Refuge-in-bag for *Bt* cotton

Mohan Komarlingam S.

In December 2016, the Ministry of Agriculture, Cooperation and Farmers Welfare, Government of India (GoI) in a notification endorsed the implementation of 'refuge-in-bag' (RIB) for *Bt* cotton and specified *Bt* trait purity standards and proportion of non-*Bt* refuge seeds in the blend[1]. A subsequent notification directed the *Bt* seed companies to implement RIB with isogenic refuge, wherever available, and implement a complete shift to isogenic refuge by December 2019. This is a welcome move with the clear intentions of phased replacement of structured refuge, which was never adopted by the cotton farmers, with RIB.

RIB is a method of delivering non-*Bt* refuge seeds into the field through blending (while packaging) a smaller but definite proportion of non-*Bt* refuge seeds with the larger proportion of *Bt* seeds in the same packet or bag, and hence the term 'refuge-in-bag'. A *Bt* cotton field with RIB will consist of non-*Bt* plants randomly interspersed among *Bt*-cotton plants in a pre-decided proportion, which is primarily determined by the control efficacy of the *Bt* product to the target pests. The recent notification specifies that the *Bt* trait purity of the blend should be between 90% and 95% and that of the isogenic non-*Bt* refuge seeds of the corresponding *Bt* hybrids between 5% and 10%. This notification is gazetted and is enforceable. Undeniably structured refuge is the superior resistance mitigating strategy, in the absence of which a RIB or 'seed-blend' of *Bt* and non-*Bt* seeds in a single bag is the next viable best option and would shift the onus of planting refuge from the farmer to the *Bt* seed producer.

Technically, a 'refuge' area comprising of plants not expressing the *Bt* protein(s) is an integral part of all insect-protected *Bt* crops planted to delay the evolution of resistance in insects which the *Bt* crop controls. With insect resistance management (IRM) in mind, the Genetic Engineering Approval Committee (GEAC) in 2002 approved commercial cultivation of *Bt* cotton (Bollgard®) with a requirement to plant 'refuge' crop of non-*Bt* cotton in five perimeter rows or 20% of total sown area, whichever is more. This is termed as 'structured' refuge[2,3]. In line with the GEAC stipulation, the seed companies provided 120 g of non-*Bt* cotton seeds (could be isogenic or a similar hybrid with matching phenotype and fibre characteristics) to be planted as refuge, as a separate packet, within every larger packet of 450 g of *Bt* cotton seeds. In 2006, the dual *Bt* gene cotton, Bollgard II, was approved for commercial cultivation with the same refuge stipulation as the single *Bt* gene cotton. Considering the superior bollworm control efficacy of Bollgard II® (=lower probability of resistance evolution), the size of refuge could have been reduced, thus possibly incentivizing planting of refuge. This did not happen and a possible opportunity was missed.

In fact, refuge planting has been the Achilles heel for *Bt* cotton in India since 2002. The farmers avoid planting refuge because they want to maximize cotton yield from their fields by planting *Bt* cotton even in the 20% area earmarked for refuge. Essentially the farmers were more focused on the short-term economic gains rather than worry about resistance development. Economically, it is more compelling for *Bt* cotton farmers with small land holdings or marginal lands, where the need to secure the available yield is high[4]. Also, farmers had to monitor the pest load on the refuge periodically to take insecticide spray decision so as to protect the yield in the refuge portion.

The low refuge compliance situation continued till 2009 without any issues. However, resistance evolution to the *Bt* protein, Cry1Ac expressed by Bollgard® was noticed in Gujarat in 2009 in pink bollworm (PBW) populations, one of the four key bollworms. Subsequently, resistance evolution to both *Bt* proteins, i.e. Cry1Ac and Cry2Ab was inferred when unusually high damage to Bollgard II® crop was experienced in several states in Central and South India since 2015 *kharif* season[5]. It is not surprising that *Bt* resistance was detected sequentially and specifically in PBW populations because conventional cotton is the only host (monophagous) on which PBW can feed and multiply in nature. In the absence or minimal amount of non-*Bt* cotton refuge across ~11 million ha (>95% of cotton area) and with minimal 'desi' (diploid) cotton area (1–2%), PBW populations evidently have been under high selection pressure to evolve resistance.

Low compliance on refuge planting is not unique to India, but is global. Refuge compliance issues have been a concern in USA, where farmers are obligated to plant structured refuge by an agreement. However, technology generators and the US Environmental Protection Agency quickly moved ahead and optimized a seed-blend refuge for *Bt* maize in which refuge seeds were delivered through blending of *Bt* (95%) and non-*Bt* (5%) seeds in a single bag. This method of refuge delivery was approved for *Bt* maize stacked with herbicide tolerance in USA and Canada in 2011 and Vietnam in 2014 (ref. 6).

RIB is not appropriate for single *Bt* toxin cotton because, first the refuge size would have to be large and, secondly, potential issues due to movement of *Helicoverpa armigera* and *Spodoptera litura* larvae between *Bt* and refuge plants. Several studies have shown increased movement of *Heliothis zea* larvae in RIB layout, and mathematical modelling has shown an accelerated resistance evolution in RIB (2–4.5-fold)[7].

Movement of bollworm larvae between *Bt* and non-*Bt* plants could possibly reduce the value of RIB

Movement of cotton lepidopteran larvae between *Bt* and non-*Bt* plants in an RIB format could influence *Bt* resistance evolution[8,9]. Among the four major lepidopteran pests of cotton in India, only larvae of *H. armigera* and *S. litura* move between plants; *Earias vittella*/*E. insulana* have limited mobility. PBW is the least mobile and completes its larval development within the seeds of the boll it enters. Generally, Indian *Bt* hybrids are bushy in architecture and the foliage of adjacent plants in a row and between rows often overlap during the peak bloom or boll-setting periods (65–100 days after sowing) in almost all the region-specific interplant spacing adopted.

Large larvae (≥ third instars) of *H. armigera* and *S. litura* which move from non-*Bt* refuge plant to an adjacent Bollgard II would feed and survive on the latter plants for a short time (causing some damage), before ultimately succumbing to the toxic effects of *Bt* protein(s). The toxic effect will be quicker to manifest in *Bt* stacks for a bollworm like *H. armigera*. However, such movements also decrease the efficacy of refuge by killing the bollworm larvae which would have matured into *Bt* susceptible moths, had they not moved away from the refuge plant. In the event of movement from *Bt* plant to non-*Bt* refuge plants, the larvae would escape receiving the lethal dose of *Bt* protein(s), and thus increase the survivorship of resistant heterozygotes which could hasten resistance development. This does not hold good for *H. armigera* because of high neonate mortality on Bollgard II plants due to the additive effect of two *Bt* proteins. Notwithstanding the challenges in RIB due to larval movement, the assured compliance on refuge planting and the greater availability of *Bt*-susceptible moths in closer proximity can be the overarching advantages of RIB.

Refuge seeds need to be isogenic non-*Bt* version of *Bt* hybrid

RIB would work best if the refuge seeds are isogenic non-*Bt* version of the *Bt* hybrid. First, the phenotypic and growth phenology match of refuge plants with *Bt* cotton plants would possibly prevent farmers from identifying and eliminating the non-*Bt* plants. Secondly, from an IRM angle, the synchrony in bloom would ensure equal probability of non-*Bt* plants being preferred for oviposition by gravid bollworm moths[10]. Thirdly, the farmer is assured of similar yield potential between non-*Bt* and *Bt* cotton plants, though the actual yield from refuge will be influenced by the intensity of bollworm infestation. Fourthly, the non-*Bt* plants will receive the same fertilizer and management inputs as the *Bt* crop, which eliminates asynchrony in larval growth and development, and importantly, moth emergence as influenced by nutritional status of plants. Lastly, the farmer can determine economic threshold level of insect infestation in a single exercise for the entire plot of *Bt* cotton with RIB.

The notification[1] on implementation of RIB is truly an endorsement of the importance of using isogenic form of refuge and could usher a new chapter in the sustenance of *Bt* cotton cultivation in India.

Producing isogenic non-*Bt* hybrid seeds is not without its challenges. With forward breeding being popular for *Bt* trait introgression among many *Bt* cotton seed producers in the past years, they need to retrieve the non-*Bt* inbred lines for refuge seed production. This involves time and the Ministry of Agriculture, GoI is providing the same. Another challenge is the relative high cost of non-*Bt* hybrid seed production because the crop would have to be protected from bollworms.

The biggest challenge before the *Bt* seed producers and regulators would be to maintain high refuge quality standards post the full implementation of RIB. Past experience does not lend this comfort. The last modification to the refuge guidelines was notified in 2009 when the use of non-*Bt* 'similar' hybrids, matching in phenotype and fibre characteristics, was permitted for refuge packing. This move was well intended to ease the high cost of production of non-*Bt* seeds and difficulties in the availability of non-*Bt* version of breeding lines. Unfortunately, with the passage of time these guidelines have been ignored, as indicated by a recently published study by the Central Institute for Cotton Research, Nagpur which indicated poor quality of refuge seeds packed by *Bt* cotton producers meant for structured refuge[10]. The refuge seed packets of various seed companies tested showed contamination with cotton seeds with single and dual *Bt* genes; seeds of *Gossypium herbaceum*; non-*Bt* seeds with varying germinability and phenology resulting in differences in bloom initiation, bloom period and boll setting between the refuge plants and the main *Bt* crop. From a bollworm productivity standpoint, the main function of a refuge is to provide, equivalence in plant phenotype, including matching bloom and boll-setting period to that of the *Bt* crop. These are the bedrock requirements to be fulfiled by a refuge. Any transgression from these refuge guidelines would diminish the IRM value of the refuge.

In conclusion, the Ministry of Agriculture, in a well-meaning move, has directed the *Bt* cotton seed producers to initiate implementation of RIB for *Bt* cotton, and has also defined trait and non-trait quality parameters in the seed blend. This certainly is a fresh opportunity to possibly remediate the ills on the refuge front. The seed producers have to take the onus of scrupulous implementation of RIB guidelines in the interest of sustaining *Bt* cotton technology which, in its run of 15 years, has brought immense monetary benefits to seed producers, farmers and in general, the cotton trade chain. Indian cotton farmers in particular cannot afford to let *Bt* cotton technology lose its value to resistance development in cotton bollworms. RIB provides a new opportunity to ensure refuge compliance and improve *Bt* resistance management.

1. http://www.indianemployees.com/gazette-notifications/details/notification-department-of-agriculture-cooperation-and-farmers-welfare-3087/
2. http://www.moef.nic.in/divisions/csurv/geac/decision-Feb-107pdf
3. http://www.envfor.nic.in/sites/default/files/geac/decision-may-93.pdf
4. Gujar, G. T., Kalia. V., Bunker, G. K. and Dhurua, S., *J. Asia-Pac. Entomol.*, 2010, **13**, 249–253.
5. Kranthi, K. R., *Cotton Statistics News*, 2015–16, **35**, 1–6; http://www.cicr.org.in/pdf/Kranthi_art/Pinkbollworm.pdf
6. USA; http://www.farmindustrynews.com/corn-hybids/refuge-bag-seed; Canada; https://www.agcanada.com/daily/monsantos-refuge-in-a-bag-corn-gets-cfia-approval; Vietnam; http://www.thanhniennews.com/business/vietnam-approves-commercial-crops-of-gmo-corn-to-cut-imports-40016.html; http://www.mard.gov.vn/Lists/appsp01_Newsletters/_Attachments/5/News_T5_2015.pdf
7. Brevault, T., Tabashnik, B. E. and Carrière, Y. A., *Sci. Rep.*, 2015, **5**, 9807; doi:10.1038/srep09807
8. Head, G. P. and Greenplate, J., *GM Crops Food: Biotechnol. Agric. Food Chain*, 2012, **3**(3), 144–153.
9. Carroll, M. W., Head, G. and Caprio, M., *Crop Protect.*, 2012, **38**, 74–79.
10. Kranthi, S. *et al.*, *Curr. Sci.*, 2017, **112**(10), 1992–1993.

Mohan Komarlingam S. lives at 775/c Annaippa Layout, Konena Agrahara, Vimanapura, Bengaluru 560 017, India.
e-mail: ksmohan775c@gmail.com