

SWOT analysis of refuge-in-bag for *Bt*-cotton in India

Mohan Komarlingam S. *

Growing a refuge crop is an integral part of transgenic crops with gene(s) from Bacillus thuringiensis (Bt) and a key requirement to delay evolution of Bt resistance in target pests. Poor refuge planting with Bt-cotton in India, for many years, is a major reason for the outbreak of Bt resistance in pink bollworm and consequent erosion in the efficacy of Bt-cotton technology. As a remedial measure, the Ministry of Agriculture, Government of India has directed complete shift to the marketing of Bt-cotton seeds blended with non-Bt refuge cotton seeds (refuge-in-bag; RIB) from this (2020) season onwards. The strengths, weaknesses, opportunities and threats of RIB Bt-cotton combination in the context of known behaviour of cotton lepidopteran pests and prior exposure to the same Bt toxins since 2002, is discussed. Bt-cotton with RIB, if implemented with strong stewardship, will have far-reaching benefits and is expected to delay Bt resistance development in target lepidopteran cotton pests. However, the relevance of RIB for Bt-resistant pink bollworm could be in question.

Keywords: *Bt*-cotton, lepidopteran pests, refuge-in-bag, SWOT analysis.

INDIAN *Bt*-cotton seed producers have begun blending non-*Bt* refuge seeds with their approved *Bt* (Bollgard II[®], BGII) hybrid cotton seeds for the market. This seed blend, termed as *Bt*-cotton with refuge-in-bag (RIB), was to be implemented in full by the end of 2019, according to directives from the Ministry of Agriculture and Farmers Welfare, Government of India (MoA-GoI)¹. This article analyses the strengths, weaknesses, opportunities and threats (SWOT) of RIB with *Bt*-cotton (Table 1), in the context of the existing levels of *Bt* resistance in cotton pests and known pest behaviour.

The refuge specifications for *Bt*-cotton, issued with the regulatory approval¹ in 2002 and with subsequent modifications^{2,3}, directed *Bt* seed producers to provide non-*Bt* refuge seeds (120 g) in a separate packet, placed into a larger packet of *Bt*-cotton seeds (450 g). The farmer was directed to sow the refuge seeds along the perimeter of the *Bt*-cotton, constituting $\geq 20\%$ of total *Bt* crop area. Refuge plants do not express *Bt* toxin(s) and constitute a key requirement to delay evolution of *Bt* resistance in the target cotton lepidopteran pests, viz. *Helicoverpa armigera* (Old World bollworm), *Pectinophora gossypiella* (pink bollworm; PBW), *Spodoptera litura* (tobacco caterpillar) and *Earias vittella* (spotted bollworm). However, in due course, cotton farmers overlooked planting of

refuge, primarily driven by the desire to maximize returns from a crop of 100% *Bt*-cotton. Moreover, refuge plants required insecticide sprays to protect cotton yield. Consequently, low-refuge conditions prevailed for several years when *Bt*-cotton acreage in India peaked at $>95\%$ (~ 11.5 million hectares; m ha) of total cotton area. With almost no refuge, a key cotton pest like PBW, which feeds and multiplies exclusively on seeds of non-*Bt*-cotton, evolved resistance to both *Bt* toxins of BGII cotton in quick succession in 2009 and 2014 (refs 4–6). The field-efficacy of *Bt*-cotton stood eroded.

Since refuge is a bedrock requirement for the durability of *Bt*-cotton technology, as an alternative approach to refuge planting, MoA, GoI, directed all *Bt* seed producers to switch over to blending of non-*Bt* refuge hybrid seeds, at 5–10% level, with *Bt*-cotton hybrid seeds (90–95%). This mode of compliance-assured delivery of refuge to *Bt*-cotton fields is a well-researched, globally accepted practice in many countries^{7,8}. RIB-*Bt*-cotton planting, a global first, is expected to be implemented fully in India from the 2020 season onwards. In this article, the term '*Bt*-cotton', refers to cotton hybrids expressing Cry1Ac and Cry2Ab insecticidal toxins (as in BGII), as *Bt* hybrids expressing a single *Bt* gene have been withdrawn from the Indian market since resistance was reported in PBW populations in Gujarat^{4,5}.

The efficacy of RIB in delaying the evolution of resistance in cotton pests to *Bt* toxins expressed by *Bt*-cotton crop, is largely influenced by the pest behaviour, genetics

Mohan Komarlingam S. lives at 775/C Annaipura Layout, Konena Agrahara, Vimanapura PO, Bengaluru 560 017, India.

*e-mail: ksmohan775c@gmail.com

Table 1. Strengths, weaknesses, opportunities and threats analysis of refuge-in-bag (RIB) for *Bt*-cotton in India

Strengths	Weaknesses
<ul style="list-style-type: none"> Compliance on refuge (non-<i>Bt</i>) planting is rendered almost absolute and not left to the choice of the farmer. Refuge seeds are sown automatically along with <i>Bt</i>-cotton seeds. Burden of compliance shifted from ~7.5 million cotton farmers to ~50 <i>Bt</i>-cotton seed producers. Easier to manage. Refuge plants will receive similar management care (water, fertilizers, and insecticides for control of sucking pests and bollworms) as those of <i>Bt</i>-cotton. The good nutritional status of refuge plants will ensure quality, abundance and better synchrony of emergence of <i>Bt</i>-susceptible moths. Close proximity of refuge plants to <i>Bt</i>-cotton plants will increase probability of mating of <i>Bt</i>-susceptible moths with the rare <i>Bt</i>-resistant moths from <i>Bt</i>-cotton. Ideal for pink bollworm which does not move between plants. Easier stewardship through implementation of harmonized guidelines and quality management processes in seed production, trait-quality assurance, product returns, packing and distribution network. Concerted educational effort on RIB awareness to farmers more feasible. 	<ul style="list-style-type: none"> Movement of mature larvae of mobile species like <i>Helicoverpa armigera</i> and <i>Spodoptera litura</i> across refuge and <i>Bt</i>-cotton plants can potentially undermine the value of refuge and hasten development of resistance. Specifically, movement of larvae from refuge to <i>Bt</i>-cotton plants will reduce efficacy and purpose of refuge. Movement of larvae from <i>Bt</i>-cotton plants to refuge will reduce the dose experienced by the larvae, increasing the proportion of larvae undergoing selection for resistance. Potential for increase in functional dominance in partially resistant seed-feeders like pink bollworm, <i>H. armigera</i> and <i>S. litura</i> due to feeding on a mosaic of sub-lethal doses of <i>Bt</i> toxins in the seeds of refuge bolls – a consequence of pollen-mediated trait flow from adjoining <i>Bt</i> plants. <i>Bt</i>-resistant heterozygote population could increase and undergo selection in such refuge bolls.
Opportunities	Threats
<ul style="list-style-type: none"> RIB will open another window of opportunity for the sustenance of <i>Bt</i>-cotton technology. Technology stakeholders and <i>Bt</i> seed producers could harmonize guidelines and uniformly implement RIB stewardship good practices to ensure the success of RIB-<i>Bt</i>-cotton. Institute participatory audits for upholding quality of refuge and <i>Bt</i> traits in cotton seeds. Formulate and implement robust RIB-<i>Bt</i>-cotton awareness programmes among <i>Bt</i>-cotton farmers. Guidelines for monitoring and implementation of RIB. Market <i>Bt</i>-cotton seeds with <i>Bt</i> traits in homozygous form to bolster 'high-dose' criteria and reduce <i>Bt</i> resistance risk in illegal F2-<i>Bt</i> seed market. Institute R&D support to monitor RIB implementation and its impact on <i>Bt</i> resistance evolution and technology sustenance. 	<ul style="list-style-type: none"> Poor-quality refuge non-<i>Bt</i>-cotton seeds. This can comprise of the following: (i) <i>Bt</i> traits in refuge seeds; (ii) low phenotypic and fibre quality match with <i>Bt</i>-cotton crop. This could tempt the farmer to selectively uproot the refuge; (iii) germination issues; and (iv) poor seed viability and trait quality management of repackaged seed-returns. Finding matching non-<i>Bt</i> hybrids as refuge for existing <i>Bt</i> hybrids in the market. Not meeting the criteria of season-long 'high dose' of <i>Bt</i> toxins in target tissues of <i>Bt</i> hybrids. Market availability and cultivation of F2-<i>Bt</i>-cotton seeds could upset the RIB-<i>Bt</i>-cotton model. Altered susceptibility of bollworms to <i>Bt</i> toxins expressed by <i>Bt</i>-cotton relative to the baseline values. Confirmed Bollgard II-resistant pink bollworm populations in cotton tracts of West, Central and South India. Refuge may have little relevance to these <i>Bt</i>-resistant populations, though possible fitness cost in <i>Bt</i>-resistant populations may counteract <i>Bt</i> resistance to some extent. Magnitude unknown. Potential risk of evolution of <i>Bt</i> resistance in <i>H. armigera</i>, <i>S. litura</i> and <i>Earias</i> spp. due to possible higher resistance allele frequency, relative to the period when <i>Bt</i>-cotton was launched. Poor RIB-<i>Bt</i>-cotton awareness among ~7.5 million <i>Bt</i>-cotton farmers.

of *Bt* resistance and insect pest management (IPM) practices aimed at breaking the pest cycle. When sown, RIB-*Bt*-cotton would result in refuge cotton plants randomly distributed among the *Bt*-cotton plants, constituting 5–10% of the plant population. Refuge plants enable *Bt*-susceptible bollworms to feed and multiply in large numbers, thus promoting a high probability of mating with any rare *Bt*-resistant moths (homozygous resistant to all *Bt* genes in the stack) emanating from the *Bt*-cotton crop. The progeny of such a mating would all succumb to the *Bt* toxins expressed by the *Bt* crop, provided that the *Bt* resistance is recessive and a high dose of *Bt* toxins is available in the *Bt* crop. Summing up, an effective *Bt* resistance management strategy would hinge on the availability of high dose of *Bt* toxins, which would kill all *Bt*-susceptible and almost all heterozygous resistant larvae (carrying a single copy of the gene conferring resistance) and a refuge size that would generate adequate numbers of *Bt*-susceptible moths.

Core strength of RIB-*Bt*-cotton lies in good stewardship of 'high dose' plus refuge strategy

This strategy emanated from *Bt* resistance risk assessment models and has been practised effectively in USA and Australia, where adoption of refuge is legally binding on the farmer. Essentially, a high dose comprises: (i) two or more *Bt* toxins (*Bt* stack) expressed in the target tissues of the *Bt* crop, and (ii) high efficacy of each of the toxins to the target pest species. At this juncture in India, when cotton lepidopteran pests have been continuously exposed to the *Bt* toxins Cry1Ac and Cry2Ab across a large area of *Bt*-cotton (~11.5 m ha) for more than a decade and half, an assessment of prevailing *Bt* resistance factors is recommended and would form the baseline for RIB-*Bt*-cotton, to be implemented fully this year. Specifically, we need to focus on evaluating the (i) efficacy of popular BGII hybrids to each of the bollworm species (insect-sensitivity assays and tissue expression of *Bt* toxins), and

(ii) frequencies of *Bt* resistance alleles prevailing in the populations of bollworm species.

From an Insect Resistance Management (IRM) standpoint, refuge would be the most effective when *Bt*-resistant homozygotes are extremely rare, heterozygote frequency is low and the *Bt*-susceptible population is large. This kind of ideal proportion of individuals with *Bt*-resistant alleles typically occurs during the initial years post-*Bt* product launch. Conversely, functional assortative mating would be the outcome of a large *Bt* resistance heterozygote and homozygote populations, undermining the very purpose of refuge. With more than 15 years of exposure to *Bt* toxins, we could expect significant deviations from the ideal frequencies of alleles in populations of *H. armigera*, *S. litura* and *E. vittella*, where field failures have not yet been reported but the *Bt*-resistant heterozygote population could have significantly increased. For instance, Cry1Ac resistance allele frequency in *H. armigera* populations in South India in 2013–14 (0.085/0.035) was 27–65-fold higher⁹ than 2002–03 (0.0013) when *Bt*-cotton was just introduced into India¹⁰. The prevailing frequency of Cry2Ab resistance alleles in *H. armigera* (or in any bollworm in India) is yet to be studied. It is pertinent to note that the initial frequency of these alleles in *H. armigera* populations in Australia was unusually high and continued to increase after the introduction of BGII. Although, Cry2Ab resistance was recessive, the concern is that such high frequency could possibly hasten the selection for Cry2Ab resistance in *H. armigera* populations in Australia¹¹. There is also a need to generate similar data in India for *S. litura* and *E. vittella*. Both these bollworms face a high risk of evolving *Bt* resistance because BGII contains just one effective toxin (Cry2Ab) to *S. litura* and often the large larvae (fourth/fifth instars) are exposed to possibly sub-lethal doses of Cry2Ab when they migrate from neighbouring soybean/greengram fields, on harvest or on receiving insecticidal sprays, to adjoining BGII fields. The spotted/spiny bollworms, *E. vittella* and *E. insulana* feed on cotton and okra fruits. Considering the relative acreages of BGII cotton and okra, across the country, these pests could possibly be under *Bt* selection pressure. PBW was among the most sensitive bollworms to Cry1Ac and Cry2Ab^{12,13}. With almost no structured refuge and very little acreage of conventional cotton as natural refuge, PBW evolved resistance to both *Bt* toxins of BGII cotton, sequentially^{4–6}. This pest has inflicted serious damage to BGII cotton in West (Gujarat and Maharashtra), Central (Madhya Pradesh) and South (Telangana, Andhra Pradesh, Karnataka and Tamil Nadu) India. In this scenario it is reasonable to expect large deviations from the initial Hardy Weinberg equilibrium of mutational forms with respect to composition of Cry1Ac and Cry2Ab-resistant homozygotes, heterozygotes and susceptible PBW individuals. Such endemic

areas could undermine the sustenance of high dose + RIB strategy and the relevance of RIB for PBW management could now be in question. Nonetheless, there is hope. There is increasing evidence that *Bt*-resistant PBW population levels are being brought within manageable levels through rigorous area-wide adoption of IPM measures¹⁴. The state government of Gujarat and non-governmental agencies have initiated special campaigns since 2015, to educate farmers and ginners on PBW management. Coordinated efforts by all stakeholders in the cotton value chain successfully managed PBW infestation on BGII cotton in 2017 and 2018 (ref. 15). The Gujarat model is now being followed in other states. A version of seed-mix refuge produced by cotton farmers in China, comprising 25% refuge and 75% *Bt*-cotton (Cry1Ac) in the form of F2 hybrids, when used over 11 years in China effectively reversed the Cry1Ac resistance in PBW¹⁶. However, this level of refuge and farmer-centric method of F2 production would not be practical in India. Refuge plants have been reported to impart a fitness cost on Cry1Ac-resistant PBW in life-history traits through the accumulation of gossypol in larval stages¹⁷. This feature could somewhat bolster the efficacy of RIB in managing *Bt* resistance in PBW in India. In contrast, several host crops of *H. armigera* like pigeon pea, chickpea, sunflower, tomato and maize continue to be ‘natural’ refuge for this notorious pest with a past history of resistance to multiple insecticides in the pre-*Bt* era^{18–20}. This could be one of the chief reasons for the continued efficacy of *Bt*-cotton to *H. armigera* in India.

Bt-cotton hybrids with BGII technology were approved for cultivation in 2006 and expressed two *Bt* toxins (Cry1Ac and Cry2Ab) for superior bollworm management. This technology was licensed by Mahyco Monsanto Biotech Ltd, Mumbai to more than 45 Indian cotton seed producers for introgression into parental lines, production of *Bt* hybrids and sale, post-approval by the regulators. The Department of Biotechnology, GoI had established a process for the evaluation and approval of *Bt*-cotton test hybrids produced by various seed companies based on the presence of the right *Bt* events, adequate expression of *Bt* proteins in target tissues across the cropping period and bollworm efficacy of the tissues. This process had approved more than 1000 BGII hybrids and ensured high trait-quality standards, including high bollworm control efficacy²¹. Post-*Bt* hybrid approval, upholding the trait quality standards became an internal process of the seed company till the *Bt* hybrids are replaced. The single *Bt* gene (*Cry1Ac*) Bollgard hybrids, in the years they were ruling (2002–10), expressed high concentration of Cry1Ac in the leaves for *H. armigera*²² and field failures of *Bt*-cotton due to this pest were not observed. Baseline susceptibility data have been generated for *H. armigera* and *E. vittella*^{23–26}, which have enabled post-commercialization assessment of shift in

susceptibility to Cry1Ac and Cry2Ab since 2002. Sensitivity of PBW to Cry1Ac and Cry2Ab was evaluated when culturing and bioassay methodologies were established in India, much later¹³. The *Bt* seed producers need to shoulder the onus of robust stewardship in marketing *Bt* hybrids with demonstrated high-dose capability. There should be no compromise on this key requirement.

Movement of larvae between refuge and *Bt* plants is an inherent but acceptable shortfall

Bt-cotton hybrids grown in India are generally bushy in architecture with well spread sympodial branches at 100+ days of growth. In a RIB format, given that *Bt* and refuge plants are adjacent, foliage overlap would allow movement of large larvae (third/fourth instars) of mobile lepidopteran species like *H. armigera* and *S. litura* between the refuge and *Bt* plants. This inter-plant larval movement could potentially hasten evolution of *Bt* resistance, but being an inherent feature, we have to work around it.

Like other heliothines, eggs of *H. armigera* are concentrated in the upper third of the cotton plant on tender terminals^{27,28}. The larvae feed on the terminals and young squares till late second instar and then move to larger reproductive tissues as squares, flowers and bolls. Thus, at any point, initial feeding leads to 70–100% damage to the developing fruiting forms in the upper half of cotton plant²⁹. Similarly, larvae of *S. litura* disperse throughout the cotton plant to feed on squares, flowers and bolls. Several studies indicate that intra- and inter-plant movement of heliothine larvae is significantly influenced by the presence of *Bt* toxin(s) in the cotton tissues. Larvae which ingest *Bt* toxins feed less but move significantly in space and time on *Bt*-cotton plants to avoid further feeding^{30–33}, and many migrate down the plant to soil exhibiting ‘spin down behaviour’³⁴. Based on the behaviour, we can reasonably assume that the availability of non-toxic food on refuge plants could deter/slowdown the migration of *H. armigera* larvae from refuge to *Bt*-cotton plants, thus sustaining the value of refuge plants. Lepidopteran moths do not exhibit ovipositional preference for non-*Bt* over *Bt* plants^{35–37}, however, if the refuge in RIB is a ‘similar’ hybrid and not isogenic of the *Bt* hybrid, kairomone profiles between the two genotypes could trigger altered ovipositional preference³⁸. Considering the possibility of some mature larvae of *H. armigera*/*S. litura* migrating from refuge to the adjoining *Bt* plants, they may not survive the combined toxicity of *Bt* toxins subject to the availability of high dose in the *Bt* plants²¹. However, any compromise on high dose, specifically in the squares and bolls of *Bt*-cotton would enable higher survival of mature larvae migrating from refuge, effectively increasing the dominance of *Bt* resistance in the heterozygotes and hastening evolution of resistance. In either case, there is loss of *Bt*-susceptible individuals re-

sulting in the erosion of refuge value. Migration of larvae from *Bt*-cotton to refuge is feasible if the *Bt*-cotton plants have not delivered a high dose to kill the neonates, including the resistant heterozygotes. Higher the proportion of survivors, larger would be the probability of migrating to the refuge plants for a possible recovery. This route will mean exposure to sub-lethal doses of *Bt* toxins and increasing the fitness of resistant heterozygotes.

Thus, for the success of RIB-*Bt*-cotton, good stewardship which ensures high dose of *Bt* proteins in *Bt* breeding lines, foundation/breeder/hybrids is extremely important and should be embodied in the processes used by *Bt* seed producers. Sporadic occurrences of mass migration in Central and South India of large larvae of *S. litura* across fields of soybean/green gram/cowpea, upon harvest or spray, into *Bt*-cotton fields could add to existing resistance selection pressure because lethal concentrations of *Bt* proteins for large larvae may not be available in the *Bt* plants. Among the two migrant species, the risk of *Bt* resistance evolution is relatively greater with *S. litura* because among the two *Bt* proteins (Cry1Ac, Cry2Ab2) expressed in BGII, only Cry2Ab2 is active on *S. litura* and tissue concentrations of Cry2Ab2 in BGII may not constitute a high dose for large larvae. It is highly desirable that future *Bt*-cotton stack should contain an additional lethality factor for *S. litura*. RIB in BGII was found suitable for a non-mobile species like PBW in terms of bollworm productivity of refuge and yielded undamaged cotton lint in trials³⁹. A combination of field data on larval movement and computer-simulated modelling of RIB in *Bt* corn demonstrated that interplant movement of larvae was less likely to increase the frequency of *Bt* resistance alleles through increased fitness of resistant heterozygotes. Hence RIB was a viable and durable alternative to the structured refuge with inherent farmer non-compliance issues⁴⁰.

Pollen-mediated trait flow could influence *Bt* resistance evolution

RIB for *Bt* corn increases the rate of adaption of ear-feeding pests^{41,42}. Cross-pollination by wind between paternal *Bt* plants and maternal refuge results in a mosaic of *Bt* toxin composition in the ears of corn. The issue may not be so serious in cotton because of the preponderance of self-pollination; however, close proximity and abundant overlap of vegetative branches in Indian cotton hybrids, in RIB format, do not rule out some cross-fertilization and corruption of the refuge. The proportion of refuge cotton seeds carrying *Bt* traits would be more if the *Bt* crop is homozygous for *Bt* genes, a desirable feature for high dose of *Bt* toxin in tissues (demonstrated with Cry2Ab)²¹, but a negative feature when it comes to outcrossing with refuge. Resistant heterozygote larvae

could avoid developing seeds with *Bt* toxin in the out-crossed boll of refuge and subsequently move and feed on non-*Bt* seeds. This would increase the heterozygote population, functional dominance of *Bt* resistance alleles and ultimately risk of resistance development. IRM for seed-feeders has this additional layer of challenge and could compromise the very purpose of refuge.

In conclusion, implementation of the much-awaited RIB for *Bt*-cotton from this (2020) season will provide a lifeline to the sustenance of *Bt*-cotton technology in India. The timing may not be ideal in view of the potential fallouts of prior exposure of cotton bollworms to *Bt*-cotton hybrids, but it is time for us to move forward with 5% to 10% assured refuge planting through RIB, which could delay resistance evolution in major cotton pests like *H. armigera*, *S. litura* and *Earias* spp. RIB would augment the natural refuge of these bollworms. The key to the success of RIB for *Bt*-cotton for these bollworms would lie in high-quality *Bt* trait stewardship in the production of refuge and *Bt*-cotton seeds. The onus of nurturing and sustaining RIB-*Bt*-cotton through a robust stewardship strategy rests on the shoulders of the *Bt*-cotton seed-producers and policy makers⁴³.

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