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Evaluation of Non-Bt Refugia Cultivation to Manage Bt Resistance in *Pectinophora gossypiella* Against Transgenic Cotton

Mahreen Hanif[1], Shafqat Saeed[1]*, Mudssar Ali[1], Muhammad Ishtiaq[1] and Zulqurnain Khan[2]

[1]*Institute of Plant Protection, MNS University of Agriculture, Multan 60000, Pakistan.*
[2]*Institute of Plant Breeding and Biotechnology, MNS University of Agriculture, Multan-60000, Pakistan.*

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MH performed the experiments, analyzed the data, and wrote the manuscript. SS supported the planning of the experiments. MA, MI and ZK assisted in planning the experiment and reviewed the article.

Key words
Boll infestation, Delaying resistance, Non-Bt refugia, *Pectinophora gossypiella*, Seed mixture, Transgenic cotton

ABSTRACT

Transgenic cotton, specifically Bt cotton, has revolutionized pest management in the USA and China, controlling pink bollworm populations. In India, practical resistance has emerged due to the widespread cultivation of Bt cotton, accelerating the development of resistance in *Pectinophora gossypiella* (Saunders). Scientists recommend the refuge strategy as an effective insect resistance management approach. The current study was conducted to evaluate the cultivation of refuge (20% non-Bt) in different layouts, including row (Ro), border (Bo), block (BL), and seed mix (SM) with transgenic cotton. The results showed that *P. gossypiella* incidence significantly decreased in treatments with refuge cultivation compared to treatments without refuge. The average range of infestation to flowers, green bolls, and open bolls in treatments with refuge was 1-1.40%, 4-68%, and 9-22%, respectively, compared to treatments without refuge. The infestation remained less in July and August in both cultivars than in September and October. But statistically, Bt was found more resistant as compared to non-Bt. The yield (Kg per ha) in treatments without refuge was lower and declined in the following year compared to the refuge treatments. These findings indicate that refugia treatment resulted in the lowest *P. gossypiella* infection in treatments (i.e. ROBt, BOBt, BLBt, and SMBt) having refuge compared to non-refugia plants. This highlights the need for refuges in insect/Bt agricultural systems due to specific insect biology requirements. Utilizing refuge strategies is a promising method for controlling *P. gossypiella* infestation in transgenic cotton and ensuring sustainable pest management practices.

INTRODUCTION

Transgenic cotton, *Gossypium hirsutum* L. (Malvaceae) has an insecticidal toxin (Cry1Ac protein) derived from soil-dwelling bacteria, *Bacillus thuringiensis* (B.) that targeted lepidopteran pests (Shelton *et al.*, 2002; Wu and Guo, 2004, 2005). It is a safe, effective, and more specific insect management tool than conventional insecticides which has given rise to the evolution of resistance in bollworms (Forrester *et al.*, 1993; McCaffery, 1998; Kranthi *et al.*, 2002; Shelton *et al.*, 2002; Wu and Guo, 2004; Walsh *et al.*, 2022). Transgenic cotton cultivation decreased insecticide use along with the management of targeted pests, increased biodiversity, lowered production cost, and improved yield by indirectly maximizing the farmer's profits (Chen *et al.*, 2005; Hutchison *et al.*, 2010; Lu *et al.*, 2012; Abedullah *et al.*, 2015; Romeis *et al.*, 2019; Dively *et al.*, 2021). It was cultivated first in the USA in 1996 and then in other countries including China, Australia, Argentina, Mexico, Colombia, India, South Africa, Brazil (James, 2006), and Pakistan in 2010. At initial, transgenic cotton was effective against american bollworm, armyworm, and PBW but later PBW adopted resistance against bollgard I due to intensive and area-wide cultivation of transgenic cotton (Liu *et al.*, 1999; Kranthi *et al.*, 2006; Tabashnik *et al.*, 2008).

Pectinophora gossypiella S. (Gelechiidae) is a monophagous pest than other bollworms, that's why PBWs start to resist transgenic Bt cotton. It is a very destructive cotton pest (Sarwar, 2017) and causes a 20-30% loss of seed cotton yield (Ahmed *et al.*, 2005; Fand *et al.*, 2019). In Pakistan, one million bales of cotton worth approximately US $ 14 billion, are affected each year by PBW (*Pectinophora gossypiella*). In the USA and China,

* Corresponding author: shafqat.saeed@mnsuam.edu.pk
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PBW has evolved resistance in the laboratory-tested progeny of many strains (Tabashnik *et al*., 2010; Wan *et al*., 2012, 2017). The PBW has also developed resistance in India (Tabashnik *et al*., 2010; Fabrick *et al*., 2014; Ojha *et al*., 2014). The PBW infestation on transgenic cotton was also reported in Pakistan (Abbas *et al*., 2016; Akhtar *et al*., 2016). Resistance may evolve as a consequence of behavioral changes, detoxification, and maturation processes (Onstad and Knolhoff, 2023).

Pyramided Bt cotton cultivars having multiple Bt toxins (i.e., Cry or Vip) targeting the same pest have been commercially planted in the United States, China, Australia, and India (Downes and Mahon, 2012; Matten *et al*., 2012; Brévault *et al*., 2013; Yang *et al*., 2013a). Relative to single gene cultivars, these pyramided Bt cotton cultivars are observed more effective against target pests and delaying the development of resistance (Roush, 1998; Zhao *et al*., 2003; Ives *et al*., 2011; Yang *et al*., 2013a, b) because of their different ways of toxicity (Monsanto, 2012). However, due to the extensive cultivation of these single and double-gene Bt cultivars, asymmetrical resistance was observed in PBWs (Tabashnik *et al*., 2009). In India and the USA, the incidence of PBWs was recorded on gene pyramiding Bt cotton having genes *Cry1Ac* and *Cry2Ab* (Kranthi, 2015; Mathew *et al*., 2018; Naik *et al*., 2018; Fabrick *et al*., 2023; Tabashnik *et al*., 2023) that showed that multi-toxin resistance permits the survival of PBWs (Fabrick *et al*., 2015).

Another reason is the cultivation of transgenic cotton 100% without following any proper refuge system recommended by Monsanto company (as the sowing of non-Bt cotton 20% without use of any microbial spray or sowing of 5% non-Bt cotton without the use of any insecticidal spray). One of the keystones of insect resistance management (IRM) for transgenic cotton is the deployment of refuge to produce a susceptible target insect population (Tabashnik *et al*., 2005; Wan *et al*., 2012; Jin *et al*., 2015). Refuge cultivation limits the selection pressure on the target pest, dilutes the resistant population that resists the transgenic cotton, and improves the life span of Bt crops (Onstad *et al*., 2011; Grettenberger and Tooker, 2015; Jin *et al*., 2015). In Pakistan, cultivation of 10% refuge (non-Bt) was recommended by the Agriculture Department of Punjab (cotton production technology manual) in 2019-2020 but not practiced, and the highest outbreak of *P. gossypiella* was recorded on Bt cotton (Agriculture Department Government of Punjab, 2020). The current study aimed to assess the impact of a refuge strategy (20%) with Bt cotton against *P. gossypiella.* Different methods of refuge, including row, border, block, and seed mix, were evaluated to determine an effective method of refuge cultivation. Refuge cultivation could be an excellent source for managing *P. gossypiella* outbreaks in transgenic cotton plants.

MATERIALS AND METHODS

Collection of cotton cultivars

Experiments were conducted using cotton cultivars Tassco-115 (Cry1Ac single gene Bt cultivar; PC-1910) and CRIS-644 (non-Bt; PC-1998), which were obtained from the Pakistan Central Cotton Committee (PCCC) (Fig. 1). This selection is made due to their genetic purity (98 to 100%).

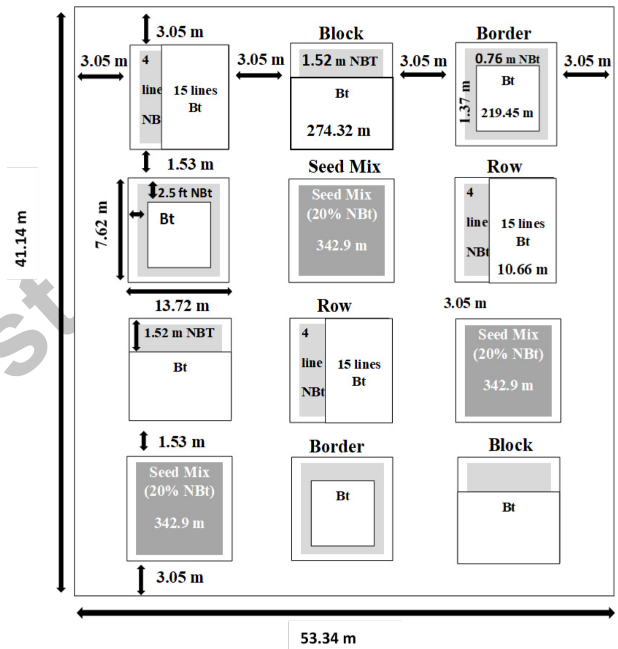

Fig. 1. Layout of Refuge field trials (four treatments and three replications) grown in field test plots in Multan, Pakistan in 2020 and 2021. Treatments include RO (Row), BO (Border), BL (Block), and SM (Seed Mix) with three replications.

Experimental design

The field trials were executed in 2020 and 2021 at the MNS University of Agriculture Multan (071.513°E and 30.255°N) using a split-plot design (recognized for its suitability in agricultural trials involving multiple treatment variables). The tassco 115 (Bt) was intercropped with 20% CRIS-644 (non-Bt) in different layouts such as row (RO), boarder (BO), block (BL), and seed mixed (SM). Each treatment was replicated three times across the experimental field (Fig. 1). Randomization was implemented using a random number generator, ensuring unbiased allocation of plots and mitigating confounding variables. This design allows for robust comparisons across refuge layouts, providing insights into their effectiveness in managing Bt resistance.

Each refuge plot encompas sed a total area of 342.9 m with a dimension of 7.62 m wide and 13.72 m long with a row spacing of 0.76 m. In row cultivation, plots had the same dimensions i.e. 7.62 m ×13.72 m (wide ×long), and had 4 lines for non-Bt plants 3.05 m and 15 lines for Bt plants 10.66 m. In border cultivation, plots had non-Bt plant areas 0.76 m long and 1.37 m wide side of the plot, although the Bt plant area was 219.45 m in the middle of the non-Bt border. In block cultivation, the non-Bt area was 1.53 m, and the Bt plot area was 274.32 m. For seed mix, non-Bt seed (20% of the total seed weight cultivated / plot) was mixed with Bt seed, and the plot area was 342.9 m. The distances between treatments and replications were purposefully kept at 1.52 m and 3.05 m, respectively, without any planted cotton. A separate Bt plot area without refuge (as control) is cultivated almost 1 hectare from the refuge field (Fig. 1).

Cotton seeds were pre-treated with a mixture of azoxystrobin (Dynasty 125FC @ 3 ml/kg, Syngenta) and thiamethoxam (Cruiser 350FS @ 6 ml/kg, Syngenta) one day before planting. During seedbed preparation, a single treatment of 1 bag per acre (50 kg) of diammonium phosphate (DAP) fertilizer was carried out using a tractor-powered bed shaper. During the whole growing season of the field tests, no additional fertilizer or insecticide was applied.

Sampling and data collection

PBW infestation was assessed at three different crop stages: flowers, green bolls, and open bolls. Sampling locations within each treatment plot were randomly selected using predefined intervals to ensure representativeness and eliminate potential bias. Infested and healthy plant parts were recorded, and infestation percentages were calculated. These sampling methods align with best practices in pest management studies, ensuring data reliability and accuracy.

PBW infestation in cotton flowers

PBW infestation (PBW larvae or its excreta) in flowers was recorded at the onset of flowering. The flowers don't open and appear rosette with PBW larvae or its excreta is considered as rosette flowers. In all treatments, nine locations were selected randomly from each plot (Bt and non-Bt cotton plants) by following the method of Shrilakshmi and Udikeri (2021) with some modifications. The total number of infested rosette flowers (with PBW and infestation) and healthy flowers (fully bloomed) was calculated for three consecutive plants per site weekly. The mean number of rosette flowers per site was calculated. The PBW infestation (%) of rosette flowers was recorded and measured by using the following formula (Shrilakshmi and Udikeri, 2021):

Flower infestation (%) = (Infested/Rosette flower)/(Total flower observed) ×100 …(1)

PBW infestation in green bolls

Samples of unopened green bolls (n = 25) from all Bt and non-Bt genotype treatments were randomly collected monthly until harvesting to record PBW infestation (%) by following the method of Shrilakshmi and Udikeri (2021) with some modifications. The collected bolls were placed separately (to avoid mixing) in a basket on the table, under controlled conditions (25±2 °C, 60-80% RH) in the laboratory. After four days, a white paper was placed on the table and the bolls were dissected with the help of a knife to observe the presence of PBW larvae (from 1st instar to 4th instar) in the bolls considered as infested boll. The PBW larvae have four growth stages or instars, first two instars of PBW larvae are creamy-white with black heads while the third and fourth instars have a pinkish color. The data was recorded monthly. The calculation of the infestation percentage was performed using the following equation (Shrilakshmi and Udikeri, 2021):

$$\text{Green boll infestation (\%)} = \frac{\text{Infested bolls}}{\text{Total bolls collected}} \times 100 \quad ...(2)$$

Open boll infestation per plant

Before cotton picking, five plants were randomly selected from all treatments by following the method of Ingole *et al.* (2019) with some modifications. All open bolls from each selected plant (Bt and non-Bt plants per treatment per replication) were checked to determine the open boll infestation (%) in the field. The open bolls having PBW larvae in the seed or bolls or lint damage of PBW as dark brown color considered as damaged open boll as compared to healthy have no larvae or its infestation damage. The average number of infested bolls from five plants (Bt and non-Bt plants) was estimated to measure open boll infestation per plant. PBW infestation (%) of the open bolls was assessed using the following formula:

$$\text{Open boll infestation (\%)} = \frac{\text{Infested open bolls}}{\text{Total open bolls}} \times 100 \quad ...(3)$$

Seed cotton yield

Seed cotton is unginned cotton that contains both seed and lint. From all treatments, the total yield of seed cotton (from Bt and non-Bt plants) was noted in (kilograms) kg after harvesting each year in 2020 and 2021.

Statistical analysis

One-way Analysis of variance (ANOVA) was used to analyze the infestation percentage of rosette flowers, green bolls, and open bolls per plant in all treatments (such as row (RO), boarder (BO), block (BL), and seed mixed (SM)) in 2020 and 2021. Tukey's honestly significant difference test (Tukey's HSD) was used to compare the means (Statistics 8.1 software). Similarly, data on seed cotton yield were analyzed using ANOVA and Tukey's HSD.

RESULTS

PBW infestation to rosette flowers, green bolls, and open bolls is described below on different layouts of refuge at different plant growth stages (i.e., 60 to 130 days).

PBW flower infestation of the non-Bt and Bt cultivars

The incidence of PBW flower infestation (%) to non-Bt plants from all treatments (RONBt, BONBt, BLNBt) was started in September, and observed non-significant in September 2020, September 2021, October 2020, and October 2021 as shown in Figure 2A. The average range of PBW flower infestation to non-Bt plants used as refuge was noted from 0.5 to 4% in 2020 and 1 to 7% in 2021 as compared to non-Bt cotton plants of other treatments (Fig. 2A).

PBW infestation to cotton flowers was checked from Bt plants from all treatments from July to October 2020 and 2021. The incidence of PBWs (%) on flowers of the Bt variety without no refuge (COBt used as control) started in August and ended in October as compared to the Bt variety with refuge that started from September and ended in October during 2020 and 2021 (Fig. 2B). PBW infestation (%) on all treatments with refuge was detected highly significant in September 2020 ($F_{4,8}$ = 35.16; P < 0.001) and 2021 ($F_{4,8}$ = 963.18; P < 0.001). In October 2021, a highly significant difference was noted in PBW infestation among treatments ($F_{4,8}$ = 166.08; P < 0.001) than in October 2020 (Fig. 2B).

The data of PBW flower infestation (%) on all treatments with refuge and treatment without refuge (Fig. 2A, B) indicated that higher infestation was observed on non-Bt variety (used as a refuge) than Bt variety (having refuge) of all treatments. The same PBW flower infestation pattern was detected from August to October during 2020 and 2021 on Bt plants (Fig. 2B) and non-Bt plants (Fig. 2A).

The lowest PBW flower infestation (%) was observed in RO cultivation (non-bt) as compared to the other three methods of cultivation in 2020 (Fig. 2B). While in 2021, the lowest PBW infestation was observed in SM. In RO cultivation, the infestation of PBW already remains similar in 2021 while its infestation was minimal in seed mix cultivation.

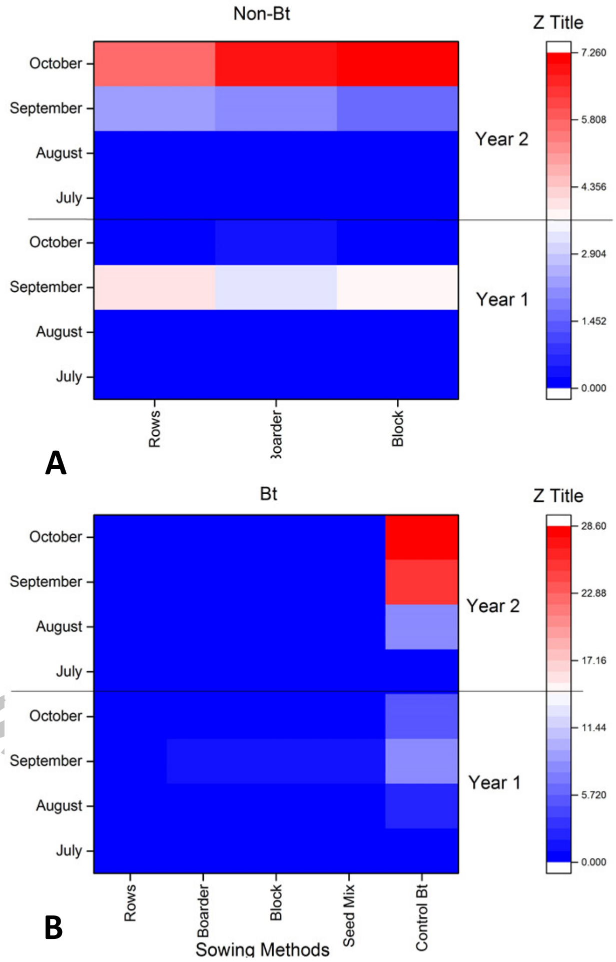

Fig. 2. Heat map of PBW flower infestation (%) to Bt and non-Bt plants in different layouts of refuge field trials from 2020 and 2021. (A) Treatments include RONBt (Row; non-Bt cotton plants), BONBt (Border; non-Bt cotton plant), and BLNBt (Block; non-Bt cotton plants). Error bars show standard errors about the means the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa. (B) Treatments include ROBt (Row; Bt cotton plants), BOBt (Border; Bt cotton plant), BLBt (Block; Bt cotton plants), SM (Seed Mix), and COBt (Control; Bt cotton plants). Error bars show standard errors about the means and the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa.

PBW infestation (%) of green bolls of non-Bt and Bt cultivars

The incidence of PBW to green bolls of non-Bt plants of all treatments was recorded as non-significant by august 2020 and 2021, september 2020, and october 2020 and 2021. However, a little bit significant difference was observed in september ($F_{2,4}$ = 18.25; P = 0.00) in 2021. The highest green boll damage was detected on non-Bt plants of all tested treatments than Bt plant treatment used as a refuge during 2020 and 2021 (Fig. 3A).

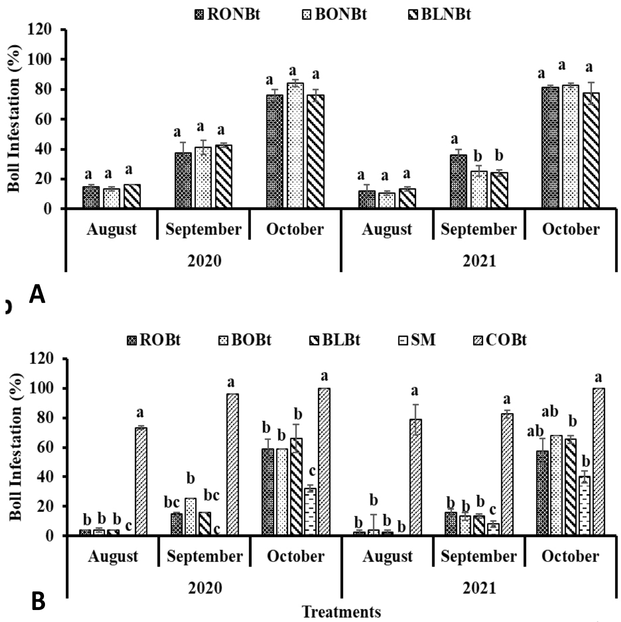

Fig. 3. PBW green bolls infestation (%) to Bt and non-Bt plants in different layouts of refuge field trials from 2020 and 2021. (A) Treatments include RONBt (Row; non-Bt cotton plants), BONBt (Border; non-Bt cotton plant), and BLNBt (Block; non-Bt cotton plants). Error bars show standard errors about the means the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa. (B) Treatments include ROBt (Row; Bt cotton plants), BOBt (Border; Bt cotton Plant), BLBt (Block; Bt cotton plants), SM (Seed Mix), and COBt (Control; Bt cotton plants). Error bars show standard errors about the means and the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa.

The PBW infestation to green bolls on Bt plants of all treatments with refuge and without refuge (COBt) was noted during 2020 and 2021 (Fig. 3B). Its infestation on Bt plants of all treatments was recorded highly significant in August ($F_{4,8}$ = 2791.00; P < 0.001), September (F= 75.02; df = 4, 8; P < 0.001) and October ($F_{4,8}$ = 21.64; P < 0.001) during 2020. Similarly, a highly significant difference was also noted on Bt plants of all treatments across the season (August (F = 52.12; df = 4, 8; P < 0.001), September ($F_{4,8}$ = 1010.91; P < 0.001) and October ($F_{4,8}$ = 9.53; P = 0.003)) in 20201. During 2020 and 2021, maximum boll infestation was recorded on treatment without refuge (COBt) than on treatments with refuge (Fig. 3B).

The PBW green bolls infestation (%) was observed highest from august to October in COBT having no refuge than the other four treatments having refuge in both years (Fig. 3B). In September and October, a similar infestation rate was observed among RO, BO, and BL as compared to SM.

Presence of PBW larvae in green bolls

The presence of PBW larvae in green bolls of non-Bt cotton plants on all investigated treatments was recorded non-significant in August, September and October during 2020 as well as in August, and October than September ($F_{2,4}$ = 18.25; P = 0.00) during 2021 (Fig. 4A). The average number of larvae per boll was same and highest on non-BT plants of all treatment used as a refuge than Bt plants.

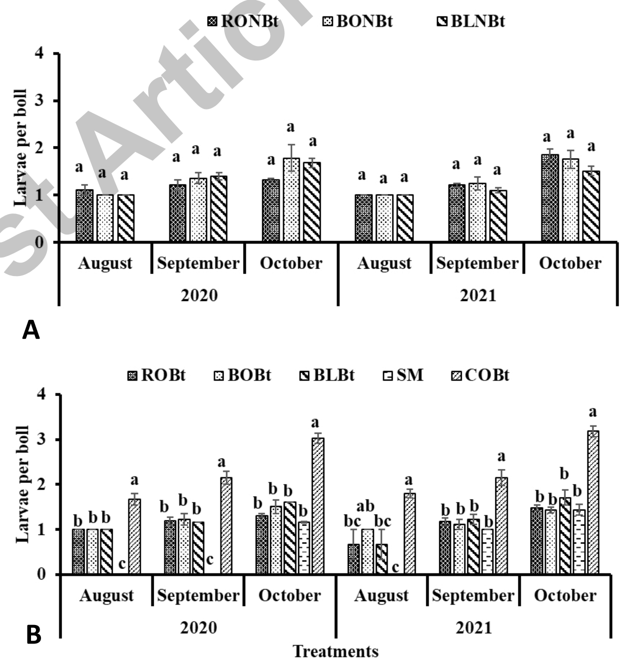

Fig. 4. Green boll infestation (%) by PBW larvae to Bt and non-Bt plants in different layouts of refuge field trials from 2020 and 2021. (A) Treatments include RONBt (Row; non-Bt cotton plants), BONBt (Border; non-Bt cotton plant), and BLNBt (Block; non-Bt cotton plants). Error bars show standard errors about the means the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa. (B) Treatments include ROBt (Row; Bt cotton plants), BOBt (Border; Bt cotton plant), BLBt (Block; Bt cotton plants), SM (Seed Mix), and COBt (Control; Bt cotton plants). Error bars show standard errors about the means and the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa.

The larval presence of PBW from picked green bolls of Bt plants among treatments with refuge and without refuge (COBt) were detected during 2020 and 2021 (Fig. 4B). A highly significant occurrence of larvae/green bolls of Bt plants was detected on all tested treatments from August to October 2020 (August ($F_{4,8}$ = 95.91; P < 0.001), September ($F_{4,8}$ = 81.05; P < 0.001) and October ($F_{4,8}$ = 24.30; P < 0.001)) as well as in August ($F_{4,8}$ = 15.09; P < 0.001), September ($F_{4,8}$ = 17.74; P < 0.001), and October ($F_{4,8}$ = 28.79; P < 0.001) during 2021 (Fig. 4B). The average of larvae per boll was ranged from 0.67-3.18 larvae/boll and lowest on all treatment with refuge and treatment without refuge (COBt).

The PBW population per boll was observed rising from august to October in COBT having no refuge than the other four treatments having refuge in both years (Fig. 4B). In august, september, and october, a similar infestation rate was observed among RO, BO, and BL as compared to SM.

Open boll infestation per plant on Bt and non-Bt cultivars

PBW open bolls infestation to non-Bt plants of all treatments with refuge was observed non-significantly different in 2020 as well as 2021 (Fig. 5A). Open bolls damage was recorded highest on non-Bt plants of treatments with refuge than Bt plant treatment with refuge both years from 2020 to 2021 (Fig. 5A, B).

Presented data (Fig. 5B) showed that PBW infestation to open bolls on Bt plants of all investigated treatments were recorded highly significantly different during 2020 ($F_{4,8}$ = 11.98; P < 0.001), and 2021 ($F_{4,8}$ = 11.70; P < 0.001). Maximum open boll damage was detected on the Bt plant of treatment without no refuge (COBt) compared to all Bt plants treated with refuge (Fig. 5B).

The PBW open boll infestation (%) was observed maximum in COBT having no refuge than the other four treatments having refuge in both years (Fig. 5B). A similar infestation rate was observed among RO, BO, BL, and SM.

Seed cotton yield

Seed cotton is unginned cotton that contains both the seed and lint. The yield data of seed cotton (kg per ha) from all tested treatments, that is, RONBt, BONBt, BLNBt, and CONBt, revealed non-significant differences among all treatments in 2020 and 2021 (Fig. 6A). Similarly, significant differences were found among all treatments (as ROBt, BOBt, BLBt, SM having refuge, and COBt without refuge) in 2020 ($F_{4,10}$ = 4.84; P = *0.01*) and 2021 ($F_{4,10}$ = 8.04; P =*0.00*) (Fig. 6B).

In 2020, the yield was highest for ROBt (1486 kg per ha), followed by BLBt (1453 kg per ha), BOBt (1157 kg per ha), SM (1165 kg per ha), and COBt (684 kg per ha). Similarly, the yield was the highest in ROBt (1279 kg per ha), followed by BOBt (1542 kg per ha), BLBt (1453 kg per ha), SM (1476 kg per ha), and COBt (725 kg per ha) in 2021. These results indicate that yield increased yearly in all tested treatments with refuge compared to the Bt plot with no refuge.

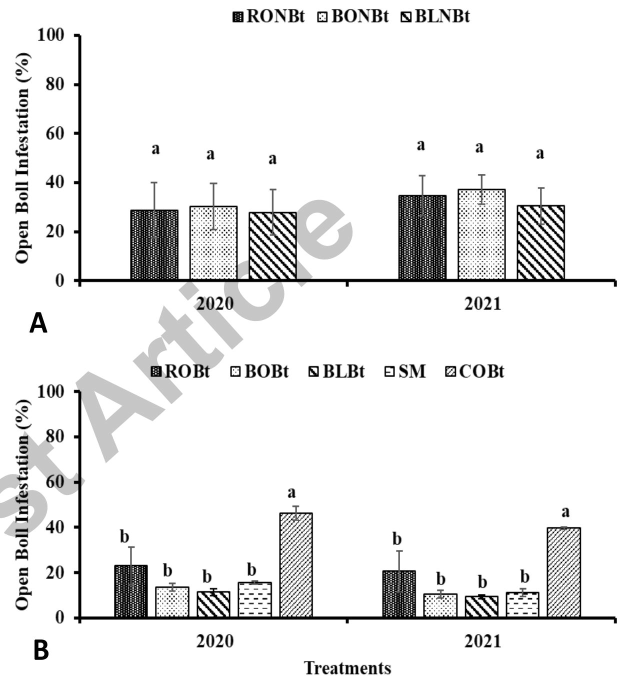

Fig. 5. PBW open boll infestation (%) to Bt and non-Bt plants in different layouts of refuge field trials from 2020 and 2021. (A) Treatments include RONBt (Row; non-Bt cotton Plants), BONBt (Border; non-Bt cotton Plant), and BLNBt (Block; non-Bt cotton Plants). Error bars show standard errors about the means the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa. (B) Treatments include ROBt (Row; Bt cotton Plants), BOBt (Border; Bt cotton Plant), BLBt (Block; Bt cotton Plants), SM (Seed Mix), and COBt (Control; Bt cotton Plants). Error bars show standard errors about the means and the different letters indicate significant differences among the different groups of samples (P < 0.05, HSD test) and vice versa.

The yield was observed maximum in all tested four treatments having refuge in both years as compared to COBT having no refuge. A statistically significant difference was observed among all treatments (i.e., RO, BO, BL, and SM) in 2020 and 2021 as the lowest yield was observed in COBt than other four treatments (Fig. 6B).

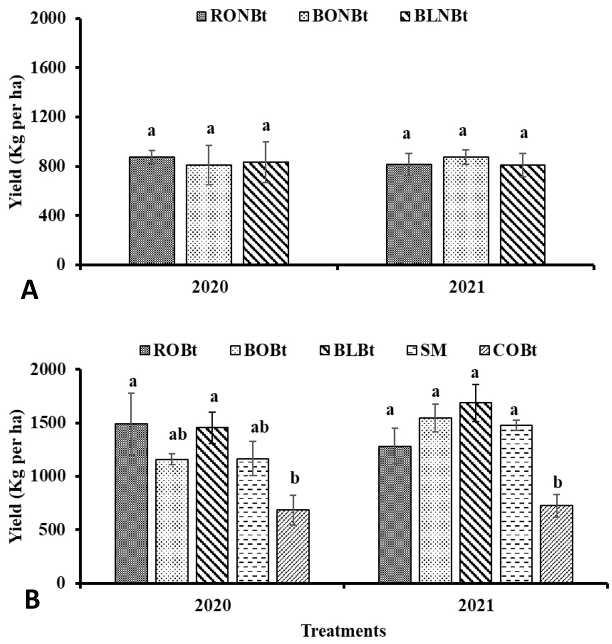

Fig. 6. Post-harvest mean cotton seed yield (kg per ha) in different layouts of refuge field trials from 2020 and 2021. (A) Treatments include RONBt (Row; non-Bt cotton Plants), BONBt (Border; non-Bt cotton Plant), and BLNBt (Block; non-Bt cotton Plants). Error bars show standard errors about the means the same letters indicate non-significant differences among the different groups of samples and vice versa (P > 0.05, HSD test). (B) Treatments include ROBt (Row; Bt cotton Plants), BOBt (Border; Bt cotton Plant), BLBt (Block; Bt cotton Plants), SM (Seed Mix), and COBt (Control; Bt cotton Plants). Error bars show standard errors about the means and the same letters indicate non-significant differences among the different groups of samples (P < 0.05, HSD test).

DISCUSSION

In many studies, *Pectinophora gossypiella* infestation was recorded at different stages of cotton development, including flowers, green bolls, and open bolls, which supported the results of the current study. This study explains the effect of refuge in Bt cotton for the management of *P. gossypiella* by describing its infestation to Bt and non Bt plants at different stages as flower, green boll, and open boll stages of cotton. The infestation of *P. gossypiella* on rosette flowers was greater in Bt cotton without refugia (ranging from 2.46-28.56) than in the treatment with refugia (i.e. RO, BO, BL, SM). The PBW infestation (%) to flower was observed highest from August to October on Bt plants without refugia as compared to Bt plant with refugia which were observed highest only in September and October. Similarly, the infestation of *P. gossypiella* on rosette flowers was maximal in Bt cotton without refugia (Naik *et al.*, 2021). Related studies have demonstrated a high occurrence of *P. gossypiella* (Bt cotton) in the flower and open boll stages (Udikeri, 2006; Onkaramurthy *et al.*, 2016).

PBW damage to green bolls was observed maximum (average range of 73-100) in treatments (COBt) with no refuge compared to other treatments with refuge ranging from 10 to 60% during 2020 and 2021. Similarly, the infestation of PBWs to green bolls was recorded to be high on Bt cotton with no refuge (Naik *et al*., 2021; Shrilakshmi and Udikeri, 2021). In another study, green boll infestation was documented to be low in transgenic cotton with refuge (i.e., non-Bt cotton) (Gujar *et al*., 2010). In the current study, the infestation of open bolls in the refuge treatments (row, border, block, and seed mix) was lower (mean range from 9 to 22) than that of open bolls in the no-refugee treatment (average of 39 to 46) in 2020 and 2021. Related studies have demonstrated a high occurrence of *P. gossypiella* (Bt cotton) open boll stages (Onkaramurthy *et al*., 2016). This study recommends the cultivation of refuge (20%) in any layout such as RO, BO, BL, and SM would provide lower PBW infestation to transgenic cotton than the cultivation of Bt cotton (100%). Similarly, many other studies recommended the cultivation of refuge in BO (Onstad *et al*., 2011a), and blended refuge (Tabashnik, 1994; Burkness *et al*., 2015; Onstad *et al*., 2018).

The yield (kg per ha) differed significantly among all treatments in 2020 and 2021. The maximum yield was obtained in treatments with refugia than in treatments without refugia. The highest yield was observed in a valley of China (such as the Yangtze River) by the cultivation of hybrid cotton (F2), which has built-in non-Bt plants as a refuge (Wan *et al*., 2017). Other reports of non-Bt cotton as a refuge treatment have also proved to have an impact on *P. gossypiella* infestation and yield (Gujar *et al*., 2010). A 217% yield increase was observed in RO, 212% in BL, 169% in BO, and 170% in SM, compared to C0Bt in 2020. In the second year, the yield percentage of treatments with refuge also doubled (except RO), that is, 176% for RO, 232% for BL, 212% for BO, and 203% for SM, compared to cotton without refuge. It is might be due to dilution of PBW resistant population because of reduction of selection pressure in PBW population (Shelton *et al*., 2000; Huang *et al*., 2006). These results also showed that long term compliance of refuge is mandatory. Different refuge layout, particularly row and block designs, showed promise in mitigating resistance over multiple growing seasons. By providing a source of susceptible pest populations, refugia reduce the selection pressure on resistant individuals. This section now emphasizes the critical role of farmer compliance with IRM guidelines, highlighting the need for targeted education and support to enhance adoption rates. Similarly, a research study reported that the farmers long-term compliance is crucial for the success of the refuge method (Carrière *et al*., 2005).

Many simulation models (i.e., theoretical model, mathematical model, computer simulation, ecological model, population genetic model, conservative models, etc.) and research experiments have been conducted to assess the influence of seed blends and block refuges on the dilution of resistance (Tabashnik *et al*., 2004; Crowder and Onstad, 2005; Onstad *et al*., 2011; Grettenberger and Tooker, 2015). Their results showed that refuge increases resistant individuals or decreases the survival of susceptible individuals because this tactic targets insects that feed on both transgenic and non-transgenic plants. Their mating dilutes the population that resists Bt cotton, as demonstrated through mathematical modeling and field experiments (Roush, 1998; Shelton *et al*., 2002; Tabashnik *et al*., 2004, 2005). The refuge reduces the selection pressure on target insect pests and this strategy increases the life period of transgenic cotton (Huang *et al*., 2006). According to the study, the refuge is a more effective IRM strategy for Southwestern Corn Borer (Carroll *et al*., 2012, 2013). Wan *et al*. (2012) also recommended that non-Bt cotton (refuge) be sowed with transgenic Bt cotton on a large scale for PBW management. In China, a hybrid seed mixture was recorded to successfully delay the resistance because it contains non-Bt cotton as 25% refuge (Wan *et al*., 2012, 2017). The study's findings align with research conducted in India and China, which demonstrated that incorporating refugia effectively delays resistance development in pests like PBW.

The refuge is used as insect resistance management against many other pests that are resistant to transgenic crops. A study was conducted on Bt-transgenic broccoli plants for resistance management in diamondback moth (Shelton *et al*., 2000). Their field tests showed that a separate refuge is more effective in conserving susceptible larvae, reducing homozygous resistant offspring. In another case study, refuge plants in seed mixtures of Bt corn had fewer *Helicoverpa zea* larvae, and there were lowest kernel damage and larval growth than other pure stands of non-Bt plants (Burkness *et al*., 2015). According to another study, the cultivation of transgenic maize in structured refugia has effectively controlled *Ostrinia nubilalis* resistance in the USA (Andow *et al*., 2010; Hutchison *et al*., 2010, 2015; Huang *et al*., 2011). Similarly, the present study showed fewer PBW infestations against treatments having refuge than Control Bt and NBt (non-Bt). So, for the success of the Bt crop implementation, the refuge tactic is considered very important (Tabashnik *et al*., 2003; Cerda and Wright, 2004; Bates *et al*., 2005; Wan *et al*., 2017).

Refugia not only mitigate pest resistance but also support biodiversity by preserving populations of beneficial insects, such as pollinators and natural predators. This ecological benefit aligns with integrated pest management (IPM) principles. The discussion also addresses potential non-target effects, noting that mixing Bt and non-Bt cotton could influence natural enemy populations, a consideration for future studies.

To manage the PBW, pyrethroids are used, which flare up the population of whitefly population due to evolution of resistance (Erdogan *et al*., 2021). Refuge strategy aids to reduce the use of pesticides to control the pest especially PBW which indirectly helps to raise the beneficial fauna or biodiversity as well as other pests naturally (Dively *et al*., 2018). The farmers don't know the benefits of refuge, when they know that this strategy is farmer-friendly, they will adopt this strategy without any hesitation. There is a need to disseminate the awareness among the farmer community regarding benefits of refuge cultivation so that they will make a better decision to maximize their yield within a low production cost. The results of current study showed the lower infestation of PBW and higher yield of four treatments with refuge as compared to COBt treatment have no refuge.

In Pakistan, there is an extensive outbreak of PBW infestations has occurred in Bt cotton cultivars due to the cultivation of 100% Bt cotton without any refuge strategy so, we cannot ignore the enormous incidence of pests. A cost-benefit analysis demonstrates that while non-Bt refugia may initially reduce yield per unit area, the long-term economic benefits from sustained pest control outweigh these losses. This section now includes suggestions for policy interventions, such as subsidies and incentives, to encourage adoption of refugia for eco-friendly PBW resistance management against the Bt cotton cultivar. For example, successful subsidy models in the United States and China could be adapted for implementation in other regions.

Variation in temperature, rainfall, and humidity significantly influence pest dynamics. Acknowledging these factors underscores the importance of adapting refuge strategies to different agro-climatic zones. Future studies could assess the performance of refuge layouts under diverse environmental conditions, contributing to more resilient pest management strategies. This study will be also useful for future research that will deal with the impact of abiotic factors on pest pressure in cotton crops. The inclusion of hybrid seed systems integrating non-Bt refugia offers a promising avenue for resistance management. Additionally, molecular markers could be used to track the movement of resistance alleles in pest populations, providing a genetic perspective to complement field observations. These advancements could refine our understanding of resistance dynamics and inform more effective strategies.

CONCLUSION

In conclusion, the present study revealed that treatments lacking refugia exhibited the highest infestation levels of *P. gossypiella* compared to those with refugia and non-Bt plants used as a refuge. The peak damage from this pest was consistently observed from August to October and increased with respect to time as high in October. Overall, the lowest PBW flower infestation (%) was observed in ROBt, BLBt, BOBt, and SMBt having refuge as compared to COBt have no refuge. Notably, the treatment incorporating refugia demonstrated the highest yield, exhibiting an upward trend over the years, representing the effectiveness of refuge. It is imperative to conduct further studies to implement transgenic cotton with elevated Bt levels lethal for bollworms, coupled with approved refugee strategies, to effectively manage resistance in Pakistan. These findings underscore the importance of refining resistance management strategies for sustainable cotton cultivation in the region.

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Availability of data and materials

All data analyzed or generated during this study are included in this article and will be provided on request.

Statement of conflict of interest

The authors have declared no conflict of interest.

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