Cry toxin expression in *Bt*-cotton hybrid seeds: impact on 'refuge-in-bag' strategy for managing resistance in bollworms

A key strategy for delaying the development of resistance among cotton bollworms to the *Bt* toxins is 'refugia'. This refers to the cultivation of a small proportion of non-*Bt*-cotton alongside *Bt*-cotton, in order to maintain a considerable population of bollworm adults that are not selected for by the *Bt* toxins[1–3]. The unselected population of bollworm adults, by means of mating with, if any, a small number of selected individuals, is expected to reduce the rate of resistance development[4,5]. Thus, with the purpose of implementing the refugia strategy, farmers purchasing *Bt*-cotton seeds have been provided with a prescribed quantity of non-*Bt* seeds for simultaneous planting. However, Indian cotton farmers, most of whom are small or marginal, have barely taken the risk of planting non-*Bt* seeds, thus leading to gross non-compliance of the prescribed refuge requirement. This non-compliance has been attributed to the development of resistance recorded among the bollworms against the *Bt* toxins[6–8].

Aiming at enforcing compliance of the refugia strategy, the Government of India has permitted purposeful mixing of non-*Bt*-cotton seeds, along with *Bt*-cotton seeds in the same commercial seed bag, which is called refuge-in-bag, or RIB. The Government has stipulated that non-*Bt* seeds may range from 5% to 10% of the total seeds in a bag[9], with a condition that the trait purity does not go below the currently prescribed level of 90%. This produces two scenarios that need to be tested before implementing the RIB. First, purposeful mixing of *Bt* and non-*Bt* seeds might not be needed if the proportion of non-*Bt* seeds in the original hybrid seed lot is in excess of 5%. This is because all cultivated *Bt*-cotton crops in India are hybrids[10], not varieties, which makes it likely to have a certain proportion of trait-impure seeds in a lot. Second, the trait purity among hybrids with Bollgard-II® technology (BG-II; containing both *Cry1Ac* and *Cry2Ab* genes) is affected not only by non-*Bt* seeds, but also by those containing only one of the two *Bt* genes, which would limit the quantity of non-*Bt* seeds that could be purposefully added to the bag. Incidentally, almost all of the *Bt*-cotton seeds that are commercially sold in India are BG-II hybrids. These scenarios make it necessary to quantify the proportion of seeds with one, or no, *Bt* genes in the commercial seed bags before implementing RIB.

Seventy-eight commercially sold BG-II seed bags were purchased in the open markets of Maharashtra, Andhra Pradesh and Karnataka. These bags represented 25 hybrids produced by 15 different companies. Each seed bag contained 450 g of *Bt*-cotton seeds from which 90 seeds were sampled ($N = 7020$ seeds). These seeds were soaked overnight before separating the endosperm from the seed coat. The endosperms were individually subjected to ELISA to determine the presence of Cry1Ac and Cry2Ab toxins (the ELISA protocol followed was the same as described by Dohare and Tank)[11]. Data were maintained bag-wise. The ELISA kit was obtained from Amar Immuno-Diagnostics Pvt. Ltd., Hyderabad, India.

Of the 7020 seeds tested for the presence of the two *Bt* toxins, the results showed that 6512 (92.76%) seeds had both Cry1Ac and Cry2Ab toxins, 74 (1.05%) seeds contained only Cry1Ac, 71 (1.01%) seeds contained only Cry2Ab and the remaining 363 (5.17%) seeds did not contain both toxins in them. The extent of trait purity (~93%) was within the permitted limits, and, interestingly, the proportion of non-*Bt* seeds in a bag met the minimum refuge size (5%) stipulated for BG-II under RIB. This suggests that RIB has been in place prior to its official implementation. The minimum trait purity prescribed by the Government, notwithstanding the implementation of RIB, allows up to 10% non-*Bt* seeds to be present in a bag. Considering that the trait impurity was in excess of 7%, adding another 5% non-*Bt* seeds would actually exceed the stipulated trait purity limits.

The development of resistance in bollworms against *Bt* toxins has been largely attributed to the non-compliance of the refugia strategy[6–8]. This study shows that farmers have been unknowingly planting non-*Bt* seeds along with *Bt* seeds in the desired proportions. Since seed companies have been following similar seed production, processing and quality assurance methods from the start of the commercialization of transgenic *Bt*-cotton in India, it is likely that the planting of non-*Bt* seeds to the extent of about 5% has been happening ever since the introduction of BG-II hybrids in India. In spite of this, the pink bollworm developed resistance to both the Cry toxins and devastated large areas of cotton across the country. Therefore, it might not be true that 5% non-*Bt* plants could slow down the rate of resistance development in bollworms. On the contrary, if this was actually true, then the act of the farmers to deliberately reject planting non-*Bt* seeds as refuge may not be the major reason for the development of resistance. There might be a need to look for reasons beyond 'refuge compliance'.

Broadly, it appears that there might not be any need for deliberate addition of 5% non-*Bt* seeds to the bags. However, the situation is different at the scale of individual bags. The study reveals a worrisome extent of variation in the trait purity and the presence of non-*Bt* seeds in a bag. Trait purity was as low as 61% in seed bags (Figure 1). There were 31 bags with <5% non-*Bt* seeds, 40 bags with 5–10% and 7 bags with >10% non-*Bt* seeds. These results call for a stricter implementation of the regulations to ensure that not only is the trait purity maintained, but also the extent of non-*Bt* seeds in a bag. As a result, it appears that companies need to ensure stringency in their seed production and seed quality assurance programmes, such that the extent of trait purity is greater than 95%. It is only under this condition that an additional 5% non-*Bt* seeds can be added to the bags.

In 2012, it was suggested in this journal that efforts may be made to include the 5% non-*Bt* seeds within the stipulated window of trait purity (i.e. 10%)[12], which has been the case in the Government's notification on RIB. While stipulating that the trait purity shall not fall below 10%, the notification maintains that the deliberately added

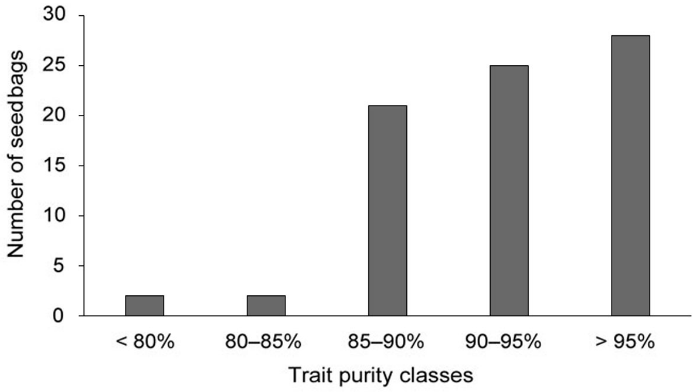

Figure 1. Frequency distribution of *Bt*-cotton seed bags with respect to the extent of trait purity. Trait purity refers to the proportion of seeds producing both Cry1Ac and Cry2Ab toxins. There were nine bags with 100% of the sampled seeds ($n = 90$) returning positive values for the two toxins. The least trait purity recorded was 61.11%.

non-*Bt* seeds shall be comparable with the *Bt* hybrid in its overall performance. However, the other potential problems expected to arise from the implementation of RIB need to be considered during implementation. As the *Bt* and non-*Bt* seeds in a bag shall be indistinguishable, and considering that this correspondence shows that RIB might not be effective in managing the development of resistance, the potential socio-economic issues arising out of the deliberate mixing of seeds have to be addressed.

1. Gould, F., *Annu. Rev. Entomol*., 1998, **43**, 701–726.
2. Tabashnik, B. E., Gassmann, A. J., Crowder, D. W. and Carrière, Y., *Nat. Biotechnol*., 2008, **26**, 199–202.
3. Tabashnik, B. E., *Proc. Natl. Acad. Sci. USA*, 2008, **105**, 19029–19030.
4. Huang, F., Andow, D. A. and Buschman, L. L., *Entomol. Exp. Appl*., 2011, **141**, 1–16.
5. Xiao, Y. and Wu, K., *Philos. T. R. Soc. B.*, 2019, **374**.
6. Dennehy, T. J., Head, G. P., Moar, W., Greenplate, J. T., Mohan, K. S., Ravi, K. C. and Parimi, S., Proceedings of the Beltwide Cotton Conference, Atlanta, GA, USA, 4–7 January 2011, p. 1063.
7. Fabrick, J. A. *et al*., *Sci. Rep*., 2015, **12**.
8. Mohan, K. S., Ravi, K. C., Suresh, P. J., Sumerford, D. and Head, G. P., *Pest Manage. Sci*., 2016, **72**, 738–746.
9. Gazette notification, 27th December 2016, Ministry of Agriculture and Farmers Welfare, Government of India. https://seednet.gov.in/SeedGO/2016/173-355_2016.pdf (last accessed on 28 February 2020).
10. Kranthi, K. R., *Cotton Statistics and News*, 2015, pp. 1–6; http://www.cicr.org.in/pdf/pop_art/Pinkbollworm.pdf (last accessed on 28 February 2020).
11. Dohare, A. and Tank, S. K., *J. Exp. Biol. Agri. Sci*., 2014, **2**(1), 43–48.
12. Muralimohan, K. and Srinivasa, Y. B., *Curr. Sci*., 2012, **102**(9), 1243.

Received 5 March 2020; revised accepted 10 April 2020

K. MURALIMOHAN*
H. M. MAHESH

Department of Entomology,
College of Agriculture,
University of Agricultural Sciences,
GKVK Campus,
Bengaluru 560 065, India
**For correspondence.*
e-mail: entomurali@gmail.com