Research Square

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Plant phenology and larval instar influence the efficacy of Bt maize against Spodoptera frugiperda (Lepidoptera: Noctuidae)

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Abstract

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is one of the most destructive pests of maize worldwide, and its management relies heavily on hybrids expressing insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*). However, the efficacy of *Bt* maize may vary according to plant developmental stage, canopy position, larval instar, and insect susceptibility. This study evaluated the mortality of first- and third-instar *S. frugiperda* larvae from laboratory and field populations fed with leaves collected from different canopy sections of VTPRO4® maize expressing Cry1A.105, Cry2Ab2, Vip3Aa20, and Cry3Bb1 proteins at the V3 and V8 phenological stages. *Bt* maize significantly increased larval mortality compared with the conventional hybrid, although mortality was incomplete under all evaluated conditions. First-instar larvae were consistently more susceptible than third-instar larvae, and the field population exhibited lower susceptibility than the laboratory population. Mortality was generally higher in leaves collected from the upper canopy at the V3 stage, whereas *Bt* efficacy declined markedly at the V8 stage across all canopy sections. These results demonstrate that *Bt*-mediated toxicity is dynamic and influenced by the interaction among plant ontogeny, canopy position, larval developmental stage, and population susceptibility. The reduced efficacy observed in older plants and against later larval instars may create survival windows for partially resistant individuals under field conditions, potentially accelerating resistance evolution and highlighting the importance of incorporating spatiotemporal variability into insect resistance management strategies for tropical maize production systems.

Introduction

Maize (*Zea mays* L.) is among the most widely cultivated cereals worldwide and plays a central role in global food security and agricultural economies. In Brazil and other South American countries, maize production has expanded under intensive succession systems, particularly in tropical and subtropical regions where second-season maize frequently surpasses the productivity of the main summer crop (Contini et al. 2019; Erenstein et al. 2022). However, the sustainability of these systems is severely threatened by insect pests, especially the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae). As a highly polyphagous and destructive herbivore, this species attacks maize throughout multiple phenological stages, causing severe defoliation and significant yield losses under high infestation levels (Overton et al. 2021; Kenis et al. 2023). Its elevated dispersal capacity, continuous occurrence across overlapping cropping systems, and remarkable adaptive plasticity have established this species as one of the most important pests in Neotropical agroecosystems (Montezano et al. 2018; Horikoshi et al. 2016).

Genetically modified maize hybrids expressing insecticidal proteins derived from *Bacillus thuringiensis* (*Bt*) have become the cornerstone of *S. frugiperda* management (Santos-Amaya et al. 2025). More recently, pyramided technologies combining multiple proteins with distinct modes of action, such as VTPRO4®, which expresses Cry1A.105, Cry2Ab2, Vip3Aa20, and Cry3Bb1 were developed to improve control efficacy and delay resistance evolution. Despite their widespread adoption, however, the

durability of *Bt* technologies has been increasingly compromised by the rapid evolution of resistance in field populations of *S. frugiperda*, particularly under tropical production systems characterized by continuous host availability and intense selection pressure (Tabashnik and Carrière 2013).

In addition to resistance evolution itself, increasing evidence indicates that *Bt*-mediated control is not spatially or temporally uniform throughout plant development. *Bt* protein expression may vary among plant tissues and decline during crop maturation, potentially creating heterogeneous exposure scenarios in which larvae encounter sublethal toxin concentrations (Bernardi et al. 2016; Chengrong et al. 2021). These “survival windows” are particularly relevant for insect resistance management because they may favor the persistence of partially resistant individuals under field conditions (Tabashnik and Carrière 2013). Furthermore, larval susceptibility to *Bt* proteins is strongly influenced by ontogeny, with later instars generally exhibiting reduced susceptibility compared to newly hatched larvae. Together, these factors suggest that *Bt* efficacy may result from a dynamic interaction among plant developmental stage, within-plant spatial variation, larval ontogeny, and population susceptibility background (Jurat-Fuentes and Heckel 2021).

Despite the recognized importance of these factors, limited information is available regarding how these variables interact to shape the consistency of *Bt*-mediated mortality throughout maize development (Chengrong et al. 2021; Jurat-Fuentes and Heckel, 2021). Therefore, this study investigated the dynamic efficacy of *Bt* maize (VTPRO4®) against *S. frugiperda* by evaluating whether larval mortality varies according to plant phenological stage, leaf position within the canopy, larval instar, and population susceptibility background. By contrasting laboratory and field populations, we aimed to determine how spatiotemporal variation in *Bt*-mediated toxicity may influence the consistency of control and potentially contribute to survival scenarios associated with resistance evolution under field conditions.

Material and Methods

Insect collection and rearing

In this study, two populations of *S. frugiperda* were used in the bioassays. The laboratory population (LP) refers to an established colony (35 generations) maintained at the Laboratory of Agricultural Entomology, Federal University of Grande Dourados (UFGD), located in Dourados, Mato Grosso do Sul, Brazil. The field population (FP) was established from larvae actively collected from maize plants at the Experimental Farm of Agricultural Sciences Federal University of Grande Dourados (UFGD), located in Dourados, Mato Grosso do Sul, Brazil. Both populations were reared under controlled conditions (25 ± 2°C; 60 ± 10% RH; 12-h photophase) and fed with artificial diet based on common beans, wheat germ, and brewer's yeast, following a previously described methodology (Kasten and Parra 1978).

Plant material and growth conditions

The maize hybrids evaluated, VTPRO4® (8480 PRO4 Technology) and 3510 RR2®, were grown in the experimental field area of the Laboratory of Agricultural Entomology, Federal University of Grande Dourados (UFGD), located in Dourados, Mato Grosso do Sul, Brazil (22°25'03" S, 54°13'57" W; 430 m a.s.l.). The region has a tropical climate with rainy summers and dry winters, according to the Köppen–Geiger classification. Sowing was performed manually in separate plots of 196 m² (14 × 14 m) for each hybrid, at a depth of 5 cm.

The VTPRO4® hybrid expresses the *Bt* proteins Cry1A.105, Cry2Ab2, Vip3Aa20, and Cry3Bb1 whereas the 3510 RR2® hybrid was used as the conventional non-*Bt* cultivar, as it is recommended for refuge areas.

To ensure the availability of leaf tissue at the V3 and V8 phenological stages synchronized with the required larval instars (1st and 3rd instars), a staggered sowing schedule was adopted. The initial sowing consisted of three rows, followed by the sowing of two additional rows every seven days to provide leaves at the V8 stage. For the V3 stage, new rows were sown 22 days after the initial planting, with subsequent sowings carried out every three days. Standard agronomic practices were followed as required; however, no insecticides were applied throughout the experimental period to avoid interference of chemical residues with the biological assays.

Bioassay procedures and experimental design

Leaf tissues from V3 and V8 stages were partitioned into upper, middle, and lower thirds, disinfected in a sodium hypochlorite solution (0.2% active chlorine) for 10 min, and rinsed in distilled water. The experimental units consisted of sterile polystyrene Petri dishes (9 cm diameter) containing a 1.5% agar-water substrate covered with filter paper to maintain leaf turgidity. Fifteen replicates were used per treatment. *S. frugiperda* larvae were individualized in each dish (approx. 3 cm² of leaf area) to preclude cannibalism. Mortality was monitored at 24, 48, 72, and 96 h, with death confirmed by the absence of response to mechanical stimulation with a fine-hair brush.

The experimental program comprised four distinct bioassays. **Bioassay I** followed a factorial design evaluating two populations (laboratory population, 35th generation; and field population, 2nd generation), three plant sections (upper, middle, and lower thirds), and two maize hybrids (*Bt* and non-*Bt*). **Bioassay II** focused on the field population across two phenological stages (V3 and V8), three plant sections, and two maize hybrids. Finally, **Bioassays III and IV** investigated the interaction between larval instars (1st and 3rd) and maize hybrids at two phenological stages (V3 and V8), utilizing exclusively the upper plant third. **Bioassay III** employed the laboratory population (36th generation), while **Bioassay IV** utilized the field population (2nd generation).

Statistical Analysis

The experiments were conducted in a completely randomized design (CRD). Cumulative mortality data at 96 h were analyzed using Generalized Linear Models (GLM) with a negative binomial distribution (log-link function) to account for overdispersion. Model fit and assumptions were verified using residual diagnostic plots with the DHARMa package. The significance of main effects (cultivar, phenology, and larval instar) and their interactions was assessed through Analysis of Deviance (Wald χ^2 tests). Post-hoc mean comparisons were performed using Tukey's HSD test ($\alpha = 0.05$) on the response scale (back-transformed). For graphical representation, data were converted to percentages, and means are presented alongside their respective standard errors (± SE). All analyses were performed in R software version 4.4.2 (R Core Team), employing the MASS and emmeans packages.

Results

Population susceptibility and leaf position (Bioassay I)

The analysis of deviance showed a significant isolated effect only for the cultivar factor ($\chi^2 = 33.82$; df = 1; $p < 0.001$). No significant interaction was observed between cultivar and population ($p > 0.05$), nor for the leaf third factor ($p > 0.05$). For the laboratory population, the *Bt* hybrid resulted in significantly higher mortality compared to the conventional hybrid across all leaf thirds ($p < 0.05$) (Fig. 1). Conversely, for the field population, mortality in the Bt hybrid (ranging from 60.0% to 80.0%) was similar to that in the conventional hybrid (20.0% to 33.3%) across all plant sections ($p > 0.05$) (Fig. 1).

Impact of plant phenology and leaf thirds on mortality (Bioassay II)

Significant effects were found for cultivar ($\chi^2 = 10.19$; $p = 0.001$) and for the cultivar vs. phenological stage interaction ($\chi^2 = 4.53$; $p = 0.033$). At the V3 stage, the *Bt* hybrid showed a trend toward higher mortality, with statistical significance confirmed only in the upper third ($p = 0.013$). Differences in the middle ($p = 0.099$) and lower ($p = 0.083$) thirds at V3 were not significant at the 5% level. At the V8 stage, no significant differences in mortality were observed between *Bt* and conventional hybrids for any leaf third ($p > 0.05$; ratio ≈ 1.0) (Fig. 2).

Larval instar sensitivity across plant phenological stages (Bioassays III and IV)

In the laboratory population (Bioassay III), both cultivar ($\chi^2 = 7.89$; $p = 0.004$) and instar ($\chi^2 = 7.89$; $p = 0.004$) factors were significant. However, Tukey's pairwise comparisons did not reveal significant differences between hybrids within specific combinations ($p > 0.05$), with a marginal trend observed only for 1st instars at the V3 stage ($p = 0.082$; Fig. 3A).

In the field population (Bioassay IV), only the cultivar factor was significant ($\chi^2 = 10.19$; $p = 0.001$), while the instar factor showed a marginal trend ($\chi^2 = 3.80$; $p = 0.050$). Pairwise comparisons indicated no

significant differences in mortality between *Bt* and conventional hybrids for any combination of phenological stage (V3/V8) or larval instar (1st/3rd) ($p > 0.05$; Fig. 3B).

Discussion

The reduced susceptibility observed in the field population of *S. frugiperda* is consistent with the extensive history of resistance evolution to *B. thuringiensis* (*Bt*) proteins documented in Brazilian agricultural systems. Resistance ratios exceeding 5,000-fold to the Cry1F protein expressed in TC1507 maize have previously been reported, and this phenotype was associated with shared autosomal recessive alleles among geographically distinct populations (Farias et al. 2014). In addition, field-derived populations retained the ability to complete their life cycle on Cry1F maize even after several generations without selection pressure, reinforcing the stability of this adaptive trait (Monnerat et al. 2015). The dissemination of resistance alleles across production regions likely contributed to the progressive erosion of field performance observed for additional Cry-based technologies. Extensive cross-resistance between Cry1F and Cry1Ab proteins has likewise been documented in Brazilian populations of *S. frugiperda* (Omoto et al. 2016). Together, these studies contextualize the response observed in the evaluated field population within the broader history of *Bt* resistance evolution in Brazil.

These historical patterns of resistance evolution are further complicated by cross-resistance among proteins expressed in pyramided hybrids. Resistance to Cry1F has been associated with high levels of cross-resistance to Cry1A.105 and Cry1Ab, likely due to structural similarities among Cry1 toxins. In contrast, the lower levels of cross-resistance reported for Cry2Ab2 reinforce the importance of pyramids composed of proteins with independent modes of action as a strategy to delay resistance evolution (Bernardi et al. 2015).

Beyond cross-resistance patterns, accumulating evidence indicates that *S. frugiperda* possesses substantial genetic and physiological plasticity that enhances its adaptive potential under selection pressure. A large genomic deletion directly associated with Cry1F tolerance has been identified in field populations, reinforcing the genetic stability of this phenotype (Banerjee et al. 2022). In addition, the species is capable of rapidly modulating detoxification pathways associated with insecticide exposure (Banerjee et al. 2022; Rodrigues et al. 2025). Although these mechanisms were not directly investigated in the present study, they collectively illustrate the considerable adaptive capacity of the fall armyworm and help explain the limited efficacy of *Bt* maize observed against the field population.

In addition to the susceptibility status of the insect population, our results demonstrate that *Bt* efficacy is strongly influenced by plant phenology and leaf position. Mortality remained detectable during the V3 stage, particularly in the upper third of the plant, whereas this effect was largely absent at V8 across all canopy sections. This pattern is consistent with documented temporal and spatial variation in *Bt* protein expression throughout plant development. Younger leaves located in the upper canopy are generally more metabolically active and tend to accumulate higher concentrations of *Bt* proteins, whereas advancing plant maturation may progressively reduce the effective toxin dose available to feeding larvae

(Chengrong et al. 2021). Similar trends have been reported in maize and cotton, where *Bt* protein accumulation varies across tissues and developmental stages, with lower concentrations frequently detected in non-foliar and reproductive structures (Balieiro Neto et al. 2013; Bahar et al. 2019). Although *Bt* protein expression was not quantified in the present study, the progressive reduction in larval mortality observed at advanced vegetative stages strongly suggests that plant ontogeny plays an important role in shaping the effectiveness of *Bt*-mediated control throughout crop development. Therefore, the observed mortality patterns should be interpreted as indirect evidence of spatiotemporal variation in *Bt*-mediated toxicity rather than direct measurements of toxin concentration.

From an ecological and resistance management perspective, the progressive reduction in mortality during crop development is particularly relevant because it may create windows of sublethal exposure throughout plant ontogeny. Within the high-dose/refuge framework of resistance evolution such conditions may increase the survival probability of individuals carrying partially resistant genotypes, thereby contributing to the maintenance and progressive increase of resistance alleles in field populations (Tabashnik and Carrière 2013; Gould and Kuzma 2018). In this context, reduced toxin expression in specific plant tissues may allow *S. frugiperda* larvae to exploit plant regions characterized by lower selection pressure, thereby weakening the effectiveness of the high-dose/refuge strategy under field conditions (Bernardi et al. 2016).

Beyond plant-mediated effects, larval developmental stage also strongly influenced *Bt* efficacy. First-instar larvae exhibited greater susceptibility to *Bt* maize, whereas third-instar larvae exhibited consistently low susceptibility regardless of plant phenological stage or cultivar. This reduction in susceptibility with larval maturation is likely associated with progressive physiological and morphological development of the digestive system. Early instars possess lower body mass and distinct enzymatic profiles that may influence the kinetics of *Bt* protein processing in the midgut (Sanahuja et al. 2011). In lepidopteran larvae, serine proteases are involved not only in protoxin activation but may also contribute to toxin degradation depending on the enzymatic composition of the digestive tract (Jurat-Fuentes and Heckel 2021). As larval development progresses, increases in body mass, digestive efficiency, and gut regeneration capacity may substantially elevate the toxin dose threshold required to induce mortality. These physiological mechanisms are consistent with previous observations of reduced mortality in third-instar larvae of *Spodoptera littoralis* fed *Bt* maize (Dutton and Bigler, 2005). A similar pattern has also been reported for *S. frugiperda* in *Bt* cotton, where first-instar larvae exhibited substantially higher mortality than third-instar larvae, reinforcing the importance of larval developmental stage as a determinant of *Bt* efficacy across different *Bt* cropping systems (André et al. 2025).

Overall, the present study demonstrates that *Bt* efficacy against *S. frugiperda* is not a static characteristic of the hybrid itself, but rather the result of a dynamic interaction among insect susceptibility, plant developmental stage, and larval ontogeny. Under field conditions, these factors may converge to progressively reduce control efficacy throughout crop development. Younger plants may still impose sufficient toxic pressure against newly hatched larvae; however, as toxin availability declines and surviving individuals rapidly develop into physiologically more tolerant instars, the probability of survival

increases substantially. This process becomes particularly concerning in populations already exhibiting reduced susceptibility to Cry proteins.

The implications of this dynamic are highly relevant for insect resistance management (IRM) programs in tropical agroecosystems. Resistance to several commercial *Bt* technologies in Brazil has been shown to exhibit functional dominance under field conditions, allowing heterozygous individuals to survive on *Bt* plants and reducing the effectiveness of the high-dose/refuge strategy (Horikoshi et al. 2016). Similarly, the performance of pyramided hybrids against *S. frugiperda* has become increasingly dependent on Vip3Aa proteins as susceptibility to Cry proteins declines in field populations (Moscardini et al. 2020). Prolonged exposure to sublethal toxin concentrations combined with inadequate refuge compliance has also been identified as a major driver of resistance evolution under field conditions (Fatoretto et al. 2017; Tabashnik et al. 2023).

Collectively, our findings support the existence of a triple challenge for *Bt*-based management of *S. frugiperda*: (i) reduced susceptibility in field populations, (ii) progressive declines in effective *Bt* toxin exposure during plant development, and (iii) rapid larval progression toward physiologically more tolerant instars. The interaction among these factors may create survival windows that accelerate resistance evolution by allowing larvae exposed to sublethal toxin concentrations to complete development and contribute resistant alleles to subsequent generations. Therefore, the long-term sustainability of *Bt* technologies will likely depend on integrated resistance management strategies capable of maximizing early-stage mortality and limiting larval survival during the initial phases of infestation.

Conclusion

This study demonstrates that the effectiveness of *Bt* maize against *S. frugiperda* depends on the dynamic interaction among insect susceptibility, plant developmental stage, and larval ontogeny rather than on the transgenic technology alone. Reduced susceptibility in the field population, combined with the progressive decline in *Bt*-mediated mortality during plant development and the lower susceptibility of advanced larval instars, may create survival windows that favor persistence of the pest and contribute to resistance evolution under field conditions. These findings highlight that the performance of *Bt* crops should be evaluated within a broader ecological context, considering both plant and insect developmental dynamics. From an insect resistance management perspective, strategies that maximize early larval mortality and minimize exposure to sublethal toxin concentrations throughout crop development will likely be essential for preserving the long-term durability of *Bt* technologies and sustaining the effectiveness of *Bt*-based pest management programs.

Declarations

Conflicts of Interest:

The author declare no conflict or competing interest.

Competing interests:

The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval:

Not applicable.

Funding information:

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Author Contribution

B.D.G. and P.L.P. conceived the study. B.D.G., N.P.S., D.P., P.L.P., and E.H.Q.N. developed the methodology. B.D.G. and N.P.S. performed the software development and formal analyses. B.D.G., N.P.S., D.P., and P.L.P. validated the results. B.D.G., N.P.S., and E.H.Q.N. conducted the investigation. B.D.G., D.P., and P.L.P. provided resources and acquired funding. B.D.G., N.P.S., and P.L.P. curated the data. B.D.G., N.P.S., and P.L.P. wrote the original draft of the manuscript, and B.D.G. and P.L.P. reviewed and edited the manuscript. B.D.G. and P.L.P. supervised and administered the project. All authors read and approved the final manuscript.

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Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

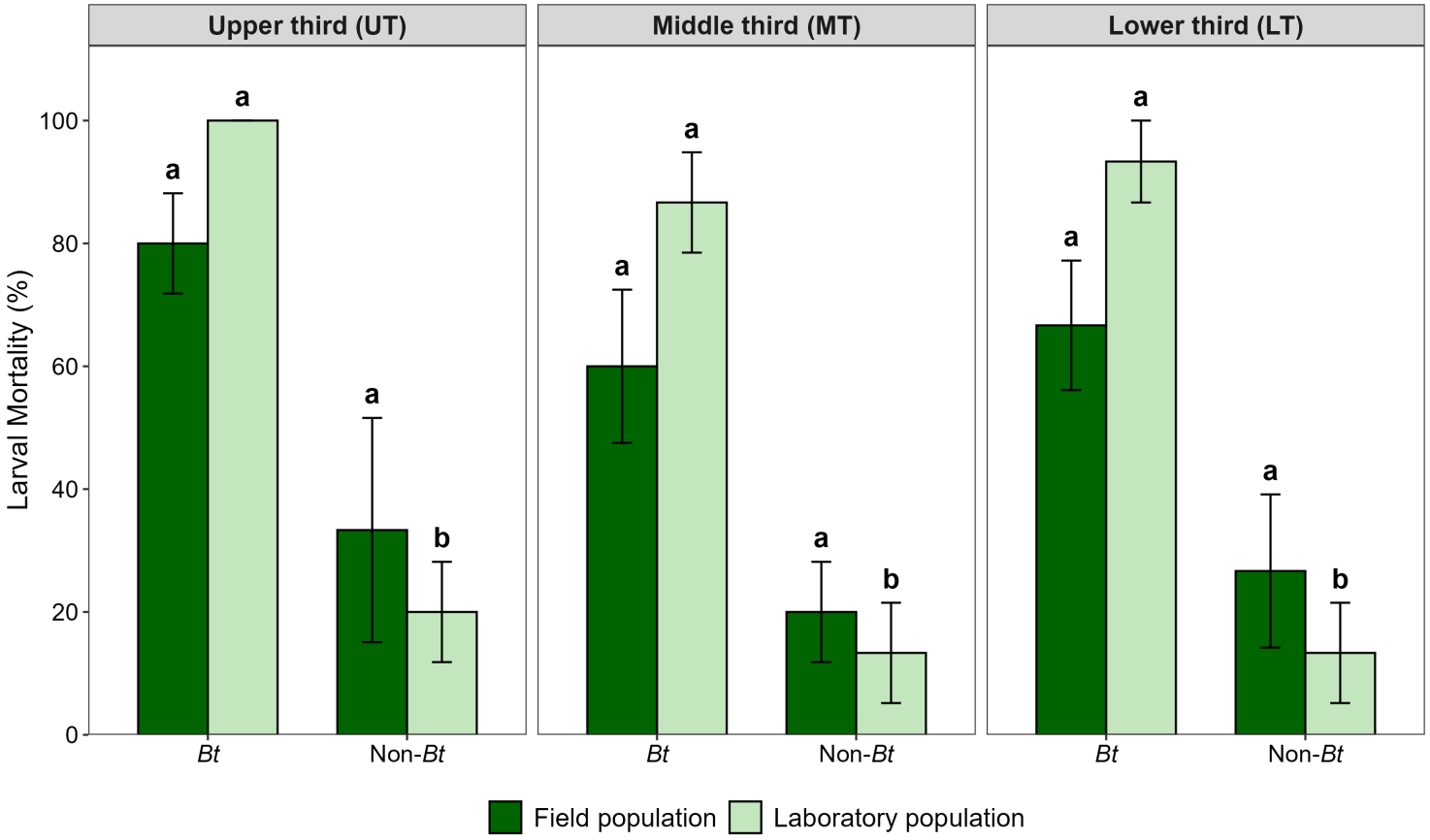


Figure 1

Larval mortality percentage (mean ± SE) of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) from field and laboratory populations fed on *Bt* and non-*Bt* maize leaf thirds. Means followed by the same letter within each leaf third (Upper, Middle, and Lower) do not differ significantly by Tukey's HSD test ($p < 0.05$).

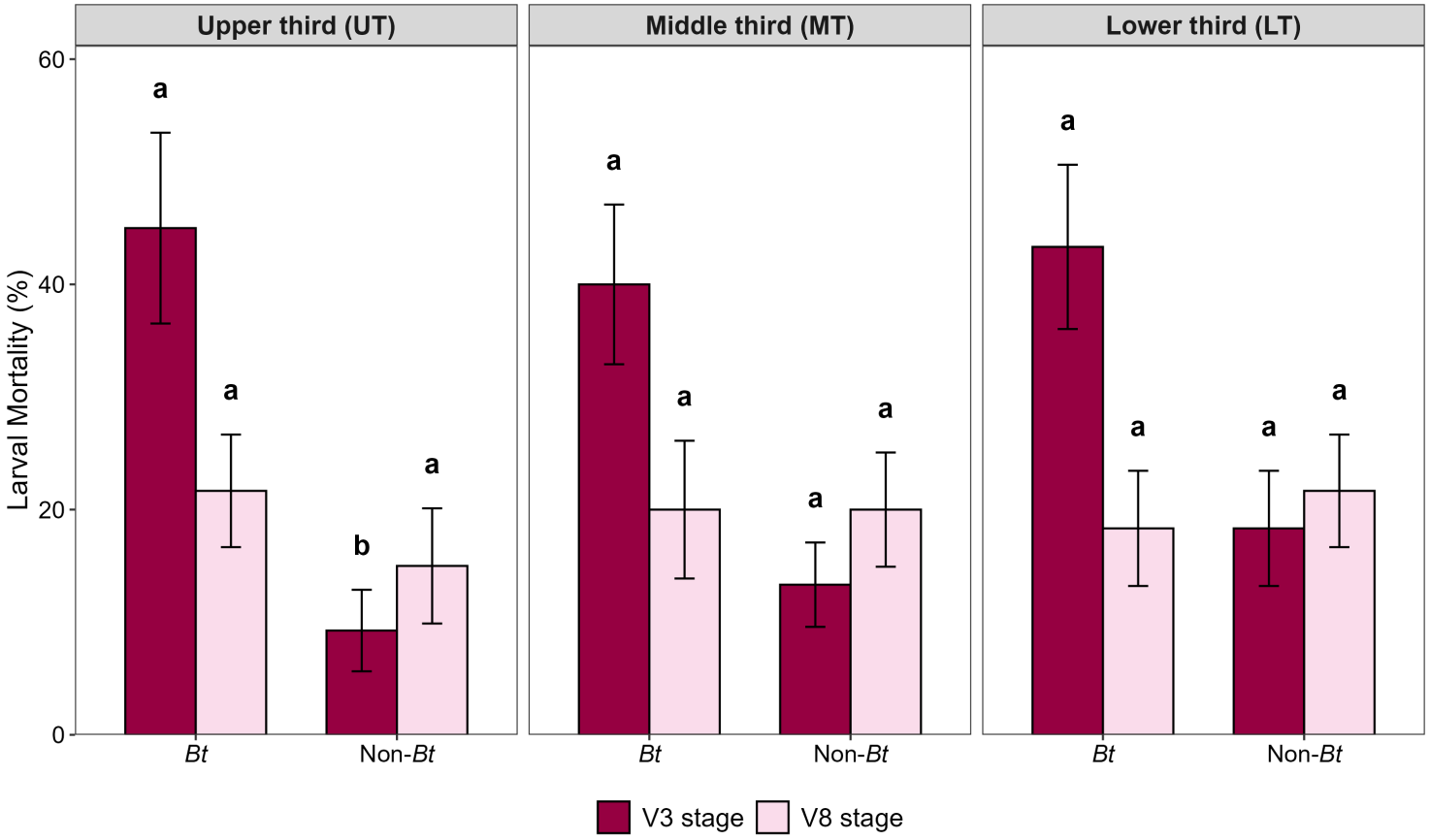


Figure 2

Effect of maize phenological stage (V3 and V8) and leaf position (upper, middle, and lower thirds) on the mortality percentage (mean ± SE) of a field population of *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Means followed by the same letter within each leaf third do not differ significantly by Tukey's HSD test ($p < 0.05$).

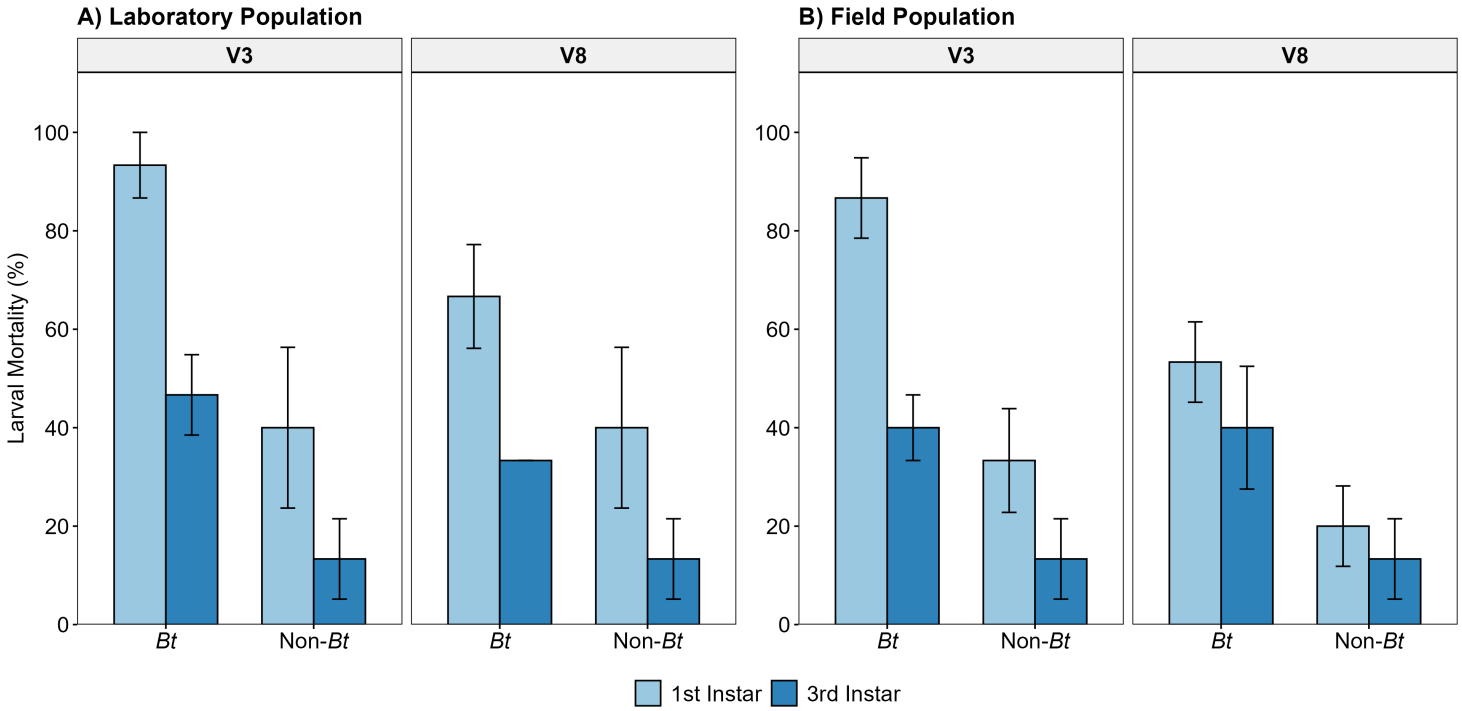


Figure 3

Larval mortality percentage (mean ± SE) of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) from **(A)**laboratory and **(B)** field populations. First (1st) and third (3rd) instar larvae were fed on *Bt* and non-*Bt* maize hybrids at V3 and V8 phenological stages. No significant differences were observed between hybrids (*Bt*vs. non-*Bt*) for any combination of phenological stage or larval instar within either population (Tukey's HSD test, $p > 0.05$).