Non-*Bt* seeds provided by seed companies in India – are they suitable as refuge for *Bt*-cotton?

Genetically modified (GM) cotton fortified with *cry1Ac* (crystal 1Ac) gene derived from the soil bacterium *Bacillus thuringiensis* (*Bt*), commonly known as *Bt*-cotton, was approved in 2002 for commercial cultivation in India[1]. The Cry1Ac protein expressed in *Bt*-cotton is toxic to the three major cotton bollworms: the American bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) and spotted bollworms *Earias vittella* (Fabricius) (Lepidoptera: Noctuidae) and *Earias insulana* (Boisduval) (Lepidoptera: Noctuidae). In 2006 GM cotton Bollgard-II® expressing two genes, *cry1Ac* + *cry2Ab* was approved for commercial cultivation[2]. The genetic engineering approval committee (GEAC) now called the Genetic Engineering Appraisal Committee, Ministry of Environment, Forest and Climate Change, stipulated that a minimum of five border rows of conventional (non-*Bt*) cotton hybrid of the corresponding *Bt*-hybrid should be planted as 'refuge' around the *Bt*-cotton field or 20% non-*Bt* cotton of the total *Bt* crop area for a given acre whichever is greater should be planted[3]. A refuge is an essential and integral part of *Bt* crop stewardship and is most important as one among the many Insect Resistance Management (IRM) components which collectively aim at delaying the development of resistance to Bt proteins in the target insects. GEAC modified the refuge requirements by approving the planting of any non-*Bt* cotton hybrid similar in duration, fibre characteristics and yield[4]. Later in 2009, the GEAC approved planting of pigeonpea as refuge crop with *Bt*-cotton[4]. Seed companies were required to provide a packet of 120 g non-*Bt* cotton seeds or 200 g pigeonpea seeds along with every packet of 450 g *Bt* cotton seeds[5]. The purpose of non-*Bt* 'refuge' was to delay resistance by serving as host for bollworms which can dilute resistance to *Bt* toxins in bollworm populations that survive on *Bt*-cotton. The efficacy of 'refuge' depends on the extent of simultaneous and synchronous flowering and fruiting of the non-*Bt* refuge with the *Bt*-cotton crop, which would facilitate equal opportunity for bollworms to get distributed on the *Bt* and the non-*Bt* plants. Also, as the refuge could be sprayed with insecticides, the proportion of bollworms would be reduced depending on the toxicity of the specific insecticides to bollworms, thus reducing the efficacy of refuge in providing '*Bt*-susceptible bollworms' for dilution of *Bt*-resistance in bollworms. Studies[6–8] indicated that refuge compliance in India has been poor thus far because of which the pink bollworm rapidly developed resistance to Cry1Ac + Cry2Ab toxins, thereby rendering BGII ineffective for bollworm control, a fact that is acknowledged by the Government of India. Ignorance of the necessity of refuge and reluctance to sacrifice 20% of the area for non-*Bt* crop which would be vulnerable to bollworms were attributed to be the main amongst several reasons for poor compliance of refuge in India. However, the present study found a few other reasons which could be responsible for poor compliance and significant reduction in the efficacy of refuge in India.

The main objective of this study was to find out the compliance levels of the seed companies in providing authentic *Bt*-cotton seeds and to assess the technical suitability of the refuge seed in serving as a non-*Bt* host to bollworms. Seeds were examined for the presence or absence of Cry1Ac and Cry2Ab proteins using Enzyme Linked Immunosorbent Assay (ELISA) and for the authenticity of transgene events Mon531 (Bollgard[R]) and Mon15985 (Bollgard II[R]) using event-specific primers. Field trials were conducted to assess germination and determine synchrony of the flowering window between *Bt* hybrids and the corresponding 'refuge'. Ninety-one *Bt*-cotton seed packets were purchased from the open market of North and Central India during April to July 2014. In 2015–2016, thirty *Bt* seed packets were procured from the open market of Central India. Details on the seed packets, viz. name of the hybrid, company producing it, event claimed, non-*Bt* refuge details, lot numbers and approved growing zone suitability, etc. were noted. A random sample of 10 seeds each out of the main packet and 10 seeds from the non-*Bt* refuge packet were drawn from each of the hybrids under study. Half of each seed was used for DNA isolation required for event-specific PCR testing to confirm the presence Mon531 and Mon15985 and the other half was used for ELISA[9,10] to test the presence of *Bt* toxin proteins. Of the 91 hybrids procured in 2014–2015, 10 belonged to the single gene category (Bollgard) and the rest were two gene products (Bollgard II). Of the 91 'refuge packets', 65 packets did not contain *Bt*

Table 1. Non compliance of regulatory guidelines by seed companies in India

Year	2014–2015			2015–2016	
Bt vs non-*Bt*	*Bt*-cotton	Refuge		*Bt*-cotton	Refuge
Species	*G. hirsutum*	*G. hirsutum*	*G. herbaceum*	*G. hirsutum*	*G. hirsutum*
Mon531 event (BG)	10/10	3/10	0/0	–	–
Mon15985 event (BGII)	81/81	23/80	0/1	30/30	12/30
Cry1Ac	91/91	26/90	0/1	30/30	12/30
Cry2Ab	81/81	23/80	0/1	30/30	12/30
Germination					
>75%	45/45	39/44	1/1	30/30	9/30
50–75%	–	–	–		14/30
25–50%	–	–	–		6/30
<25%	–	5/44	–		1/30
Flowering window					
≈45–70 days	3/40	–	–		
≈55–80 days	27/40	17/38*	–		
≈65–90 days	10/40	21/38	1/1		

*One refuge seed packet carried redgram seeds.

seeds but 26 packets contained *Bt* seeds (Table 1). Three out of 26 packets had only Cry1Ac and 23 packets had seeds with Cry1Ac and Cry2Ab. The 30 packets procured in 2015–2016 showed the presence of Cry1Ac + Cry2Ab toxins (Mon15985 Bollgard II) in the 450 g *Bt* seed packets. However, of the 30 non-*Bt* refuge seed packets tested, 12 were found to contain Cry1Ac and Cry2Ab seeds.

A field trial was laid out at ICAR–Central Institute for Cotton Research (CICR), Nagpur with 45 BGII hybrids along with the corresponding refuge seeds approved for release in Central India. The replicated trial was sown at 90 × 60 cm with plot size of 164 m^2 during *kharif* 2014. Observations on per cent germination and synchrony in flowering and fruiting of the refuge non-*Bt* cotton and *Bt*-cotton were recorded. Seed packets procured in 2015–2016 demonstrated germination percentage of more than 75 and while the corresponding refuge packets demonstrated variable germination percentages with 21 refuge seed packets showing less than 75% germination (Table 1). Refuge seeds of five *Bt* hybrids recorded less than 5% germination. One of the BGII hybrid seed packets carried *Gossypium herbaceum* seeds as refuge. GEAC guidelines are not clear on the use of *Gossypium* species other than *Gossypium hirsutum* as refuge.

Of the 40 *Bt* hybrids that were assessed in the 2014 field trial, 3 hybrids were early in maturity with the date of first flower between 45 and 70 days after sowing (DAS) and were asynchronous with respective refuge. Of the 27 hybrids that recorded 55–80 DAS to first flower, 10 hybrids flowered earlier than the corresponding refuge by about 10 days and 17 hybrids flowered synchronously to their corresponding refuges. Refuge plants of 11 *Bt* hybrids that took 65–90 days for first flower were completely asynchronous with the corresponding *Bt* hybrids, with the former reaching days to first flower stage at least 20 days after the corresponding *Bt* hybrid (Table 1).

Such asynchrony clearly diminishes the value of having a refuge where the purpose is to maintain susceptibility in target pest populations. Synchrony of flowering and boll formation is important as the target pest *H. armigera* prefers to feed on squares and bolls over leaves and the pink bollworm larvae feed on developing seeds of green bolls. When cotton plants, whether *Bt* or non-*Bt*, enter the reproductive stage they serve to attract and build up moth populations which can accelerate the development of resistance in the absence of synchrony between refuge and *Bt* crops. Problem of asynchrony especially during the reproductive stage between *Bt*-cotton and corresponding refuge can be overcome through the use of isogenic lines either as structured refuge or through the use of refuge in bag.

Seed companies charge Rs 45 for 120 g of non-*Bt* refuge seed production in pricing of *Bt*-cotton seed packets[5]. It is therefore essential that they provide non-*Bt* seeds as specified on the packet in compliance with regulatory guidelines set by the competent authorities. With anomalies like these, it is clear that seed companies are violating the guidelines, thereby putting the existing concept of refuge at serious risk. On the other hand if 'refuge in bag (RIB)', a proposed concept of refuge with seed mix of 5% non-*Bt* + 95% *Bt* seed in a 475 g seed packet is approved, the farmer would not have the choice of avoiding refuge planting. In light of the current findings it is difficult to presume that seed companies may strictly adhere to the guidelines of 5% non-*Bt* + 95% *Bt* as seed mix. Further with the existing methods of testing 10 seeds per packet, monitoring the correct percentage of non-*Bt* seeds in a bag of *Bt* seeds will be difficult. Stakeholders will have to maintain high standards of ethics if RIB is permitted by the regulatory body. There is an urgent need to develop proper testing methods in the country, especially to ensure compliance and monitoring of regulatory guidelines with reference to genetically modified crops.

The existing practice of providing non-*Bt* seeds to be used as refuge is fraught with problems of seed admixture with *Bt*, either Cry1Ac and/or Cry2Ab; poor germination of non-*Bt* seeds and providing non-*G. hirsutum* species as refuge; asynchrony between the *Bt* and corresponding non-*Bt* refuge, are in contravention to the existing regulatory guidelines. It is important to strengthen seed testing and monitoring systems to ensure that regulatory guidelines are followed ethically year after year to facilitate and ensure sustainability of the *Bt* technology.

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ACKNOWLEDGEMENT. We are grateful to the GEAC Standing Committee for authorizing CICR to carry out event detection in commercial seed packets available in open market.

Received 4 June 2016; revised accepted 7 February 2017

S. KRANTHI[1,*]
U. SATIJA[1]
P. PUSADKAR[1]
RISHI KUMAR[2]
C. S. SHASTRI[1]
S. ANSARI[1]
H. B. SANTOSH[1]
D. MONGA[2]
K. R. KRANTHI[1]

[1]*ICAR-Central Institute for Cotton Research,*
Post Bag No. 2,
Shankar Nagar P.O.,
Nagpur 440 010, India
[2]*ICAR-Central Institute for Cotton Research,*
Regional Station,
Sirsa 125 055, India
**For correspondence.*
e-mail: sandhya.kranthi@gmail.com