planting to emergence; (2) from emergence to first fruiting branch; (3) from emergence to first square (floral bud); (4) from square to white bloom; (5) from emergence to peak bloom; and (6) from white bloom to open boll. The transitions between these stages are not always sharp and clear. Each stage may also have different physiological processes operating within specific requirements (table 1).

For example the lint yield is adversely affected if *G. hirsutum* is planted too early (due to cold temperatures) or too late (due to a shortened growing season) (Kittock et al., 1987).

Table 1. The average number of days and day degrees required for various growth stages of cotton*

Growth Stage	Days	Day degrees**
From Planting to Emergence	8 to 10	25 – 35
From Emergence to First Fruiting Branch	15 to 20	165 – 190
From Emergence to First Square	15 to 20	235 – 265
From Square to White Bloom	20 to 25	165 – 195
From Emergence to Peak Bloom	50 to 60	770 – 795
From White Bloom to Open Boll	45 to 55	415 – 610
From Emergence to a Mature	180 to 200	1165 – 1250

**Compiled from: Anonymous, 2006; Boyd et al., 2004; Kerby et al., 1987; Young et al., 1980*

*** Day degrees, or heat units, are calculated progressively during the season from the number of days with a temperature over 12^{0}C using the formula:*

$$\text{Day degrees} = \frac{(\text{daily max. temp} - 12) + (\text{daily min. temp} - 12)}{2}$$

3. Cotton production, EU demand and crop cultivation

Cotton is the most used natural fibre and the third largest source of vegetable oil (FAOSTAT, 2010). It ranks sixth worldwide among cultivated crops and it is important to note that of the fifteen most important crops in the world, cotton is the only one that did not acquire its value by being part of the staple diet (Wendel et al., 2010).

Cotton accounts for around 35 percent of total world fibre use. While some 80 countries from around the globe produce cotton, the United States, China, and India together provide two-thirds of the world's cotton. The United States, which ranks third in production behind China and India, is the leading exporter, accounting for over one-third of global trade in raw cotton.

Cotton production is a labour intensive commodity crop. As a result, the agricultural production of cotton is limited to countries having cheap labor or in countries, where production is completely mechanized (Hartman, Flocker et al. 1981).

Although cotton is mainly valued for its fibre, cotton by-products are used in human food (mainly cottonseed oil) and animal feed (cottonseed meal). Cottonseed income can account for between 10% and 15% of the value of a cotton crop (Service, U.N.A.S, 2015). For example, production of cottonseed that is acceptable for human consumption could conceivably produce enough protein to feed half a billion people annually (Watkins, C., 2013). However, cotton is expensive to grow: conventionally grown cotton uses significant amounts of pesticides, fertilisers, fossil fuels and water (Truscott, 2010) that are not only expensive, but can be detrimental to the environment and animal populations and cotton is also associated with high labour input. For example the Carbon Trust (2011) estimated that global cotton production up to ginning generates global emissions of 220t carbon dioxide equivalents (CO_2), accounting for 3.6–4.3% of greenhouse gas (GHG) emissions from agriculture or 0.4% of overall global emissions. Production of a tonne of cotton lint results in the emission of 4 – 12 of CO_2. Consequently, in recent years, efforts have been made to improve the yield of cotton while, at the same time, decreasing the growing costs and environmental impact.

To address the economic and environmental challenges a new category of agricultural technologies (known as precision farming) that adjusts application levels of agricultural inputs to accommodate variations within fields and also to climatic and other variations within seasons was introduced. Gemtos et al. (2003) and Markinos et al. (2004) reported potential of precision farming for small cotton farms in Greece. For similar purposes in 2009 the Regional Government of Andalucia, Spain published an analysis of energy efficiency of cotton cultivation in Andalucia[9]. The May 2011 meeting of the Cotton Advisory Group to DG AGRI[10] reported a reduction in the use of pesticides, fertilizers and excess use of irrigation water as a result of the adoption of integrated production models.

As more efficient alternatives to expensive and heavily impacted conventional production of cotton were introduced biotechnological and organic practices for cotton cultivation.

In 2012 GM cotton was grown on 24.3 million acres in 15 different countries and constituted 81% of the cotton crop (ISAAA, 2014). The traits expressed included insect resistance, herbicide resistance and combined resistance to insects and herbicides. GM cotton was third most planted biotech crop by area. In 2014, 52 GM cotton events were sown in 21 countries, making cotton the crop with the second highest number of events after maize (ISAAA, 2014).

In 2009/10, organic cotton was grown in 23 countries and world production amounted to 241,697 tons, 38% higher than in 2008/09. The main leader of organic cotton production is India, followed by Syria, Turkey and China (Textile Exchange 2010). In 2010/11, organic cotton production dropped by 37% to 151,079 tons, and for 2011/12 was about 143,600 tons (Textile Exchange 2011, 2012). Despite this high fluctuation in the amount of production, organic cotton has been less than 1% of global cotton production since its introduction.

9 http://www.juntadeandalucia.es/agriculturaypesca/portal/export/sites/default/comun/galerias/galeriaDescargas/cap/servicio-estadisticas/Estudios-e-informes/desarrollo-rural-sost/eficiencia_energxtica_algodxn_andalucxa.pdf

10 http://ec.europa.eu/agriculture/consultations/advisory-groups/cotton/2011-05-27_en.pdf

In the EU, cotton is produced currently only in three Member States on around 300,000 ha[11]. Greece is the main cotton grower, with 80% of European cotton area, followed by Spain (mainly the region of Andalucía) with a share of 20%. Bulgaria produces cotton on less than 1000 ha. Cotton production ceased in Italy in 1991 and in Portugal in 1996.

Although cotton represents less than 0.2% of the value of European agricultural production, it has strong regional importance in the two main producing Member States.

Cotton is a major agricultural crop in Greece, accounting for more than 8 percent of total agricultural output. More than 75,000 farmers grow cotton. Thessaly, Macedonia, and Mainland Greece are the major cotton-producing areas. Most cotton is irrigated and machine harvested. For the marketing year 2013/14 Greece's cotton production was estimated at 298,000t , 14.6% up from the previous season thanks to exceptional yields (around 3.22 t per ha) and more effective pest control.

Domestic spinners in Greece consume approximately 10% of lint production and the remainder is exported. About 58% of cottonseed production is exported (mainly to Italy), and the remainder is crushed for oil and oilseed cake, or retained for seed. Greece is therefore a major cotton exporter in the EU. During marketing 2012/13, Turkey was the main destination for Greek cotton, accounting for approximately 37% of total exports.

Spain is the EU's second largest cotton producer after Greece. In Spain, the large majority of cotton production is concentrated in Andalucía, especially in the provinces of Cadiz and Seville. There is some minor cotton cultivation in other Spanish regions such as Extremadura and Murcia. This crop has critical environmental, social and economic implications in the areas where it is grown as it contributes to job creation and it is grown in areas where crop alternatives are limited. The large majority of cotton (over 90 percent) is grown under irrigation. For the marketing year 2013/14 the Spanish cotton production was estimated at 145,400 t, with an average yield of around 2.27 t per ha. Ninety nine percent of produced cotton seed is used for domestic feed, and just 1 % is crushed for oil. The economic value of this feed is an important income for the Spanish cotton producers. Spanish cotton lint exports, which exceed imports, are mainly directed to other EU MS, followed by China, Morocco and Bangladesh. Turkey and Pakistan are the main sources of cotton lint imports into Spain.

In Bulgaria cotton is produced in the South-Western part of the country in the provinces of Stara Zagora, Plovdiv, Haskovo and Blagoevgrad (NAAS).

EU cotton production has declined by about 50% since the 2006 CAP reforms. However, between 2010/11 and 2012/13, following the intermediate cotton reforms from 2009 the EU-27 planted area and production have increased by 41.2% (from 249,000 to 354,000 tonnes). Currently a small area (about 200-250 ha) is cultivated with organic cotton in Europe. There is no authorization for cultivation of GM cotton in the EU.

In the EU, most farms growing cotton are characterised by their small size (Greece: 2-10 ha and Spain: 10-20 ha) and large number (approx. 65,000 in Greece and 4,500 in Spain). Most Greek farmers grow under 3 ha of cotton (47.8 %) and only 3.25 % of producers grow more than 20 ha of cotton. The average farm size in Spain is 10.9 hectares. Just over 36 % of Spanish farms are smaller than 5 ha; 44.41 % are between 5 and 10 ha.

11 http://ec.europa.eu/agriculture/cotton/index_en.htm (accessed March 2015)

4. Existing systems for segregated and Identity-Preserved cotton production

4.1. Cotton seed production

Cotton seed production in the EU occurs in some regions of Greece (mainly Thessaly and Macedonia-Tracia), Spain (in Andalusia) and Bulgaria (area of Chirpan).

The production of certified cotton seeds in Greece for 2012 accounts 3535.9 tons[12]. In 2005 US cottonseed or seed using US genetics accounted for 60-80% of the sown area of cotton in Greece (USDA, 2005 – not in reference list, is it http://apps.fas.usda.gov/gainfiles/200511/146131562.pdf?). Productivity, earliness, resistance to *Verticillium* wilt and high lint quality are the main breeding targets.

In 2012 Spain produced 2310.1 tons[14] certified seeds. Most varieties developed for the Spanish market are also derived from US varieties. Both the Andalusian government and private seed companies provide certified seeds.

For Bulgaria, cotton breeding is located at the Research Institute for Cotton and Durum Wheat, Chirpan. Certified cotton seeds produced during 2012 were 51 tons[14]. Breeding in Bulgaria is focused on early-maturing (vegetation period of 110-112 days) and high-yielding varieties. The varieties developed by this institute cover 100% of the Bulgarian needs.

EU production of cotton seed has been declining over time because of the reduced area of cotton planting and in some years there have been further decreases due to reliance on imported seeds and, occasionally, high seed stocks from previous years.

The proportion between domestic and imported seed supply for Greece is about 60% domestic vs. 40% imported; for Spain it is 59% domestic vs. 41% imported; while in Bulgaria there is 100% domestic cotton seed production. Regardless of their origin all cotton seeds used in the EU are from *Gossypium hirsutum*.

Council directive 2002/57/EC[13] on the marketing of seed of oil and fibre plants in EU established minimum separation distances between cotton fields for production of certified and basic seeds of 200m and 400m respectively.

The OECD seed schemes recommend separation distances of 200 m for production of certified seed of *G. hirsutum* and 600 m for *G. barbadense*, and separation distances of 600 m and 800 m respectively for basic (i.e. foundation) seed (OECD, 2008).

This OECD standard has been adopted by seed companies in Australia (Cotton Seed Distributors, 2007). QSEED specify 600 m for *G. barbadense* and 200 m for G. hirsutum (QSEED, 2004).

The isolation distances required by California Crop Improvement Association between cotton types of the same species (including GM and non-GM varieties) for foundation or registered cotton seed production in California, USA are 201 m. The seed production of cotton types from different species should be separated of 402 m.

In Brazil the isolation distances between cotton crop fields, depending on the objective, are a distance of 250 m or 800 m (Freire, 2005).

In India the Minimum Seed Certification Standards require an isolation distance of 50 meters for production of foundation seed of varieties or hybrids (Tunwar and Singh, 1998).

12 http://www.escaa.org/index/action/page/id/9/title/certified-seed-quantities

13 http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32002L0057&from=EN

4.2. Schemes for Identity-Preserved (IP) cotton production

Over the last decade a number of projects have been launched to improve agricultural practices for cotton in developing countries as well as the level of social and environmental responsibility in developed countries. The major initiatives are: organic cotton (Textile exchange)[14] , Fairtrade cotton (FT)[15], Cotton made in Africa (CmiA)[16], the Better Cotton Initiative (BCI)[17] and Bayer's e3[18]. While organic cotton focuses mainly on the farming system and environmental sustainability, FT, CmiA, BCI and Bayer's e3 are more focused on tackling rural poverty. Organic, BCI and Bayer's e3 cotton can be cultivated in any producing country, while FT cotton production is localized in developing countries and CmiA focuses on African countries. Total cotton production across these alternative initiatives amounted to about 2% of world cotton production in 2011/12. The countries with the largest production of IP cotton are Brazil (321,010 t), India (255,738t) and Pakistan (157,000t), jointly accounting for 64% of global production of identity-preserved cottons.

The organic cotton, FT and CmiA exclude the possibility for GM cotton cultivation, while BCI and Bayer's e3 initiatives are open for both production systems. For 2012/2013 the cotton production under BCI and Bayer's e3 programmes comprised about 75% of total world production of identity-preserved cotton, while BCI by itself produced 66% of identity cotton.

The International Cotton Advisory Committee (ICAC), at its 71st Plenary Meeting in October 2012, established the Task Force on Cotton Identity Programs in order to enhance cooperation, improve transparency, and exchange experiences as well as information about sustainability of cotton production. Cotton Identity Programs are defined as those organizations that support or promote various cotton production initiatives.

4.2.1. Organic cotton

Organic cotton means certified organic cotton. If the production and processing systems are not certified, it is illegal to claim the produce as organic. The organic production requires full physical segregation and traceability from field to final product assuring that it remain unchanged, since fibre properties of organic cotton are the same as those of conventional cotton grown in the same geographical region.

The organic cotton initiative is led by a non-profit organization, the Textile Exchange, which developed two global organic cotton standards: the Organic Exchange (OE) 100 Standard and the OE Blended Standard. The organic content standard refers to national and International Federation of Organic Agriculture Movements (IFOAM) organic farm standards. The IFOAM is the worldwide umbrella organization for the organic agriculture movement.

The organic cotton production system aims to satisfy all three components of sustainability: economic, social and environmental. While social and environmental variables are important pillars of the organic movement, only the economic viability of a system can assure its survival. Organic cotton is not contracted, i.e. cotton farmers gain access to the market for organic products by obtaining the organic certification, but there is no guarantee that they will actually be able to sell their organic cotton and receive a price premium over conventional cotton. In 2011/12, the average premium paid for organic cotton over conventional cotton of similar quality amounted to 25-50 cents (US currency) per pound weight, although quotations were much higher. Moreover, producers sometimes sell part of their organic cotton production as conventional (in particular when conventional cotton prices are high). The fact that a significant number of organic cotton farmers had difficulties selling their cotton at a premium contributed to the decline in organic area and organic cotton currently represents less than 1% of global cotton production.

India is the only country to have introduced a national standard for organic textiles, the Indian Standards for Organic Textiles (ISOT)[19], with the goal of ensuring the integrity of Indian organic textiles from cultivation up to labelling and distribution. Over the past 5-6 years, India has significantly increased organic cotton production and has emerged as the global leader in organic cotton production, surpassing Turkey, Syria, China and the USA, who were the leading organic cotton producers until 2007. In 2009/2010 India produced 81% of world organic cotton (Textile Exchange 2010), with 80-85% of the cotton cultivated area in the country under GM cotton. The main tool to maintain coexistence between GM and organic cotton production systems in India is to ensure an isolation distance of at least 50 meters from Bt cotton fields, by selecting such villages where this is possible.

14 http://textileexchange.org/node/963

15 http://www.fairtrade.org.uk/en/farmers-and-workers/cotton

16 http://www.cottonmadeinafrica.org/en/

17 http://bettercotton.org/about-bci/

18 http://www.e3cotton.us/

19 http://apeda.gov.in/apedawebsite/organic/ISOT_Textiles_Standard.pdf

The 2010 Guidance Document for Co-existence between Organic and GMO Cotton in India[20] recommends maintaining a minimum 3m buffer distance from farm boundary to organic cotton and to plant a buffer crop (not cotton) in the buffer zone. In addition to the spatial segregation in this guidance there are also recommended measures to avoid adventitious admixture arising from:

- mixing of seed;
- planting of GM seed;
- mixing during harvest;
- mixing in storage;
- mixing during transport;
- mixing in storage of gin.

4.3. Modelling scenarios for coexistence between GM and non-GM Cotton in Andalusia, Spain

Although no GM cotton varieties are authorised for cultivation in the EU, a study was commissioned in 2006 to evaluate *ex ante* scenarios for coexistence between conventional and GM cotton in European cultivation conditions.

The case study of cotton coexistence was designed and coordinated by the Institute for Prospective Technological Studies (IPTS) of the Joint Research Centre (JRC) of the European Commission in cooperation with the Regional Government of Andalucía, Spain (Messean et al., 2006). The aim of the study was to analyse how the different cotton production systems can coexist in Andalucía, Spain. As the cultivation of GM cotton is not allowed in the EU, the study adopted a prospective approach based on existing knowledge for cotton behaviour and the GM varieties cultivated in other countries and the cultivation practices and the structure of farms in Andalucía.

The scope of the study included:

- The whole production cycle from farm to ginning factory;
- GM cotton and non-GM cotton based farming production systems;
- Simulation of various scenarios for seeds and crop producing farms:
  - Presence of GM cotton in the region and on the farm level of: 10% and 50%;
  - Thresholds of GM cotton presence in non-GM production: 0.1% and 0.5% for seed production, and 0.1% and 0.9% for fibre production;

To simulate different scenarios the study considered two types of farm:

- conventional farms, producing only non-GM cotton; and
- mixed farms, called “coexistence farms”, producing both GM and non-GM cotton (10% or 50%, in the two scales),
- with different sizes:
- small farms (using shared machinery); and
- large farms (with own machinery).

The analysis of the entire production process: from planting of the crop to the entry of the product into the ginner, identified eight possible points as potential sources of admixture:

- Seeds/crop from the previous year’s harvest;
- Seeds for sowing, which may contain GM cotton seeds as an impurity;
- Seed storage, both in private warehouses and on the farm itself:
- Sowing;
- Cross-pollination;
- Harvesting;
- Transport;
- Intermediate storage.

The levels of adventitious presence of GM cotton in non-GM cotton caused by all possible sources of admixture were predicted for the different types and sizes of farm under each scenario.

The most pronounced difference between the predicted levels of admixture of GM cotton in non-GM cotton was associated with the farm size (1.82% for small farms and for large farms 0.92%).

The general conclusion of this study is that the identification of coexistence practices that could keep the adventitious presence of GM cotton under the threshold of 0.9% or 0.1% is feasible. To achieve the threshold of 0.1% a set of coexistence practices stricter than those required to achieve 0.9% is proposed, mainly consisting of not allowing the sharing of machinery between GM cotton and non-GM cotton fields for all farm types. The economic effect at farm level of these coexistence measures for both thresholds of 0.9% and 0.1% is predicted as negligible.

20 from http://www.organicandfair.org/oftcc/Publications/Publications.php

5. Review of the available information on sources of adventitious GM presence in cotton crop production

5.1. Seed impurities

The purity of cotton seeds is of significant importance for the purity of cotton harvests. It is evident that the purity of the seed stock must equal or exceed the purity standards of the final product. Therefore the presence of GM seeds in conventional seed lots is a critical factor and must be managed to achieve coexistence. The best approach to manage this is the use of certified cotton seeds that comply with legal EU regulation.

The two important parts of EU legislation covering the purity requirements of cotton seeds are the Council Directive 2002/57/EC on the marketing of seed of oil and fibre plants and Directive 2001/18/EC on the deliberate release into the environment of GMOs. In annex II of the Council Directive 2002/57/EC the conditions which must be satisfied by marketed cotton seeds are laid down. Basic and certified seeds of *Gossypium spp*. must have minimal analytical purity for spp. of 98%.

In terms of adventitious GM presence, there are no tolerance thresholds (for authorised or unauthorised GM events) for conventional cotton seeds marketed in the EU, therefore marketed conventional cotton seed complying with EU legislation should not be a significant source of adventitious GM presence in the final crop.

In order to avoid GM admixtures, official controls of conventional seeds are regularly applied by MS of EU. For example in Greece, the Ministry of Rural Development has performed regular inspections since 2002, in accordance with national legislation. During the first years, inspections detected admixtures derived mainly from non-EU countries. In the last year admixture cases have begun to be identified in cottonseed produced in Greece. Thus seed inspections should continue and be reinforced to exclude impurities.

5.2. Cultivation

Cotton gene flow can occur in two distinct ways: by spreading pollen and seeds. Hardly any gene flow occurs from seeds directly from the field, as they are large, covered with large quantities of fiber and rarely carried by animals (Llewellyn and Fitt, 1996). To germinate cotton seeds require large amounts of water and, when they do germinate in non-agricultural environments, formed plants have little chance of survival because of cotton's poor colonizing ability (Wozniak, 2002).

Eastick and Hearnden (2006) hypothesized that the introduction of resistance genes for major pests, may increase the potential invasiveness of GM cotton to modify their suitability compared to conventional varieties, because of that they evaluated the invasiveness of GM cotton, in terms of germination, survival and dispersal. The results of this study do not provide any evidence about differences in survivality of GM cotton and their conventional counterparts, even in areas of high risk such as wet areas conducive to the establishment of cotton.

5.2.1. Out-crossing to wild relatives

Whilst compatible wild cotton species are present in Asia, Africa, Oceania and America, this is not the case in Europa. In general, wild tetraploid cotton species of genus *Gossypium* are candidates for gene exchange with *G. hirsutum* cultivated in the EU. No other species is closely enough related to cultivated cotton to enable out-crossing. Gene transfer to unrelated plant species is highly improbable because of pre-and post-zygotic genetic incompatibility barriers that are well documented for distantly related plant groups. No evidence for vertical gene transfer from cotton to other plant taxa has been identified.

In southern Europe, *G. herbaceum* and *G. hirsutum* have been grown since the 19th century, giving rise to occasional feral plants in the same area (Todaro, 1917, Davies, 1967, Zangheri, 1976 and Tutin et al., 1992). Therefore, the plant-to-plant gene transfer from GM cotton is restricted to cultivated and occasional feral cotton populations. However, because of the size and distribution of feral cotton, it does not represent a coexistence concern as an intermediate link for gene flow from GM to conventionally cultivated cotton.

To limit the likelihood for vertical gene transfer in some regions of Australia (GMAC, 2001), USA (EPA, 2001) and Brazil (Barroso et al., 2005), where native wild species of cotton are present, the cultivation of GM varieties is restricted. In Mexico, where no restrictions are applied for cultivation of GM cotton in the centres of origin and diversity of G. *hirsutum*, gene flow over long distances has been confirmed (Wegier el al.2011).

5.2.2. Out-crossing between GM and non-GM cotton

Gene flow caused by pollen dispersal in cotton is a complex process dependent on many factors (Messeguer, 2003, Barton and Dracup, 2000 and Ellstrand et al., 1999), including:

- Environmental conditions, crop location, climate and season (Hokanson et al., 1997, Elliott et al., 2004, Van Deynze et al., 2005, Zhang et al., 2005, Llewellyn et al., 1996 and Llewellyn et al., 2007);
- Crop variety (Van Deynze et al., 2011);
- Agricultural practices, such as pesticide spraying and plant density (Vincenza and Marina, 2001 and Hamilton et al., 2002). As a result of standard agronomic practices using pesticides to control insect pests in cotton, no bees and very few insects could be observed (Van Deynze et al. 2005 and Bozdek et al., 2008);
- Number and behaviour of pollinators (Xanthopoulos and Kechagia, 2000, Van Deynze et al., 2005 and Zhang et al., 2005);
- The transformation events, which affect the agronomic practices for GM crops (such as application of pesticides), especially for insect-resistant GM plants such as *Bt* cotton. As the advent of *Bt* cotton has reduced insecticide applications (Carpenter and Gianessi, 2001), pollinator activity in fields may have increased in recent years (Betz et al., 2000). This in turn could affect pollinator numbers and behavior and subsequently pollen transmission.

In predominantly self-pollinated crops like cotton, the effect of open pollination (by wind or insects) is negligible (Wang et al., 1997 and Vincenza and Marina, 2001). When self-pollination is prevalent, it favours "own" pollen grains in competition with "foreign" ones (Robert et al., 1991). When out-crossing occurs in cotton, cross-pollination is mainly mediated by activity of pollinators (e.g. bees; Insecta, *Apidae*) and not by wind (Green and Jones, 1953, McGregor, 1976 and Van Deynze et al., 2005), because of the characteristics of cotton pollen (heavy and sticky, for more information check chapter 1.3. *Flower, pollen and seed morphology*).

Wind impact

In 2013 Zhu et al., however attempt to estimate the particular impact of wind flow (directed and indirected) on pollen-mediated gene flow from *Bt* cotton (*Gossypium hirsutum L.*) in greenhouse conditions and thus excluding completely the insect polination. In this study, because of the different micro-environmental conditions (with fluctuation of wind speed), in different parts of the tested plot, created by clash of air flow with serial row of the cotton, the frequency of gene flow is not downward with increased distance of the pollen source, but is with random fluctuation. The highest cross pollination rate detected in these conditions is with conventional counterpart, while – with other *Gossypium hirsutum L.* cultivar is observed significant reduction of it, highlighting the quite low fertilization efficiency of wind as pollination agent, even its capacity to carry on fertile cotton pollen gains over several tens of meters. Therefore, even the outcrossing of cotton is primarily caused by insects in some specific environmental conditions the wind could contribute it as well.

For example Zhang et al., 2005 observed greater movement of pollen toward the north and west presumably related to the movement of pollinators and was consistent with the prevailing wind direction at the trial sites.

A directional effect on gene flow is reported by Llewellyn and Fitt (1996) for conditions in Australia. The "hot spots" in cross-pollination have been noted in other pollen dispersal studies (Hockanson et al., 1997) and suggest that localized events, such as a windy day, can result in a major change in the rate of gene flow.

To assess the precise impact of air flow on cross-pollination, Zhu et al (2013) pointed out that the closed greenhouse environment caused an increase in the pollen density and the high internal greenhouse temperatures can exacerbate pollen proliferation rates. In similar greenhouse conditions He et al. (2013) found that the maximal distance of gene flow caused by air flow is 25.6 m to conventional counterparts and 19.2 m to different *Gossypium hirsutum L.* cultivars.

Insects and out-crossing

When the cotton pollen is carried by insects, the insect prevalence significantly influences out-crossing rates for cotton (Elfawal et al. 1976, Pheloung 2001 and Llewellyn et al. 2007), and varies with location and time (Moffett et al. 1975, Elfawal et al. 1976 and Moffett et al. 1976). However, insect visitation rates may overestimate cross-pollination rates because many potential pollinators preferentially

target nectaries rather than the pollen (Moffett et al. 1975 and Rao et al. 1996).

Globally, the most important insects for cross-pollination are those belonging to the order *Hymenoptera*. Of the various hymenopteran species that act as cotton pollinators, bees are the most significant (McGregor, 1959 and Umbeck et al., 1991). The most frequently mentioned bees are bumble bees (*Bombus spp.*) (Llewellyn et al., 2007, Van Deynze et al., 2005 and Zhang et al., 2005) and honey bees (*Apis* spp., such as *Apis dorsata*, *A. indica*, *A. mellifera*, and *A. florea*) (Llewellyn and Fitt, 1996, Van Deynze et al., 2005, Zhang et al., 2005 and Llewellyn et al., 2007). Numerous types of bees, including *Melissodes* species and bumblebees (*Bombus* spp.) have been mentioned in multiple studies as the primary pollinators of *Gossypium hirsutum L.* varieties (Moffet et al. 1976, 1980 and McGregor et al., 1976). By comparison, honeybees have been described as secondary pollinators (Waller et al., 1985 and Waller, 1972). Other insects such as wasps, flies, and hibiscus beetles can also serve as pollinators. In Europe both wild bees and honey bees are frequently associated with cotton (Christidis, 1965, Xanthopoulos and Kechagia, 2000, Loureiro et al., 2013 and Loureiro et al., 2016 and Bozdek et al., 2008). Loureiro et al. (2013 and 2016) noted that in Spain pollinators from the insect orders *Coleoptera* and *Diptera* are also present.

The frequency of out-crossing depends on how many and how often the pollinators visit the different flowers: the higher the pollinator population and activity, the higher the rate of out-crossing. Insect pollinator species and their population densities vary geographically and seasonally (Llewellyn et al., 1996).

Higher estimates of inter-row cross-pollination (Llewellyn et al., 2007) have been reported (e.g., Oosterhuis and Jernstedt, 1999), particularly when more active insect pollinators, like bumblebees (*Bombus* spp.), are present in large numbers.

While bees are the only well-documented outcrossing agent of cotton, it is possible that cotton plants could be cross-pollinated by physical contact between flowers, but only of adjacent plants, or through transportation of pollen on tractors (Heuberger et al., 2008).

Overview of cross-pollination studies

An overview of world-wide studies on cross-pollination of *Gossypium hirsutum L.* is presented in tables 2A – 2F and table 3. The studies cited in tables 2A – 2F measured out-crossing through buffer rows of cotton, while the cross-pollination rates presented in table 3 are measured for different distances of bare land. These studies captured a broad range of geographical regions and field conditions from 6 continents – Europa (table 2A), Asia (table 2A and 2D), North America (table 2B), South America (table 2E), Australia (table 2C) and Africa (table 2F) as the experimental design of most of them included non-GM cotton planted contiguously with an adjacent plot of GM cotton.

Table 2A. Cross-pollination rate between cotton varieties, measured through buffer rows of cotton in Europe - Asia

Growing location	Test system & Experimental conditons	Distance	Cross-pollination rate	Reference
Greece	morphological marker - glandless	1m (adjacent rows) 2 m 10 m	1.67-2.67% 1.42% < 0.1	Xanthopoulos and Kechagia, 2000
	morphological marker - red leaf	1m (adjacent rows) 2m 10 m 15m	3.85% 2.79% 0.31% 0.1%	
Greece	morphological marker	1m (adjacent rows) 4m 20m	7.5% 1.4% almost 0	Christidis, 1965
Spain	GM and non-GM (Cry1F Cry1Ac & pat protein)	1 m 2 m 10 m 25 m	3.03% 0.61% 0.19% 0,06%	Loureiro et al.,2013 and Loureiro et al., 2016
	GM and non-GM (2mespsps gene)	1 m 2 m 10 m 25 m	0.17% 0.23% *0.19%* 0.01%	
Turkey	morphological marker-glandless (heavy pesticide usae)	0m 10 m	8.1% - 8.9% near zero	Bozdek et al., 2008
	morphological marker - red leaf (heavy pesticide use)	0m 10m	0% - 3% near zero	
Turkey	morphological marker – glandless or red leaf (low insecticide use)	0.7m (1st rows) 5.6m (8th rows) 6.3m (9th rows) 8.4m (12th rows) 9.1m (13th rows)	3.75% - 5.60% 0.32% 0.0% 0.25% 0.10%	Sen et al. , 2004

Table 2B. Cross-pollination rate between cotton varieties, measured through buffer rows of cotton in North America

Growing location	Test system & Experimental conditions	Distance	Cross-pollination rate	Reference
USA, California (Shafter)	GM and non-GM (without bees)	0.3 m 1.0m 3.0m 9.0m 30m	4.86 % 0.3% 0.03% 0.03% 0.03%	Van Deynze et al. 2005
USA, California (Kearney)	GM and non-GM (with artificially added beehives at every corner of tested cotton field),	0.3m 1.0m 3.0m 9.0m 30.0m	7.65% 3.1% 1.6% 0.67% 0.32%	
USA, California (Kearney)	GM and non-GM (with artificially added beehives at every corner of tested cotton field),	1.0m 3.0m 6.0m 7.5m 10.0m 15.0m	1.44% 0.66% 0.25% 0.13% 0.05% 0%	Van Deynze et al. 2011
USA, Mississippi	GM and non-GM	1m 16m 24m	1.89% 0.13% undetectable	Berkey and Savoy, 2002
USA, Mississippi	GM and non-GM (NptII gene)	1m 7m 22m	5.7% <1% 0.7%	Umbeck et al., 1991

Table 2C. Cross-pollination rate between cotton varieties, measured through buffer rows of cotton in Australia

Growing location	Test system	Distance	Cross-pollination rate	Reference
East Australia	morphological marker - red leaf	<3m 3m 4m - 8m	5% 0.01% 0%	Thomson, 1966
North Australia	GM and non-GM (NptII gene)	1 m 3 m 5m 10m 15 m 20m	0.15% -0.4% 0.13% - 0.08% 0.0% - 0.1% 0.0% - 0.06% 0.0% 0.0 %	Llewellyn and Fitt, 1996
West Australia (Kununurra)	GM and non-GM (Cry1Ac gene) (with artificially added beehives at 20m from edges of cotton field)	0m 9m 20m	10.5% 1.29% 0.13%	Llewellyn et al., 2007

Table 2D. Cross-pollination rate between cotton varieties, measured through buffer rows of cotton in Asia

Growing location	Test system	Distance	Cross-pollination rate	Reference
China	GM and non-GM (*tfd* A gene) (complemented with bee farm in surrounding area)	1m 2m 9m 20m 50m	10.13% 3.29% 0.3% 0.17% 1 of 2680 cotton plants was crosspolinated	Zhang et al., 2005
	GM and non-GM (*cry* IA gene) (complemented with bee farm in surrounding area)	1m 20m 50m	5.24% - 10.05% 0.08% 0%	

Table 2E. Cross-pollination rate between cotton varieties, measured through buffer rows of cotton in South America

Growing location	Test system	Distance	Cross-pollination rate	Reference
Brazil	GM and non-GM Bolgard ® Roundup Ready® marker with methylene blue morphological marker-glandless	 border row border row 1m 10m 1m 10m	 0.85% 11.7% 29% - 54% 0% 3.9% - 42.1% 0%	Freire, 2002
Argentina	GM and non-GM	1m 2m 5m 10m 20m	32% 26% 22% 20% 0.15%	Kareieva et al., 1994

Table 2F. Cross-pollination rate between cotton varieties, measured through buffer rows of cotton in Africa

Growing location	Test system	Distance	Cross-pollination rate	Reference
Sudan, Gezira	morphological marker, okra leaf-shape	0m 5m 10m 15m 20m 25m 30m	1.33% – 3.32% 1.20% - 1.33% 0.70% - 0.96% 1.10% 0 % 0.33% 0%	Ali et al., 2006
Burkina Faso	GM and non-GM (Cry1Ac & Cry2Ab) proteins (maximum presence of insect pollinators was assured)	2m 5m 10m 15m	4.36% 1.45% 0.99% 0%	Bourgou et al., 2013

Table 3. Cross-pollination rate between cotton varieties, measured for different distances of bare land

Growing location	Test system	Distance	Cross-pollination rate	Reference
USA, Oklahoma	morphological marker	5.0m 10.0m 25.1m	6.0% 4.7% 0.6%	Green and Jones, 1953
Egypt	outcrossing from *Gossyoium Barbadense*	1.1m 35.2m	7.8% 0.16%	Galal et al., 1972
Western Australia (Kununurra)	GM and non-GM (*Cry1Ac* and *Cry2A* genes) (with natural abundance of honeybees), (with artificially added beehives at 20m from edges of cotton field),	1m 12.6m 25.2m 48.6m 1m 10m 25m 50m	7.9% 1.0% 0.88% 0.79% 15.3% - 30.1% 0.97% - 2.2% 0.69% - 1.1% 0.55% - 0.97%	Llewellyn et al., 2007

Despite the differences in cultivars and locations where the experiments were conducted, the results reported (table 2A – 2F) confirm that distance is an important factor in reducing cross-pollination. The correlation between distance and out-crossing rates varies depending on the location, the time period, and how measurements are taken. For example several methodologies have been applied to estimate the rate of cross-pollination in cotton (table 2A – 2F and table 3). They can be classified into three types, according to the marker used: morphological (Xanthopoulos and Kechagia, 2000, Christidis, 1965, Berkey and Savoy, 2002, Sen and Cicek, 2003, Thomson, 1966 and Freire, 2002), bioassay labelling (Freire, 2002) and molecular or protein detection (Loureiro et al., 2016, Umbeck et al, 1991, Llewellyn et al., 2007, Zhang et al., 2005, Llewellyn and Fitt, 1996, Bourgou et al.al., 2013, Van Deynze et al. 2005 and Van Deynze et al. 2011). Morphological and molecular or protein detection measure the cross-pollination rate directly, while bioassay labelling in the approach utilized by Freire (2002) estimated the number of flowers in recipient fields visited by pollinators after overhaul of donor flowers, which could lead to overestimation of actual cross-fertilization.

All of the studies in tables 2A - 2F reported a decrease in out-crossing with increasing number of rows away from the GM cotton field. This follows the same pattern seen in almost all other pollen dispersal studies in plants (Umbeck et al.,1991, Llewellyn and Fitt, 1996, Lavigne et al., 1998, Amand et al., 2000, Van Deynze et al., 2005 and Weekes et al., 2005) with a higher rate of gene flow over short distances from the source and a long tailed distribution at greater distances of stochastic pollination events (a so-called leptokurtic distribution; Lavigne et al., 1998); and secondly, that the rates of gene flow are influenced by pollinator abundance which varies with local geography and climate (Hockanson et al., 1997; Amand et al., 2000; Van Deynze et al., 2005).

Correlation analyses of the data (table 2A - 2F) on the decline of pollen-mediated gene flow with distance revealed that it follows an exponencial function (Llewellyn and Fitt, 1996, Messeguer, 2003, Van Deynze et al., 2005 and Loureiro et al., 2016); a power curve model (Zhang et al., 2005) or an inverse function model presented as a hyperbolic best-fit curve (Van Deynze et al., 2011) depending on the environmental conditions. For example, in California, USA Van Deynze et al. (2005) detected that in the presence of honeybee activity, cross-pollination follows the exponential function, being 7.65% at 0.3m and rapidly declining with increased distance, droping to less than 1% beyond 9 m. The extension of this exponential decline in pollen-mediated gene flow is illustrated by the detected cross pollination of 0.04% (1 out of 2,250 plants) at 1625 m from pollen sources in commercial fields. This cross-pollination rate of 0.04% was predicted at 30 m by Van Deynze's et al. (2011) hyperbolic curve model and calculated to decline to 0.004% (1 in 26,000 seeds sampled) at 800m. Zhang et al. (2005) also reported the same cross-pollination rate of 0.04% but at 50m with the power curve model.

The above research data make evident that the rate of natural cross-pollination of cotton over distance for a variety of environmental conditions is equally well accounted for by either an exponential or power curve model. Van Deynze et al. (2005) reported that a negative exponential curve explained over 99% of the variance in cross-pollination at the study site. The correlation coefficients of the exponential

models built by Loureiro et al. (2016) are 0.97 - 0.98, and the power curve models of Zhang et al. (2005) have correlation coefficients of 0.998 - 0.999.

All of these best-fit models concur with the downward trend of naturally occurring cross-pollination in cotton with increased distance. While this conclusion is valid not only for short distances (10m – 20m) but for distances further than 30m – 50 m, where the levels of pollen dispersion are very low and there is no clear cut-off distance beyond which these levels reach zero.

Regional differences of insect impact on out-crossing rates of cotton

All of the studies cited in tables 2A – 2F reported a rapid decrease in outcrossing as distance from the pollen source increased. However, there are differences in the magnitude of the pollen-mediated gene flow reported for different locations. In decreasing order they are: China (Asia); Sudan, Burkina Faso (Africa); USA (North America); Brasil and Argentina (South America); Greece, Spain, Turkey (Europe and Asia); Australia.

The difference in observed outcrossing rates may be related to differences in pollen and nectar feeding habits of insects, which are mainly responsible for out-crossing of cotton. As insect pollinator species and their population densities vary geographically and seasonally, so outcrossing rates vary regionally.

For example, outcrossing in China is further and larger from GM plants than that in the United States or Australia. One of the reasons for this difference according Zhang et al. (2005) may be the different environmental conditions, especially the type, number, and behavior of the pollinators. In China, the primary pollinators are bumble bees and honey bees; secondary pollinators are diurnal hawk months and butterflies (Tian et al., 2004). Zhang et al. (2005) reported that their entire study took place on a bee farm and there were frequent visits by honey bees to the cotton field.

The study of Bourgou et al. (2013) (table 2F) for cross-pollination of cotton in Burkina Faso, was carried out in areas with an abundance of pollinator insects, such as bumble bees, honey bees and butterflies, with no insecticides applied to maximise presence of pollinator insects. Ali et al. (2006) reported for conditions of Gezira, Sudan cross-pollination rates of cotton with lover presence of aphids, the total number of aphids was not exeding 0.075 - 0.15 per plant, even in 50% of experimental condition they were completely absent.

Llewellyn and Fitt (1996) reported that the difference in pollen-mediated gene flow recorded for the trials in the United States and Australia was probably a reflection of differences in pollinator species. In the United States, the bumble bee, honey bee, and Melissodes bee are considered most important as pollinators of cotton (McGregor, 1959). However, in Australia, Llewellyn and Fitt (1996) did not observe any pollinators, including bees, except for small numbers of wasps and flies in the field during both field trails. The absence of efficient pollinators, especially bumble bees, may be the reason for the low frequency of pollen-mediated gene flow of transgenic cotton in the Australia trials (Llewellyn and Fitt, 1996). Llewellyn (2007) found that gene flow from GM plots into adjacent conventional cotton was much higher in northern Australia than in eastern Australia, most likely due to higher honey bee (*Apis mellifera*) numbers, often deliberately enhanced to aid the surrounding horticultural industries.

In a trial conducted in Andalucia (Spain) Loureiro et al. (2013) provided data that indicated the presence of 0.011 - 0.050 *Apidaes* per flower. Similar data for insect scouting in cotton fields in Turkey (mediterranean coast) are reported by Sen et al. (2004): 0.046 – 0.062 wild bees and 0.005 - 0.0077 honeybees were counted for each flower. The wild bee population acounted for 88.9% - 90.2% and honeybee populations varied from 9.8 % to 11.1% of total bees visiting cotton flowers. This finding shows that under natural European conditions the honey bees are not the primary pollinators in terms of flower visits, as also indicated by Moffet et al. (1980).

For such a pollinator background (table 2A), in Spain, Loureiro et al., 2016 reported 0.17% - 3.03% outcrossing in cotton, which significantly declined with distance to 0.19% at 10m and 0.01% - 0.06% at 25m. Similar data were reported by Christidis (1965) for Greece: 4.7% – 9.7% outcrossing, which was greatly affected by the distance; from about 7.5% in adjacent rows; it decreased to 1.4% after 4 rows spacing (4m), and after 20m it fell almost to zero. Xanthopoulos and Kechagia (2000), again in Greece, conducted two experiments using glandless and red–leaf traits as genetical markers to determine the extent of outcrossing in cotton. They found that, in the first experiment where estimation of natural crossing was based upon gland status, the percentage ranged from 1.67% to 2.67% in adjacent rows, dropped to 1.42% in plants 2m apart, and declined to almost zero after 10m. In the second experiment, where the red-leaf marker gene was used, the mean of natural crossing was 3.85% in adjacent rows, which dropped to 2.79% in plants 2m apart and progressively diminished to 0.31% after 10m. Bozek et al. (2008) and Sen et al. (2004) reported similar results for conditions of Turkey. Bozek et al. (2008) and Sen et al. (2004) also revealed that distances >10 m between cotton plants can effectively prevent natural crossing.

Sen et al. (2004) concluded that 12 to 13 rows (8 to 9 m) can provide good separation for isolation distance in cotton production in Turkey. Xanthopoulos and Kechagia (2000) concluded that distances greater than 10m between cottons were sufficient to minimize outcrossing. Loureiro et al. (2016) concluded that in Spain the distance required to achive the 0.9% threshold is between 1m - 1.8m and 10m are sufficient in practice to limit cross-pollination almost to zero.

Conclusions from outcrossing studies using cotton buffer rows

All the data summarized in tables 2A - 2F presented a rapid decrease of outcrossing rate as the number of rows away from the pollen source increased. The differences in magnitude of cross-pollination rate among the reported studies are a reflection of variation in agroecological and agricultural conditions where they were conducted.

The extensive analysis of world-wide data on cross-pollination rates in cotton (tables 2A - 2F), including data particular to European conditions (table 2A), indicated that cross-pollination beyond 5m of buffer zone will efficiently prevent GM presence above 0.9%. Furthermore, since the correlation function for short distances away from the pollen source has a steep slope, small increases in buffer zone size are reflected in high reductions of cross-pollination rate. To limit cross-pollination to 0.1% almost all studies are agree that, given naturally occurring populations of pollinators, 10m buffer zone will be sufficient.

The placement of beehives in the vicinity of GM cotton fields will affect cross-pollination rates. In the absence of such a study under conditions prevailing in European environment, the studies of Van Deynze et al. (2005) and (2011) and Llewellyn et al., (2007) on impacts of artificially added beehive activities in surounding areas of cotton fields in USA and Australia must be considered. In the presence of an artificially increased abundance of honeybees the restriction of cross pollination below 0.9% was achieved with a 10m buffer zone and a 20 m buffer zone limited it to 0.1%.

The definition of "increased concentration of honey bees" (which could be a reason for enlarging buffer zones in order to limit adventitious admixture to target threshold) is not easy, because it is a dynamic value, which is influenced by competition between the present pollinators and the availability of accessible alternative food sources. Van Deynze et al. (2005) is again pertinent here as this study involved installation of 11 beehives per hectar of cotton with beehives placed at the beginning of the flowering and pollination period to ensure ample bee activity and represent a worst-case environment to investigate insect-mediated pollen flow, such as might occur with a cotton field adjacent to a bee-pollinated crop (Van Deynze et al., 2011).

Of course the number of beehives required to consider increased pollinator activity is relative and could vary greatly for different agro-environmental and landscape conditions, where there may be competition and migration of introduced bees to neighbouring crops (that may be preferred by the bees for pollen or nectar collection). However, the similarity between the conditions in California and European areas where cotton cultivation take place is enough reason to propose utilization of this selection criterion.

It should also be noted that all data on the cross pollination of cotton (table 2A – 2F) are from measurements of out-crossing rates at a particular distance. However, in considering the GM presence in the combined harvest from a whole cotton field, i.e. including the crop beyond the buffer zone, the downward trend in cross-pollination across the field will serve as dilution factor. The value of this dilution factor is dependent on the size and shape of the recipient field and the correlation function between the cross-pollination rate and distance from the donor source. For European conditions there are not enough quantitative data for calculation of such a factor given the many possibilities for shapes and sizes of cotton fields. However, it is clear that the out-crossing rates summarised in these tables will be substantially reduced when considering the total harvest of the field.

Outcrossing measured through bare land distances

All the data discussed before are applicable for use of cotton rows as buffer zone to limit out-crossing. Less empirical evidences are available about the cross-pollination rate between cotton plants separated by bare ground (table 3). In general the cross-pollination rate might be expected to be higher in the absence of buffer rows for the same distance of total separation. Llewellyn (2007) observed in Australia the movement of cotton pollen over bare ground to nearby crops at a number of locations. This study suggested that pollinator insects were flying across the bare ground but did not often venture far into the neighbouring fields, rather remaining near the outer edges and showed a rapid decline in cross-pollination with increasing distance from the GM plots similar to that observed in all other studies of gene flow in cotton. Bees have a tendency to forage around the edges of the crop with random visits into the centre (Rhodes, 2002). These foraging behaviours are consistent with the predominance of cross-pollination observed at the leading edges of the conventional cotton near the GM plots. It is of interest to note, that having traversed the gap between the cotton fields, the rate of cross-pollination moving into the sink field starts out similar to that right next to a GM source and then drops very rapidly with distance, similar to the results seen in conjoined GM plots and buffers (Reboud, 2003).

Green and Jones (1953) demonstrated in Oklahoma, USA (table 3) that out-crossing in the absence of a buffer decreased from 6.0% at 5.0 m, to 4.7% at 10.0 m, and to 0.6% at 25.1 m. In Egypt, Galal et al. (1972) measured out-crossing from *Gossypium barbadense* and also demonstrated a rapid decline with distance over fallow ground from an average level of 7.8% at 1.1 m to 0.16% at 35.2 m.

From the data in table 3 it can be concluded that to limit potential out-crossing to 0.9% 30m of bare land will be sufficient given a natural abundance of native pollinators and artificially added beehives in the vicinity. To achieve 0.1% out-crossing at least 100m isolation distance of bare land seems needed. A more precise definition of the isolation distances will require further research. Again, the final

admixture in the harvest will be determined by the dilution factor of the bulk field harvest.

5.2.3. Seed-mediated gene flow

Seed-mediated gene flow has received less attention than pollen-mediated gene flow (Heuberger et al., 2010, Beckie and Hall, 2008 and Mallory-Smith and Zapiola, 2008) mainly because the cotton seeds are large, covered with thick fibres and enclosed in a tough boll that retains most of the seeds on the plant (Llewellyn and Fitt 1996). However, during harvesting some cotton seed may be lost from the plants into the fields. Some dispersal of cotton seed may also occur in areas where cotton seed is stored. Seed is stored on farms in various ways (for example in sheds) that maintain its quality and protect it from animals and weather, thereby limiting dispersal. Wider dispersal of cotton seed may occur during transport, stock feeding, adverse weather conditions and through the actions of animals, each of these is discussed below.

Rache et al. (2013) detected six samples of seed gene flow in fields planted with conventional cotton varieties. These fields were located approximately 4-10 m away from fields planted with transgenic varieties, and Rache et al. (2013) concluded that the gene flow could be due to transgenic seed falling inadvertently in these fields, or seeds that were disseminated or scattered by the wind. This pattern has also been shown by Messeguer (2003) and Heuberger et al. (2010). Gene flow observed in conventional fields located further away from fields planted with transgenic cotton could be due to contamination of seeds at source (Van Deynze et al., 2005), inadvertent planting of seeds during harvest (Messeguer, 2003), or as a result of human errors committed during planting, harvesting or processing of the seed (Heuberger et al., 2010). However, it should be noted that factors such as dumping of seed in production areas or during transport and their use for feeding livestock may also be involved as potential causes for gene flow via seeds.

Use of farm-saved cotton seeds is another possible route for gene flow, given their high vulnerability to accumulate gene flow (Gaines et al. 2007). In contrast to the situation in the EU, farm saving of cotton seeds is common in developing countries, despite industry and government efforts to promote commercial seed use (Bellon and Berthaud, 2004, Huang et al., 2009 and Tripp, 2009). Furthermore, seed distribution companies in developing regions often purchase seed from small farms where gene flow could be prominent.

Seed processing may also be important in the contamination of fields planted with non-GM cotton (Heuberger et al., 2008 and Heuberger et al., 2010).

Heuberger et al. (2010) provided some evidence that adventitious Bt cotton plants (that had resulted from seed gene flow) diminished the association between neighboring Bt cotton fields and pollen-mediated gene flow, suggesting that the two pollen sources may compete to out-cross non-Bt cotton plants, i.e. the effect of pollen from adventitious GM presence in seed and pollen from a neighbouring GM crop was not additive. When the adventitious Bt cotton plants acted as a source of pollen-mediated gene flow further into a field, e.g. at 20m where the influence of neighbouring crops would be expected to be negligible, the adventitious Bt cotton plants did contribute to out-crossing (Heuberger et al., 2008, Goggi et al., 2006 and Bannert and Stamp, 2007).

5.2.4. Volunteers

Climate conditions in Greece, Spain, Bulgaria and Portugal are suitable for growing cotton. Seeds from cotton cultivars do not possess dormancy and will germinate in autumn if conditions are favourable. In addition, seeds will usually not survive in humid soil. In regions with mild and dry winters, cottonseeds may overwinter and germinate in spring if adequate moisture is available. The occurrence of volunteer cotton is limited by soil moisture content and frost.

Cotton seed in commercial trade must be handled properly to preserve germination quality. In humid environments, seed left in the field will not usually survive until the next season (Jenkins, 2003).The existence of a soil seed bank seems unlikely because dispersed seeds that do not germinate are rapidly weathered, leading to significant decreases in their viability (Halloin, 1975, Woodstock et al. 1985).

Cotton seeds can show induced dormancy by low soil temperature and/or low soil moisture. In addition to induced dormancy, cotton seeds collected immediately following fruit maturation can display ‘innate dormancy’ (Taylor and Lankford, 1972) – an inherent condition of the mature seed/embryo that prevents the seed from germinating, even when exposed to appropriate environmental conditions.

Volunteer crop plants compete for essential nutrients, water, and light with the crop and can cause harvest issues. However, the survival of volunteer cotton seedlings has clear trends indicating that the habitat into which seeds were sown affected survival. Survival at sites located near cattle yards or adjacent to water bodies was consistently high, probably because of high soil nutrients and/or soil moisture. Both factors are clearly critical for survival of cotton seedlings, but the relative importance of each is unknown. The result highlights field observations that the occurrence of naturalised and volunteer cotton appears to be limited by the availability of adequate soil moisture. Significantly, the nutrient-enhanced experimental sites were the only habitats in which a second generation of seedlings was recruited from the original batch of seeds that was sown.

If they survive, the cotton volunteers can be controlled mechanically or chemically. Tillage is one of the most effective tools for managing volunteer cotton in fallow situations or prior to planting any crop, various herbicides also provide excellent volunteer cotton control during either the fallow period or growing season.

5.3. Process management during sowing, harvesting, transportation and use as stockfeed

5.3.1. Sowing

To avoid possible mixing during sowing, seed planters should be cleaned between seed lots. Cleaning recommendations depend on the type of seed planter and seed metering mechanism. For specific procedures for individual planters, operators have to refer to the operation manuals. However, it is hard to predict how the remaining seeds will exit the seed planter, individually over a long distance or as a concentrated lot at an unknown time and location. Experience with an individual planter over time will help to find where seed may be lodged.

Heuberger et al., 2008 identified the possibility for transportation of pollen stuck on tractors performing regular agro-technical and/or agro-chemical activities from one cotton field to other, which could result in some cross-pollination, but without providing any data about the extend of this impact.

5.3.2. Harvesting and seed dispersal

Harvesting is the most critical step, as combine harvesters are in general an important source of on-farm grain comingling, due to their complex construction.

It is difficult to estimate exactly how much of this remaining material will end up in the next crop, although a thorough cleaning of the combine harvester is recommended if the harvester is used for non-GM cotton after harvesting of GM cotton. Choosing the appropriate technique for combine clean-up should be based on the desired level of purity of the grain.

The small size of cotton fields in Greece forces farmers to share the same machines, often in a short period of time that depending on the local weather conditions each year, which is unpredictable. Consequently, there may be no opportunity for cleaning harvest machinery each time before the processing of each field. For example[21], in 2012 the proportion was 24.9 fields per sowing machine and 130.4 fields per harvesting machine.

5.3.3. Transportation

The amount of cotton seed being transported and the distances transported depends on the amount of the cotton grown each year and its end use. This can be highly variable, for example, cotton seed is used as a supplementary food for cattle and sheep, so transport to these areas would increase potential seed dispersal (Knights 2007).

There are three sources of transported seed that may be distributed onto roadsides (Addison et al., 2007). These are:

- Seed cotton (as harvested from the plant) being spilled during transport from the field to the gin;
- Seed which had been ginned being spilled during transport away from the gin to oil crushing facilities or for stock feed. In the case of *G. hirsutum* this is commonly called 'fuzzy seed' as it is still coated with linters;
- Seed (for planting) being spilled during transport to cotton farms for sowing. For *G. hirsutum* this seed is delinted and is often called black seed.

Although cotton seeds are quite big and this characteristic should ease the handling during these steps, the cleaning of trucks and trailers is required when non-GM grains are handled after GM lots.

5.3.4. Via use as livestock feed

Cotton seed is fed to both sheep and cattle as a protein supplement. The quantity of cotton seed used is generally limited to a relatively small proportion of the diet, and must be introduced gradually to avoid potential toxic effects due to the presence of anti-nutrients (gossypol and cyclopropenoid fatty acids) in cotton seed (Farrell and Roberts, 2002). Such seed has been observed in seed storage areas, along paths in feed lots and grazing paddocks.

In addition to seed dispersal during feeding, a small percentage of cotton seed consumed by livestock can pass through the digestive system intact and is able to germinate (Eastick, 2002). Whole seed may be excreted in faeces in cattle yards, or in fields where animals graze after being fed and this could, under suitable conditions, germinate.

21 Source: Ministry of Rural Development and Food of Greece, Directorate of Arable Crops

6. Occurrence of cotton pollen in honey

Nectar production by the cotton plant has historically been of economic importance to commercial beekeepers in a number of countries such as USA (Martin and McGregor, 1973 and Waller, 1982), former USSR (Kuliev, 1958), Egypt (Wafa and Ibrahim, 1959 and El-Banby et al., 1985), Greece and Spain although there are no recent references of its current status, particularly since the increased use of modern synthetic pesticides.

In Greece, cotton is one of the main crops that bloom during the summer; hence approximately 1,000,000 of 1,800,000 beehives[22] are placed at the edges of cotton farms (zero distance). Cotton honey accounts for 15 % of total honey production in Greece (> 3000 t). The production of cotton honey might be increased due to the application of the Regulation (EU) No 485/2013 that restricts the use of neonicotinoids for seed[23].

However, out of the 200 nectar-producing plants listed by Crane (1975), cotton is in the lowest class, with 0-25 kg honey/ha. Honey yields have been estimated to be 0.12 - 0.76 kg/d/ha (Butler et al. 1972 and El-Banby et al., 1985).

Waller et al. (1981), McGregor and Todd (1956), and Butler et al. (1972) reported that *G. barbadense* is a better nectar producer than *G. hirsutum*, by reporting an estimated 44 kg and 28.6 kg honey yield per hectare from *G. barbadense* and *G. hirsutum*, respectively.

Honey bees avidly collect cotton nectar but often forage at extra-floral nectaries and thus fail to enter the corollas and contact pollen. Those bees that do enter flowers are said to only "rarely" (Moffet et al., 1975) or "seldom" (McGrecor, 1959) collect upland cotton pollen and instead tend to groom and rid themselves of pollen grains before returning to colony. This observation is confirmed by palynological data (Tsigouri et al., 2004) on the content of *Gossypium hirsutum* pollen in Greek cotton honeys which is in the range of 1.2% to 16.5% of the total pollen, with 75% of the samples containing <10%. This quantitative palynological analysis places cotton honey in Maurizio's Class II, "important minor pollen" (3-15%) (Louveaux et al. 1978).

Pollen analysis conducted by Karabournioti (2000) also shows low percentages of cotton pollen grains in cotton honeys produced in Greece: in 36.4% of samples (n=4), pollen cotton was a minor constituent (1-3% of total pollen) and in another four samples it was an important minor type (3-15% of total pollen).

Similar data to those found in Greece about the presence of cotton pollen grains in cotton honey have been produced in Egypt (Hamdy et al., 2009): 16.25% ± 3.18 of pollen grains found in cotton honey samples were from cotton.

Karabournioti (2000) concluded that because of the very low presence of cotton pollen, it is difficult to be used for authentication of "cotton honey". Therefore the organoleptic characteristics seem to be crucial for identification of this type of honey. All of the examined samples of cotton honey (n=11, collected directly from beekeepers in north and central Greece) contained pollen of *Trifolium sp.* and eight out of eleven (72.75%) pollen of *Brassicaceae* and *Apiaceae* which appeared predominant in one sample (9.1%) each. The presence of *Apiaceae* can be explained in the same way as in *Helianthus* honey. In every sample pollen from *Chenopodiaceae* was also detected. *Chenopodiceae* plants, according to Louveaux et al., (1978), are nectarless but entomophilous. The combination of *Apiaceae* and *Chenopodiaceaei* pollen grains according Karabournioti (2000) may be characteristic to Greek cotton honey.

Even assuming the highest reported data for the proportion of cotton pollen in honey produced in an area with a high percentage of cotton cultivation (15% of total pollen) and considering the requirement for water-insoluble content in marketed honey in the EU (<0.1%, as also is stated in the Codex Alimentarius standard for honey[24]), the calculated maximal content of cotton pollen in total honey will never exceed 0.023% (Rizov and Rodriguez-Cerezo, 2013).

22 according to Federation of Greek Beekeepers' Association

23 http://www.efsa.europa.eu/en/topics/topic/beehealth?wtrl=01

24 Codex Alimentarius standard for honey – CODEX STAN 12-1981 and Council Directive 2001/110/EC

7. Detection of GM events in cotton crop and honey

A number of methods for the detection of GM cotton have been developed. These include:

- PCR-based methods, both qualitative and quantitative (Khadye V. S. and Sahasrabudhe A. V., 2012, Vidhya et al.,2012, Yang et al., 2005 and Lee et al., 2007); and
- Protein-based methods (Kamle et al., 2013 and Wang et al., 2007).

The European Union Reference Laboratory for GM food and feed (EU-RL GMFF) has validated quantitative PCR methods for identification and quantification of several GM cotton events and methods for DNA extraction mainly from cotton seeds. The PCR methods can be found in the EU Database of Reference Methods[25], maintained by the Joint Research Centre (JRC) in collaboration with the European Network of GMO Laboratories (ENGL). DNA extraction methods are also available at JRC database[26].

Depending on the transformation event, newly introduced proteins can be detected in raw fibres of GM cotton. For example, with western blot analyses Sims et al. (1996) reported presence of CryIA(c) protein in raw linters at 0.17 µg/g and CP4 EPSPS protein presence in combed lint at the levels below 0.5 µg/g. Inactivation of this protein in the first processing step (cleaning, bleaching and dying or alkaline wash, heat and bleaching) for lints or lint indicates that the protein will not be present in cotton fibre or cellulose products, thus making protein-based detection methods unsuitable for analysis of processed cotton products.

Extraction of high quality genomic DNA for PCR amplification from Gossypium (cotton) fibres is difficult due to high levels of polysaccharide and other interfering substances. Though Liang M. and Shu Kin So S. (2015) published method for genotyping of mature cotton fibres and textiles under USA patent US8940485 B2. DNA is extracted from the mature cotton fibres and subjected to PCR techniques which enable the identification of the cultivar of a particular cotton species utilized in the textile or cotton material of interest. The extracted genomic DNA from the mature cotton fibres comprises chloroplast DNA. The DNA isolated by this protocol is of sufficient quality for screening of 35S promoter of Cauliflower Mosaic Virus and NOS terminator of *Agrobacterium tumefaciens* with detection limit of 0.1%.

For detection of the crosspollination occurred in fields Cardenal et al. (2013) selected the bolls from parental plants, resulted negative to PCR analysis. Therefore, progeny analysis to evaluate pollen mediated gene flow was carried out with seeds produced by fields in which all parental plants were negative with Immunostrip™ and PCR. Positive results with both Immunostrip™ and PCR are interpreted as seed mediated gene flow.

Another approach to detect crosspollination rate mediated by pollen gene flow is based on zygosity of plants (Heuberger et al., 2008). Marketed Bt cotton varieties are homozygous for the Bt gene (Adamczyk and Meredith, 2006 and Jayaraman, 2005) and the sequence coding for production of Bt toxins is dominantly inherited in cotton (Heuberger et al., 2008, Sachs et al., 1998 and Zhang et al., 2000). The hemizygous seeds (i.e., containing one copy of the transgene) are result of crosspolinaton (Heuberger et al. 2011). Plants with both seed types had a mean of 78% Bt seeds (95% confidence interval, 70 to 86%), which fits the expected 3:1 ratio of Bt expression in self-pollinating Bt-hemizygous plants (Zhang et al., 2000).

To distinguish seed-mediated from pollen-mediated gene flow Heuberger et al. (2010) tested seeds and fruit wall (maternal pericarp tissue) from the conventional cotton plants which could have been cross contaminated. Cross-pollination with pollen of GM cotton does not affect the maternal pericarp tissue of the conventional counterpart. Therefore the presence of GM out-crossing is identified by bolls with transgenes detected in some of the seeds but not in the fruit wall.

25 http://gmo-crl.jrc.ec.europa.eu/gmomethods/search?db=gmometh&q=cotton&jumpMenu2=.%2Fsearch%3Fdb%3Dgmometh%26q%3Dac%253A

26 http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx

Adventitious GM plants (resulting from seed-mediated gene flow) can be further sorted by whether they contained only GM seeds or both GM and non-GM seeds. GM plants producing both seed types are hemizygous and average 75% seeds with the GM trait when they self-pollinate (Zhang, 2000). Calculating the relative proportions of hemizygous versus homozygous plants yields insight into the source of adventitious plants, as hemizygous plants result from cross-pollination events between GM and non-GM cotton in previous generations (Heuberger et al., 2008).

Currently a practical and robust PCR protocol able to quantify GM pollen relative to total pollen in honey is not available. This is becasue in all honeys, even those classified as unifloral; the pollen fraction consists of pollen from several species (for details please check Rizov and Rodriguez-Cerezo, 2013).

8. Best practices for coexistence in cotton production

The TWG for Cotton analysed the possible sources for potential GM admixture in cotton production, which are summarised in the previous sections and agreed on the following best practices for the coexistence of GM cotton cultivation with non-GM cotton harvests and honey production.

The thresholds for coexistence which were analysed were the legal labelling threshold for authorised events (0.9%) and the limit of quantification (about 0.1%), which is required by private operators in some markets. The potential adventitious admixture in the commodity produced is cumulative through every one step of production and this has been taken into consideration with the aim of the final product meeting the threshold targets.

8.1. Best practices for ensuring seed purity

The use of certified cotton seeds that comply with EU legislation is the best practice because according to EU legislation any seed lot containing traces of GM material must be labelled and therefore can be easily identified.

Where cultivation of both GM and non-GM varieties is planned on the same farm, the seeds of GM varieties should be transported to the farm and stored upon arrival in their original packaging, separately from non-GM varieties. Label information should be retained with the seeds.

8.2. Best practices for reducing cross-pollination from GM fields

8.2.1. Buffer zones

Buffer zones are a feasible and effective coexistence measure to reduce adventitious presence of GM cotton in conventional and organically produced cotton, even if they are the only measure applied (worst case scenario). All available information from literature and pre-existing segregation systems shows that, given a natural background of pollinators, 5 m buffer zone would be sufficient to limit adventitious GM presence caused by cross pollination to 0.9%, and that to achieve thresholds of 0.1%, a 10 m buffer zone should be sufficient. Moreover, the combining of the harvest batches from different parts of the field works as dilution factor and makes this adventitious presence at field level significantly lower than the point estimation of GM presence at the frontier of the buffer zone. Where there is a significantly increased abundance of honeybees, the limitation of cross pollination to below 0.9% could be achieved by a 10m buffer zone and to achieve 0.1%, 20m buffer zone is sufficient. Currently this “increased concentration of the honeybees” data comes from experiments placing 11 or more beehives in the vicinity of every hectare of cotton field.

8.2.2. Isolation distances

If instead of buffer rows of cotton bare land is used, 30m isolation distance is recommended to limit potential admixture to 0.9%, which should be sufficient both for fields with a natural abundance of pollinators and for situations with artificially added beehives in the vicinity. To reduce admixture to 0.1% at last 100m bare ground isolation distance is needed. More precise definition of the isolation distances will require further research.

Farmers should be able to choose the best spatial isolation (buffer zones or isolation distances) according to the particular landscape and agro-tehnical conditions.

When the establishment of refugi, to delay development of pest resistance is required (*Bt* cotton), the non-GM buffer zone could serve both as refuge and a coexistence measure. Conversely, when the local landscape provides borders of GM cultivated fields with empty spaces or non-compatible species, the bare-land isolation distance has economical advantages. For example, the bare ground isolation is preferable at the ends of rows in fields where the planting of buffers is technically demanding because of the frequent changes of seed type required during mechanical planting of individual rows.

8.2.3. Temporal isolation

The replacement of spatial isolation with temporal isolation, achieved by planting of different maturity classes of cotton, is not feasible in European conditions as it will not provide enough insurance to avoid flowering coincidence. This is due to the limited gene pool from which cultivated varieties are derived and the small window of optimal climatic conditions available in Europe.

8.3. Best practices during sowing, harvesting and on farm storage

The equipment utilization and maintenance should be done in economically sound manner. The use of dedicated equipment for different production systems eliminates the risk of admixture or to use them for non-GM crops prior to GM crops would have a similar effect. The equipment used for processing of GM crops should be cleaned thoroughly before it can be used for processing of non-GM crops. The specific protocols for cleanout depend on the type of equipment and its construction; hence is recommended that operation manual for the specific piece of equipment is consulted. It is important to consider where the greatest chances are for previous grain to enter and commingle with the new grain. Experience with individual equipment over time will help to determine common areas where seed may be lodged. Choosing the appropriate technique for equipment cleaning should be based on the desired level of purity of the grain.

GM crops should be clearly labelled and stored separately from non-GM crops. The storage space must be thoroughly cleaned out and inspected after being used for GM crops and prior storing of non-GM crops. As a general rule, if it is possible to tell what has previously been stored, it is not clean.

8.4. Best practices for coexistence with honey production

There is no available empirical data to establish a statistical relationship between cotton pollen content in honey and distance of beehives to cotton crops.

Cotton pollen is rarely collected by honeybees; hence its transfer to beehives is quite limited. Even honey produced from cotton nectar naturally contains such low levels of cotton pollen that it can not be used for its autentification. For example, if consider the maximum pollen content (number of grains) in commercial honey and the average weight of cotton pollen grains, the weight fraction of cotton pollen in honey will be below 0.1%.

In conclusion the current practices in honey production and marketing in Europe in line with quality legislation are sufficient to ensure that adventitious presence of GM cotton pollen in honey is far below the legal labelling threshold and even below 0.1 %.

9. Economic analyses of best practice

No empirical data are available to estimate the costs for implementation of these coexistence best practices by EU farmers intending to grow GM cotton. However, data for the economic impacts of GM cotton cultivation in a similar agrotechnical and environment conditions world-wide could provide an indicative estimation of the impact of implementing these measures on the gross margins obtained by farmers.

Ex-ante the cost of coexistence can be approximated roughly to the difference in the gross margins of GM and alternative crops or as the utilization of coexistence measures on part of the farm. The gross margins obtained by farmers can be defined as the difference between a farmer's income and variable costs, i.e. costs that depend on production, such as, costs of seeds, fertilizers, pesticides, costs of fuel used for machinery, labour costs, etc. The above-mentioned coexistence measures for spatial segregation and machinery maintenance and cleaning are accounted in partial farm budgeting as variable costs. Messean et al. (2006) in the modelling scenarios for coexistence between GM and non-GM Cotton in Andalusia, Spain calculated that the cost for additional cleaning of drilling machinery, harvesters and trailers for small farms would be 39.94 €/ha and for large farms would be 34.10 €/ha. The cost of cotton seed, fertilizer (commercial fertilizers, soil conditioners, and manure), and chemicals accounted for 54% of the total budget per planted hectare (Bilbao et al., 2004). Additionally, on top of the costs relating to the restriction of GM cultivation due to the coexistence measures in place, so-called opportunity costs should be considered. These stem from the management of two different production systems within a field or farm, which obviously comes at an extra cost. The reported (Messean et al. 2006) gross margin for small farms was 943 €/ha and 1007 €/ha for large cotton-producing farms in 2004.

Ceddia et al. (2008) analysed ex-*ante* the effects of introduction of *Bt* cotton in Andalusia, Spain and reported that the gross margin would increase by 6.7% per ha. The range of gross margin advantage of *Bt* cotton reported in other world-wide studies varies from 73% in some regions of India (Morse, Bennett, & Ismael, 2005) to 2.2% in the United States (Fernandez-Cornejo & McBride, 2002). The high increase in India and in developing countries in general, is due to lower availability of insecticides and the significant effective yield improvement associated with the adoption of *Bt* varieties in these countries (e.g., Shankar & Thirtle, 2005). In Andalusia, where pesticide access is good, the main predicted benefits of *Bt* cotton are related to reduced spraying costs and therefore will be more limited compared to developing copuntries. Additionaly when pest pressure and/or weed concentration is high, it is predicted that specific GM event will economically outperform conventional cotton, based on the cost of chemicals and their application.

Gutierrez et al. (2015) performed more holistic analyses by using biological models of the cotton/pink bollworm system to examine irrigated and rain-fed cotton in finer detail, underlining that econometric analyses often ignore the underpinning ecology of the system and disregard underlying agro-ecological principles of yield formation. Gutierrez et al. (2015) concluded that as a percentage of the total revenue, the costs of the *Bt* and insecticide technologies decrease with increasing yield making it an acceptable assurance option in high-yield areas.

Quiao (2015) performed a descriptive analysis of the economic benefit of *Bt* cotton cultivation and its dynamics, which is presented by showing the quantities of pesticide cost, seed cost, labour use and cotton yield and their dynamics since *Bt* cotton adoption in China, 15 years ago. This study showed that the economic benefit of *Bt* cotton did not diminish, but remained stable and continuous in China.

Basically there is no information on the cost of using bare land as an isolation measure. In contrast the use of buffer zones is widesprean in *Bt* cotton cultivation as a refuge to delay pest resistance development so it seems economically reasonable to select this measure for coexistence as well.

Isolation distance cost could be defined as the lost profit on the area bordering a crop plot on which farmers are not able to raise a crop (Gustafson, 2002). By dividing of the total value of the lost area with the amount of crop yield sold to place, it assessment is adjusted per unit basis. Additionally, the isolation distance is a particular measure since it does not affect all farmers equally. Fields are not randomly distributed on a common physical landscape. Farmers whose neighbouring fields lie beyond isolation distance will have no constraints in their decision-making of planting GM varieties or not and will experience no economic impact at farm level. However farmers, intending to use GM varieties but with neighbouring non-GM cotton fields falling within isolation distances will be constrained in their choice. At farm level,

this will have a monetary cost equivalent to the difference in gross margin between the GM and non-GM cotton varieties. At regional level, the economic effect will depend on the physical landscape of the area affected (Messean, 2006).

In general, the costs of coexistence for GM cotton farmers would have to be offset by monetary or non-pecuniary benefits of growing GM cotton varieties.

Ultimately, farmers will consider both monetary and non-monetary benefits of GM adoption versus coexistence costs in their decision making process to select what kind of variety to adopt.

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