Influence of elevated CO$_2$ on development and food utilization of target armyworm *Mythimna separata* fed on transgenic *Bt* maize infected by nitrogen-fixing bacteria

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**Key words**
elevated CO$_2$; transgenic *Bt* maize; azotobacter; *Mythimna separata*; growth and development; food utilization

**Summary statement**
Elevated CO$_2$ effect on development and food utilization of target armyworm *Mythimna separata* fed on *Bt* maize infected by azotobacter, *Azospirillum brasilense* and *Azotobacter chroococcum*
Abstract

Bt crops will face a new ecological risk of reduced effectiveness against target-insect pests owing to the general decrease in exogenous-toxin content in Bt crops grown under elevated CO₂. How to deal with this issue may affect the sustainability of transgenic crops as an effective pest management tool especially under future CO₂ raising. In this study, azotobacters, as being one potential biological regulator to enhance crops’ nitrogen utilization efficiency, were selected and the effects of Bt maize and non-Bt maize infected by Azospirillum brasiliense and Azotobacter chroococcum on development and food utilization of target Mythimna separate were studied under ambient and elevated CO₂. The results indicated that azotobacter infection significantly increased larval life-span, pupal duration, RCR and AD of M. separata, and significantly decreased RGR, ECD and ECI of M. separata fed on Bt maize; There were opposite trends in development and food utilization of M. separata fed on non-Bt maize infected with azotobacters compared with the buffer control regardless of CO₂ level. Presumably, the application of azotobacter infection could make Bt maize facing lower field hazards from the target pest of M. separate, and finally improve the resistance of Bt maize against target lepidoptera pests especially under elevated CO₂.
Introduction

With increased fossil fuel combustion and drastic changes in land utilization, the concentration of atmospheric carbon dioxide (i.e., CO$_2$) has increased by more than 40%, from 280 to 400 ppm, between the industrial revolution and now (Ciais et al., 2013). The recent forecast indicated that atmospheric CO$_2$ concentration will increase to approximately 900 ppm by 2100 (IPCC, 2014). Increasing atmospheric CO$_2$ concentration alone can be very significant in crop production because of its direct effect on plant physiology and biochemistry (Cornelissen, 2011), and indirect effect on tri-trophic interactions involving plants, herbivores, and predators or pathogens (Robinson et al., 2012; Trębicki et al., 2017). Elevated atmospheric CO$_2$ also affects the crop production via direct or indirect impact on the physiology and feeding behavior of phytophagous insects (Zvereva & Kozlov, 2006; Massad & Dyer, 2010; O'Neill et al., 2010). These changes may then lead to more severe and frequent outbreaks of pest insects in agricultural ecosystems (Percy et al., 2002).

Several studies have shown that the elevated CO$_2$ increased lepidopteran insect feeding and damage severity in agricultural crops (Ainsworth & Schurr, 2007; Lindroth et al., 2001), because of the increased proportion of C: N in host plant tissue and lower nutritional quality caused by elevated CO$_2$ (Ainsworth & Schurr, 2007). For example, larvae of Helicoverpa armigera fed on wheat grown in elevated CO$_2$ showed the extended larval life span and increased consumption with reduced growth rate (Chen et al., 2004). Transgenic maize that expresses insecticidal Cry proteins derived from the soil bacterium Bacillus thuringiensis Berliner (Bt) has been used to control target lepidopteran insects (Carrière et al., 2010; Huang et al., 2014; Walters et al., 2010), e.g., European corn borer Ostrinia nubilalis (Hübner), Asian corn borer O. furnacalis (Guenée) (Lepidoptera: Crambidae) and corn armyworm Mythimna separata (Lepidoptera: Noctuidae) (Guo et al., 2016; Zhang et al., 2010; Jia et al., 2010). Transgenic Bt maize has recently been adopted worldwide (Cattaneo et al., 2006; Huang et al., 2005; Hutchison et al., 2010; Lu et al., 2012). It was anticipated that the primary effect of elevated CO$_2$ on Bt toxin production would be due to differences in N concentration in plant tissues (Coviella et al., 2002). Biologically relevant changes in plant defensive chemistry of Bt maize are expected to have measurable effects on the target lepidopteran pests under climate change.

Additionally, many researchers found that the nitrogen metabolism of transgenic Bt crops could affect the expression of Bt toxin protein (Stitt & Krapp, A, 1999; Pang et al., 2005; Gao et al., 2009). Nitrogen plays the most important role for plant growth, and it is an important complement of enzymes catalyzing and controlling reactions in plants for normal physiological processes (Richardson, 2009). While most of nitrogen in the environment is found in a form of nitrogen gas (N$_2$) which approximately amounts to 78% in the atmosphere, plant available nitrogen found in soil is generally derived from fertilizer augmentation. As plants cannot use N$_2$ directly, soil-inhabiting microbes play a significant role in nitrogen uptake by plants as they change the nitrogen gas into ammonia (Yamprai et al., 2014). Azospirillum sp. and Azotobacter sp are the two major free-living soil microbes (Biari et al., 2008; Sastro et al., 2014), that are economically important nitrogen-fixing bacteria in maize crop production system (Yamprai et al., 2014). Thus, optimization of soil-nitrogen management offers significant potential in the utilization of soil azotobacters to increase Bt-crop nitrogen utilization to affect the expression of Bt toxin.
In this study, we examined the influence of elevated CO\textsubscript{2} on larval development and food utilization of target armyworm \textit{M. separata} fed on \textit{Bt} maize (line IE09S034 with \textit{CryIle} gene) versus its non-\textit{Bt} parental line (cv. Xianyu335) infected with \textit{A. brasilense} and \textit{A. chroococcum} under ambient and elevated CO\textsubscript{2}.

Materials and Methods

Setup of CO\textsubscript{2} levels

A two-year study (2016-2017) was conducted in six open-top chambers (i.e., OTCs; Granted Patent: ZL201120042889.1; 2.5m in height \times 3.2m in diameter) (Chen et al., 2011) at the Innovation Research Platforms for Climate Change, Biodiversity and Pest Management (CCBPM; http://www.ccbpm.org) field laboratory in Ningjin County, Shandong Province of China (37°38′ 30.7″ N, 116°51′ 11.0″ E). Two CO\textsubscript{2} levels, ambient (375 μl/L, hereafter referred to as \textit{aCO}_2) and elevated (750 μl/L or double-ambient, hereafter referred to as \textit{eCO}_2) were applied continuously from 10 June to 7 October in both years. Three OTCs were used for each CO\textsubscript{2} treatment, and the CO\textsubscript{2} concentrations in each OTC were monitored continuously and adjusted using an infrared CO\textsubscript{2} analyzer (Ventostat 8102; Telaire Company, Goleta, CA). The OTCs of elevated CO\textsubscript{2} treatments were inflated with canned CO\textsubscript{2} gas with 95% purity and automatically controlled by the same type of infrared CO\textsubscript{2} analyzer (Chen et al., 2004). Actual mean CO\textsubscript{2} concentrations (2016-\textit{aCO}_2: 372.6 μl/L, \textit{eCO}_2: 744.4 μl/L; 2017-\textit{aCO}_2: 374.5 μl/L, \textit{eCO}_2: 748.8 μl/L) and temperature (2016-26.06°C; 2017-26.11°C) throughout the entire experiment are measured in both 2016 and 2017.

Plant materials

The \textit{Bt} maize cultivar (Line IE09S034, hereafter referred to as Bt) and its non-\textit{Bt} parental line (cv. Xianyu335, referred to as Xy) were both obtained from the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences. Both \textit{Bt} and non-\textit{Bt} lines used in this study possessed identical growing periods (approximately 102 d) and were well adapted to the growing conditions of northern China (Guo et al., 2016; Zhang et al., 2010; Jia et al., 2010; Ling, 2010). Both maize accessions were planted in plastic buckets (diameter \times height=30 cm \times 45 cm) filled with 20kg autoclaved soil and 10g compound fertilizer (N: P: K=18: 15: 12), then placed them into chambers on 10 June each year.

Soil nitrogen-fixing bacteria and infection of maize seeds

Lyophilized \textit{Azospirillum brasilense} (strain number ACCC 10103) and \textit{Azotobacter chroococcum} (strain number ACCC 10006) were provided by Agriculture Culture Collection of China (ACCC) in plastic tubes (3 cm in diameter and 15 cm in height) with bacterial growth medium. Both species of azotobacters were grown in liquid medium at 28°C under continuous shaking (200rpm) until they reached an absorbance of 1.008 (\textit{A. brasilense}) and 1.005 (\textit{A. chroococcum}) at a wavelength of 600nm. Before inoculation, the culture was centrifuged, and the supernatant was discarded, and the pellet of cells was re-suspended in the liquid medium to a density of 10^8 copies per milliliter. The seeds of both \textit{Bt} and non-\textit{Bt} maize were infected with \textit{A. brasilense} and \textit{A. chroococcum} cultures each, and the inoculation doses were all adjusted...
to a final volume of 10 ml. After inoculation, all the treated seeds were maintained under sterile laminar air flow for 2h at 28°C (Cassán, 2009). Bacteria inoculation treatments consisted of three types of azotobacter infection, including (1) seeds infected with A. brasilense (referred to as AB); (2) seeds infected with A. chroococcum (referred to as AC); and (3) non-infected seeds (control) treated with a final volume of buffer solution (referred to as CK). The entire experiment, thus, consisted of twelve treatments, including two CO2 levels (aCO2 and eCO2), two maize cultivars (Bt and Xy), and three azotobacter infections (AB, AC, and CK), replicated six times. Specifically, six buckets for each maize cultivar (Bt and Xy) and three azotobacter inoculations (6 buckets per cultivar treatment × 2 cultivars x 3 inoculation treatments=36 buckets) were placed randomly in each CO2 chamber (ambient and double-ambient CO2), and 3 maize seeds were sown in each bucket at 2 cm soil depth. No pesticides were applied during the entire experimental period and the manual weeding keep the maize buckets weed-free during the experiment. The rhizosphere soil was sampled from each bucket 1 day before planting, 14 days after planting, and at harvest and measured the relative density of AB (Sequence specific primers of A. brasilense NifH gene: F'-CAAGGGCACCATCCCGAC; R'-CTGCTGCTCCTCCTCGAC) and AC (Sequence specific primers of A. chroococcum nifH gene: F'- GTGACCCGAAAGCTGACTCC; R'-CCACCTTTCACGTCTTCC) using RT-PCR (Table 1).

**Insect source and rearing**

The colony of armyworm *M. separata* was originated from a population collected in maize fields in Kangbao County, Hebei province of China (41.87°N, 114.6°E) in the summer of 2014, and fed on artificial diet and maintained for more than 10 generations in climate-controlled growth chambers (GDN-400D-4; Ningbo Southeast Instrument CO., LTD, Ningbo, China) at 26 ± 1°C, 65 ± 5% RH, and 14:10h L/D photoperiod. The same rearing conditions were maintained for the following experiments. Newly-hatched larvae were randomly selected from the above colony of *M. separata* and fed on artificial diet (Bi, 1983) until the 2nd instar larvae, and then the 3rd instar larvae were individually fed on excised leaves of the experimental plants from CO2 chambers. Feeding trials were conducted in plastic dish (6 cm in diameter and 1.6 cm in height) and the experimental leaves were randomly selected from 6 buckets for each of the 12 experimental treatment combinations (two cultivars x two CO2 treatments x 3 bacteria inoculations) during the heading stage until pupation. Sample size for the *M. separata* larval feeding trial consisted of 20 individuals (sample unit size) with 5 replicates for each of the 12 treatment combinations (i.e., 1200 larvae evaluated for the entire study). Because of the cannibalism among the late instar larvae of *M. separata* (Jiang et al., 2016; Ali et al., 2016; Liu et al., 2017), the sampled individuals were reared separately in the Petri dish until pupation.

**Development and reproduction of *M. separata***

Larval development was evaluated from 3rd instar to pupation by way of observing each individual petri dish every 8 h and recording the timing of larval ecdysis, pupation, and emergence of *M. separata* moths. After eclosion, the newly emerged moths were paired (female: male=1: 1) for mating in a metal frame screen cage (length × width × height=35 cm × 35 cm × 40 cm), and the paired moths were fed with a 10% honey solution provided on a large cotton wick in a single plastic cup (diameter × height=8 cm × 20 cm).
covered with cotton net yarn butter paper for ovipositing. The cotton net yarn and butter paper were replaced every day. Moth survivorship and oviposition were recorded daily until both moths from each pair died.

**Food utilization of the larvae of *M. separata***

Each 3rd instar test larvae of *M. separata* was weighed at the initiation of the feeding trial by using an electronic balance (AL104, METTLER-TOLEDO, Switzerland). Total accumulated feces from 3rd instar until the larva entered pupal stage (6th instar), 6th instar larval weight, and the remaining leaves were also weighed. The food utilization indices of *M. separata* included the relative growth rate (RGR), relative consumption rate (RCR), approximate digestibility (AD), efficiency of conversion of ingested food (ECI) and efficiency of conversion of digested food (ECD) (Chen et al., 2005a; Chen et al., 2005b). Formulas for calculation of the measured indices were adapted from Chen *et al.* (2005b).

**Data analysis**

All data were analyzed using the statistical software SPSS 19.0 (2015, SPSS Institute, Chicago, IL). Four-way analysis of variance (ANOVA) was used to analyze the effects of CO$_2$ levels (elevated vs. ambient), transgenic treatment (*Bt* maize vs. non-*Bt* maize), azoto infection (AB and AC vs. CK), sampling years (2016 vs. 2017), and the interactions on the measured indices of growth, development, and reproduction, including larval life-span, pupation rate, pupal weight, pupal duration, adult longevity and fecundity of *M. separata*. The measured food utilization indices were analyzed by using an analysis of covariance (ANCOVA) with initial weight of *M. separata* (i.e., 3rd instar larva) as a covariate for RCR and RGR, while food consumption was a covariate for ECI and AD to correct the effect of variation in the growth and food assimilation of *M. separata* (Raubenheimer & Simpson, 1992); food assimilated was also used as a covariate to analyze the ECD parameter (Hägle, 1999). The assumption of a parallel slope between covariate and dependent variable was satisfied for each analysis. Treatment means were separated by using the Duncan-test to examine significant difference at $P<0.05$.

**Results**

**Effects of CO$_2$ level, transgenic treatment and azoto infection on the rhizosphere soil densities of *A. brasilense* and *A. chroococcum* in different sampling period**

Significant effects of azoto infection ($P<0.001$) were observed on the measured rhizosphere soil densities of *A. brasilense* (AB) and *A. chroococcum* (AC) 14 days after maize planting. Compared with ambient CO$_2$, elevated CO$_2$ significantly increased the rhizosphere soil densities of both *A. brasilense* and *A. chroococcum*; compared with the control buffer solution (CK), azoto infection significantly increased the rhizosphere soil densities of *A. brasilense* and *A. chroococcum* ($P<0.001$; Table 1). CO$_2$ level and azoto infection ($P<0.001$) both significantly affected the measured rhizosphere soil densities of *A. brasilense* and *A. chroococcum* at maize harvest (Table 2).

**Effects of CO$_2$ level, transgenic treatment and azoto infection on the development and reproduction of *M. separata***
CO$_2$ level and transgenic treatment both significantly affected the larval life-span, pupation rate, pupal weight and duration, adult longevity, and fecundity in *M. separata* fed on both *Bt* and non-*Bt* maize infected with *A. brasilense* and *A. chroococcum* ($P<0.001$). However, the azoto infection significantly affected the larval life-span, pupal duration ($P<0.05$) and fecundity ($P<0.001$) of *M. separata* fed on both cultivars and at both CO$_2$ levels (Table 3).

Compared with ambient CO$_2$, elevated CO$_2$ significantly prolonged the larval life-span (+6.21%), pupal duration (+5.56%), and significantly decreased the pupation rate (-18.08%), pupal weight (-8.12%), adult longevity (-6.06%) and fecundity (-22.58%) of *M. separata* ($P<0.05$; Table 4). Also, compared with the buffer control, azoto infection with *A. brasilense* and *A. chroococcum* both significantly shortened the larval life-span (-5.20% and -5.70%), pupal duration (-3.68% and -3.81%) and fecundity (-10.20% and -9.53%) of *M. separata* ($P<0.05$; Table 4). Moreover, *Bt* maize significantly prolonged the larval life-span (+13.67%) and pupal duration (+7.54%), shortened the adult longevity (-10.41%), and decreased the pupation rate (-75.55%), pupal weight (-13.54%) and fecundity (-75.46%) of *M. separata* compared to that for non-*Bt* maize ($P<0.05$; Table 4).

**Impacts of CO$_2$ level, transgenic treatment and azoto infection on the food utilization of *M. separata***

There were significant effects of CO$_2$ level, transgenic treatment, and azoto infection ($P<0.01$ or $P<0.001$) on food utilization of *M. separata* fed on both *Bt* and non-*Bt* maize infected with *A. brasilense* and *A. chroococcum* at both CO$_2$ levels in both years of the study (Table 3).

Compared with ambient CO$_2$, elevated CO$_2$ significantly reduced the RGR (-9.95%), ECD (-16.05%) and ECI (-17.95%), and significantly enhanced the RCR (+10.44%) and AD (+5.59%) of *M. separata* ($P<0.05$; Table 4). Compared with the buffer control, azoto infection with *A. brasilense* and *A. chroococcum* both significantly decreased the ECD (-9.28% and -7.48%) and ECI (-9.22% and -7.91%), and significantly increased the RGR (+4.75% and +5.56%), RCR (+6.78% and +7.53%) and AD (+5.28% and +4.93%) in *M. separata* ($P<0.01$; Table 4). Moreover, significant decreases in RGR (-13.85%), ECD (-41.25%) and ECI (-31.97%), and significant increases in RCR (+16.60%) and AD (+7.88%) were found when *M. separata* fed on *Bt* maize compared to that on non-*Bt* maize ($P<0.05$; Table 4).

**Interactive influence of CO$_2$ level, transgenic treatment, and azoto infection on growth, development and reproduction of *M. separata***

In addition to the significant main effects of CO$_2$ level, transgenic treatment, and azoto infection, there were significant two-way and three-way interaction of these three main effects on larval life-span, pupation rate, pupal weight and duration, adult longevity, and fecundity of *M. separata* fed on *Bt* and non-*Bt* maize infected with *A. brasilense* and *A. chroococcum* under both CO$_2$ levels in both years of the study ($P<0.05$, $P<0.01$ or $P<0.001$; Table 3).

**Cultivar × CO$_2$**

Similar trends were found in the measured growth, development and reproduction indexes of *M. separata* fed on both *Bt* and non-*Bt* maize cultivars grown under elevated CO$_2$ in contrast to ambient CO$_2$, infected with *A. brasilense* (AB) and *A. chroococcum* (AC) as well as the control buffer (CK) in 2016 and 2017.
Compared with ambient CO\textsubscript{2}, elevated CO\textsubscript{2} significantly prolonged the larval life-span (\textit{Bt maize}: +6.44%; \textit{non-Bt maize}: +8.39%) and pupal duration (\textit{non-Bt maize}: +7.27%) and shortened the adult longevity (\textit{non-Bt maize}: -6.19%), and significantly decreased the pupation rate (\textit{non-Bt maize}: -20.81%), pupal weight (\textit{Bt maize}: -7.03%; \textit{non-Bt maize}: -13.73%) and fecundity (\textit{Bt maize}: -29.43%; \textit{non-Bt maize}: -18.85%) when \textit{M. separata} fed on \textit{Bt} maize and \textit{non-Bt} maize (\(P<0.05\); Fig. 1 A-F).

\textbf{Cultivar \times Azoto}

Inverse trend was found in the measured growth, development and reproduction indexes of \textit{M. separata} fed on \textit{Bt} maize and \textit{non-Bt} maize, which were infected with \textit{A. brasilense} (\textit{AB}) and \textit{A. chroococcum} (\textit{AC}) under ambient and elevated CO\textsubscript{2} in 2016 and 2017 (Fig. 1 G-L). Compared with the buffer control (CK), azoto infection significantly prolonged the larval life-span (\textit{AB}: +7.63%; \textit{AC}: +8.45%), pupal duration (\textit{AB}: +4.53%; \textit{AC}: +5.08%) and shortened the adult longevity (\textit{AB}: -4.88%; \textit{AC}: -6.94%), and decreased pupation rate (\textit{AB}: -20.83%; \textit{AC}: -30.81%), pupal weight (\textit{AB}: -7.24%; \textit{AC}: -10.65%) and fecundity (\textit{AB}: -48.36%; \textit{AC}: -64.60%) when \textit{M. separata} larvae fed on \textit{Bt} maize and \textit{non-Bt} maize (\(P<0.01\); Fig. 1 G-L); and azoto infection significantly shortened the larval life-span (\textit{AB}: -7.97%; \textit{AC}: -10.15%) and pupal duration (\textit{AB}: -5.36%; \textit{AC}: -6.08%) and prolonged the adult longevity (\textit{AB}: +3.95%; \textit{AC}: +5.62%), and increased pupation rate (\textit{AC}: +9.73%), pupal weight (\textit{AB}: +6.27%; \textit{AC}: +8.16%) and fecundity (\textit{AB}: +9.75%; \textit{AC}: +16.16%) when \textit{M. separata} larvae fed on \textit{non-Bt} maize (\(P<0.01\); Fig. 1 G-L).

\textbf{CO\textsubscript{2} \times Azoto}

Similar trends were found in the larval life-span, pupation rate, pupal weight and pupal duration, while inverse trends were observed in adult longevity and fecundity of \textit{M. separata} under ambient and elevated CO\textsubscript{2} (\textit{AB}) as well as the control buffer (CK) in 2016 and 2017 (Fig. 1 M-R). Compared with the buffer control (CK), azoto infection significantly shortened the larval life-span (\textit{AB}: -4.98%; \textit{AC}: -7.84%) and pupal duration (\textit{AB}: -5.01%; \textit{AC}: -5.01%) of \textit{M. separata} under ambient CO\textsubscript{2}, and significantly shortened the larval life-span (\textit{AB}: -4.98%; \textit{AC}: -5.11%) and decreased the pupal weight (\textit{AC}: -4.77%) under elevated CO\textsubscript{2} (\(P<0.05\); Fig. 1 M-P); and azoto infection significantly decreased the adult longevity (\textit{AB}: -4.63%; \textit{AC}: -5.09%) and fecundity (\textit{AB}: -22.90%; \textit{AC}: -22.58%) of \textit{M. separata} under elevated CO\textsubscript{2} (\(P<0.05\); Fig. 1 Q and R).

\textbf{Cultivar \times CO\textsubscript{2} \times Azoto}

There were opposite trends in the measured growth, development and reproduction indexes of \textit{M. separata} fed on \textit{Bt} maize and \textit{non-Bt} maize infected with \textit{A. brasilense} (\textit{AB}) and \textit{A. chroococcum} (\textit{AC}) compared with the buffer control (CK) in 2016 and 2017 regardless of CO\textsubscript{2} level (Fig. 2). In comparison with the buffer control, azoto infection with \textit{A. brasilense} and \textit{A. chroococcum} both significantly prolonged the larval life-span and pupal duration of \textit{M. separata} fed on \textit{Bt} maize, and significantly shortened the larval life-span and pupal duration of \textit{M. separata} fed on \textit{non-Bt} maize under the same CO\textsubscript{2} level; and azoto infection with \textit{A. brasilense} and \textit{A. chroococcum} both significantly reduced the pupation rate, pupal weight, adult longevity and fecundity of \textit{M. separata} fed on \textit{Bt} maize, and significantly...
enhanced the pupation rate, pupal weight, adult longevity and fecundity of *M. separata* fed on non-Bt maize under the same CO$_2$ level. Moreover, compared with ambient CO$_2$, there were opposite trends in the larval life-span, pupal weight, pupal duration, adult longevity and fecundity of *M. separata* fed on Bt maize infected with *A. brasilense* and *A. chroococcum* compared with the buffer control under elevated CO$_2$ in both years; compared with ambient CO$_2$, elevated CO$_2$ significantly decreased the pupation rate of *M. separata* fed on non-Bt maize, and decreased the pupation rate, pupal weight, adult longevity and fecundity of *M. separata* fed on non-Bt maize infected with *A. brasilense* and *A. chroococcum* compared with the buffer control in both years.

**Interactive effects of CO$_2$ level, transgenic cultivar treatment, and azotobacter infection on food utilization of *M. separata***

In addition to significant main effects of CO$_2$ level, transgenic cultivar, and azoto infection, two- and three-way interactions of these factors influenced the RGR, RCR, AD, ECD and ECI of *M. separata* larvae fed on Bt maize and non-Bt maize infected with *A. brasilense* and *A. chroococcum* under ambient and elevated CO$_2$ in both years (*P*<0.05, *P*<0.01 or *P*<0.001; Table 3).

**Cultivar × CO$_2$**

Similar trends were found in the measured food utilization indexes of *M. separata* fed on Bt maize (Bt) and non-Bt maize (Xy) grown under elevated CO$_2$ in contrast to ambient CO$_2$, infected with *A. brasilense* (AB) and *A. chroococcum* (AC) as well as the control buffer (CK) in 2016 and 2017 (Fig. 3 A-E). Compared with ambient CO$_2$, elevated CO$_2$ significantly decreased the RGR (non-Bt maize: -7.34%), ECD (Bt maize: -9.67%; non-Bt maize: -10.25%) and ECI (Bt maize: -8.53%; non-Bt maize: -8.89%), and significantly increased the RCR (Bt maize: +9.69%; non-Bt maize: +6.37%) when *M. separata* larvae fed on Bt maize and non-Bt maize (*P*<0.05; Fig. 3 A-E).

**Cultivar × Azoto**

Inverse trend was found in the measured food utilization indexes of *M. separata* fed on Bt maize and non-Bt maize, which were infected with *A. brasilense* (AB) and *A. chroococcum* (AC) under ambient and elevated CO$_2$ in 2016 and 2017 (Fig. 3 F-J). Compared with the buffer control (CK), azoto infection significantly enhanced the RGR (AB: +9.53%; AC: +11.78%), ECD (AB: +11.61%; AC: +19.79%) and ECI (AB: +10.08%; AC: +15.79%), and significantly decreased the RCR (AC: -6.52%) and AD (AC: -6.19%) when *M. separata* larvae fed on non-Bt maize (*P*<0.001; Fig. 3 F-J); and azoto infection significantly decreased the RGR (AB: -9.62%; AC: -10.41%), ECD (AB: -34.32%; AC: -41.55%) and ECI (AB: -20.16%; AC: -25.28%), and significantly increased the RCR (AB: +14.99%; AC: +19.06%) and AD (AB: +9.60%; AC: +10.79%) when *M. separata* larvae fed on Bt maize (*P*<0.001; Fig. 3 F-J).

**CO$_2$ × Azoto**

Similar trends were observed in RGR, RCR and AD, while inverse trends were shown in ECD and ECI of *M. separata* under ambient and elevated CO$_2$, which fed on Bt maize and non-Bt maize infected with A.
brasilense (AB) and A. chroococcum (AC) versus control buffer (CK) (Fig. 3 K-O). Compared with the buffer control (CK), azoto infection significantly decreased ECD (AB: -20.71%; AC: -22.07%) and ECI (AB: -12.77%; AC: -12.89%) of M. separata larvae under elevated CO₂, and significantly increased ECD (AB: +5.35%; AC: +8.04%) and ECI (AB: +7.43%; AC: +9.85%) of M. separata larvae under ambient CO₂ (P<0.05; Fig.3); and azoto infection significantly enhanced RGR (AB: +3.32% and +7.40%; AC: +5.14% and +8.67%), RCR (AB: +9.78% and +5.29%; AC: +11.32% and +5.93%) and AD (AB: +7.34% and +4.18%; AC: +7.92% and +4.66%) under elevated and ambient CO₂, respectively (P<0.01; Fig. 3 K-O).

Cultivar × CO₂ × Azoto

There were opposite trends in the measured food utilization indexes of M. separata larvae fed on Bt maize (Bt) and non-Bt maize infected with A. brasilense (AB) and A. chroococcum (AC) compared with the buffer control (CK) in both years regardless of CO₂ level (Fig.4). In comparison with the buffer control, azoto infection with A. brasilense and A. chroococcum both significantly decreased RGR, ECD and ECI of M. separata fed on Bt maize, and significantly increased RGR, ECD and ECI of M. separata fed on non-Bt maize under the same CO₂ level; and azoto infection with A. brasilense and A. chroococcum both significantly enhanced RCR and AD of M. separata fed on Bt maize, and significantly reduced RCR and AD of M. separata larvae fed on non-Bt maize under the same CO₂ level. Moreover, compared with ambient CO₂, elevated CO₂ significantly increased RCR and AD, and significantly decreased RGR, ECD, and ECI of M. separata larvae fed on same type of maize cultivar infected with A. brasilense and A. chroococcum in both years (P<0.05; Fig.4). Furthermore, there were significant decreases in RGR, ECD, and ECI, and significant increases in RCR and AD of M. separata larvae fed on Bt maize in contrast to non-Bt maize infected with same type of azoto species within the same CO₂ level in both years.

Discussion

Insects are sensitive to environmental variations, and environmental stresses can cause changes on their growth, development, fecundity, food utilization and the occurrence and distribution of populations as a result of metabolic rate fluctuation (Bloom et al., 2010). Elevated CO₂ negatively affected the larval survival, weight, duration, pupation, and adult emergence of cotton bollworm, Helicoverpa armigera (Akbar et al., 2016), and reduced the egg laying by Cactus moth Cactoblastis cactorum (Stange et al., 1997) and Achaea Janata (Rao et al., 2016). In this study, elevated CO₂ significantly prolonged larval and pupal duration and decreased pupation rate and pupal weight of M. separata compared to ambient CO₂. Relative growth rates (i.e., RGR) of Gypsy moth (Lymantria dispar) were reported to be reduced by 30% in larvae fed on Quercus petraea exposed to elevated CO₂ (Hattenschwiler & Schafellner, 2004). Relative consumption rate (i.e., RCR) was significantly higher for H. armigera larva fed maize grown at 375 and 750 ppm CO₂ in contrast to ambient CO₂ condition, and elevated CO₂ significantly decreased the efficiency of conversion of ingested food (i.e., ECI), the efficiency of conversion of digested food (i.e., ECD), and the RGR of H. armigera larvae compared with ambient CO₂ (Yin et al., 2010). In this study, elevated CO₂ significantly increased the RCR (+10.44%) and the approximate digestibility (+5.59%) (i.e., AD), and significantly reduced the RGR (-9.95%), ECD (-16.05%) and ECI (-17.95%) of M. separata
larvae compared with ambient CO$_2$. According to the “Nutrition compensation hypothesis”, elevated CO$_2$

332
can affect the development fitness of herbivores by changing the nutritional components, above and
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below-ground biomass, and photosynthetic rate of host plants indirectly (Ainsworth & Rogers, 2007;
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Jackson et al., 2009; Zavala et al., 2013), including increased C/N ratio and decreased nitrogen content etc.
335
Declined growth rate, reproduction, and survival rate were found in the chewing mouthparts insects (e.g.
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*Helicoverpa armigera*, *Spodoptera exigua*, *Mythimna separata*), and the food consumption of which
337
increased so that they could obtain necessary nutrition to survive (Bottomley et al., 1993; Rogers et al.,
338
2006). Yin *et al.* (2010) reported that elevated CO$_2$ increased the food consumption and prolonged the
devlopment time of *H. armigera*, which due to the reduced nutritional quality of maize leaves, as a result
of reduced nitrogen content and increased C/N ratio. Elevated CO$_2$ significantly reduced the food
conversion rate and enhanced the food ingestion of *H. armigera*, which might reduce nitrogen
content of the cotton, Simian-3 (Chen et al., 2005a; Chen et al., 2005b). Thus, Chen *et al.* (2005a; 2005b)
339
inferred that elevated CO$_2$ might be unfavorable to *H. armigera*. Our results in maize system appear to be
similar to the study by Chen *et al.* (2005a; 2005b) in a cotton system.

340
Although the transgenic corn, *Zea mays* L., hybrids expressing the Cry insecticidal protein from
*Bacillus thuringiensis* (Bt) were developed to control *Helicoverpa zea*, *Ostrinia nubilalis*, *Spodoptera
frugiperda* and *Mythimna separata* (Ostlie et al., 1997; Koziel et al., 1993; Armstrong et al., 1995;
341
Jouanin et al., 1998; Lynch et al., 1999), few studies focused on the defense responses of transgenic
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cry1Ie maize to corn armyworm under elevated CO$_2$, especially on the growth, development and food
utilization of the pest insects. Prutz & Dettner (2005) reported that the transgenic *Bacillus
343
thuringiensis*-maize could result in decreased growth rate and increased mortality, which might attribute to
the termination of larval metamorphosis. Most studies showed that adverse effects on life-table parameters
of different herbivores were direct by the Cry protein (Lawo *et al.*, 2010), which might be due to the
interaction of feeding inhibitors and growth inhibitors (e.g. secondary plant substances) (Smith & Fischer,
1983). Effects of elevated CO$_2$ on the plant nutrition, metabolism and secondary defense metabolism
344
might adverse for the growth, development and nutrition utilization of herbivores (Akbar *et al.*, 2016).
345
The insects possessed more nutrients to meet their growth needs and prolong the food digestion time in
the midgut so that the RCR and AD increased (Reynolds, 1985). In this study, we found that some
346
negative effects of transgenic cry1Ie maize (Bt) and Xianyu335 (Xy) grown in elevated CO$_2$ on the food
utilization indices (including RGR, ECD and ECI) of *M. separata* larvae and some positive effects on the
347
RCR and AD, which indicated that the resistance responses of Bt maize might persist under elevated CO$_2$
348
and *M. separata* might ingest more food to get enough nutrition for surviving in limited developmental
time under elevated CO$_2$. Meanwhile the Bt maize and its parental line (Xianyu 335) prolonged their
larval life-span and pupal duration, decreased growth rate and increased mortality that might result in
lowering of pests’ occurrence. According to the “carbon nutrition balance hypothesis” (Gebauer *et al*.,
1997), elevated CO$_2$ would increase the fixed organic matter in plant while increase C-based secondary
metabolites and decrease N-based secondary metabolites, thus affecting the insects resistance of plants.
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Robinson *et al.* (2012) indicated that elevated CO$_2$ increased 19% phenols, 22% condensed tannins and 27%
350
flavonoids, while the terpenoids and NBSC decreased by 13% and 16% respectively. Coviella (2002)
anticipated that the primary CO$_2$ effect on Bt toxin production would be due to differences in N concentration within the plant. In a meta-analytical review of 33 studies that simultaneously increased carbon dioxide conditions compared to ambient conditions, Zvereva & Kozlov (2006) showed that nitrogen concentration in plants was reduced under elevated CO$_2$, and this decrease was stronger for woody compared to herbaceous plants. If conditions of increased carbon (e.g. elevated CO$_2$) allow plants to allocate significantly more resources to condensed tannins and gossypol, then the enzyme composition in the insect herbivore is expected to also change. Similarly, if Bt toxin production changes due to elevated CO$_2$, then the insect herbivore’s body enzymes should also be changed in this circumstance.

Most of nitrogen, however, is found in the form of nitrogen gas (N$_2$) which approximately amounts to 78% in the atmosphere. As plants cannot use this form of nitrogen directly, some microbes can change the nitrogen gas into ammonia. Most free living microbes in soil which can fix nitrogen and whose activities in enhancing the growth of plants are bacteria namely Azotobacter sp. and Azospirillum sp. These two bacteria are particularly important in maize production system due to their greater nitrogen fixing ability. Azospirillum acquires carbohydrate directly from sieve tube as a resource of carbon which promotes its growth (Olivera et al., 2004). Azospirillum can be used to promote the growth of sprouts under normal and arid conditions (Alejandra et al., 2009). Azospirillum also provides more flexibility to cell wall which enhances the growth (Pereyra et al., 2010) and increases products of wheat in waterless plot of land (Martin et al., 2009). Furthermore, azospirillum had the highest efficiency in nitrogen fixation at the root of sweet corn and it would reach the highest point of nitrogen fixation in the week 4 amounting to 0.20 mgNhr$^{-1}$m$^{-2}$ (Toopakuntho, 2010). Azospirillum can also create auxin, a substance promoting growth of maize, of 53.57 mg/ml (Phookkasem, 2011). Therefore, we used techniques of azotobacter (A. brasilense & A. chroococcum) inoculation of maize seeds to stimulate plant N uptake to increase in biomass N relative to C under elevated CO$_2$, increase Bt toxin production for transgenic cry1Ie maize and create a substance promoting maize plant growth. In this study, we found that elevated CO$_2$ significantly enhanced the rhizosphere soil densities both A. brasilense and A. chroococcum. We hypothesize that the elevated CO$_2$ increased the maize root bifurcation and soil nutrition (e.g. carbohydrates, amino acids and multi-trace elements) for azotobacter to provide the living space and nutrition. Other researchers have also shown positive effects of elevated CO$_2$ on the bacterial community in the rhizosphere of maize (Chen, 2012). Moreover, significant adverse effects on the growth, development, reproduction and food utilization of M. separata was observed when the host substrate maize was exposed to azotobacter treatments, which might be attributed to azotobacter stimulating plant N uptake to increase Bt toxin production for transgenic cry1Ie maize and promoting growth of its parental line (Xianyu335).

There was no significant year-to-year variation in our field research data. Therefore, the overall results clearly indicate that increasing CO$_2$ had negative effects on M. separata. Resistance performance of transgenic cry1Ie maize decreased under elevated CO$_2$ as shown by decreased RGR, ECD, and ECI. The azotobacter treatments (A. brasilense & A. chroococcum) had positive effects on improving the effectiveness of Bt maize on target Lepidoptera pest management via decreased RGR, ECD, and ECI of M. separata that fed on transgenic cry1Ie maize and promoting growth of Xianyu 335 via increased RGR, ECD, and ECI of M. separata. Under future predicted climate changes (e.g. elevated CO$_2$), it is
particularly important to understand the field insect resistance traits of resistant crops to target pests. In an environment of accelerated greenhouse effect, Bt maize may have decreased resistance performance in the field with inhibiting effect on the development and food utilization of insects. Therefore, we used techniques of azotobacter (A. brasilense & A. chroococcum) inoculation of maize seeds to stimulate plant N uptake to increase in biomass N relative to C under elevated CO$_2$, increase Bt toxin production for transgenic cry1Ie maize, and create a substance promoting maize growth. This study demonstrates that the use of azotobacter (e.g., A. brasilense and A. chroococcum) as pest control enhancer especially under elevated CO$_2$ is significantly more beneficial in transgenic Bt maize system compared to that in non-transgenic system.

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Competing interests

The authors have no competing interests to declare.

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Data availability

All data are included within the manuscript.
References


40. 155.


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Table 1: The rhizosphere soil densities of azotobacters, *Azospirillum brasilense* (i.e., AB) and *Azotobacter chroococcum* (AC) inoculated in the potted soil of transgenic *Bt* maize (i.e., Bt) and its parental line of non-*Bt* maize (Xy) grown under ambient and elevated CO$_2$ in 2016 and 2017

<table>
<thead>
<tr>
<th>Measure matters</th>
<th>Azoto infections</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampled soil before maize planting (AB; AC copies/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCO$_2$-Bt</td>
<td>5.53 ± 0.24 10$^5$; 4.47 ± 0.12 10$^5$</td>
<td>5.61 ± 0.11 10$^5$; 4.33 ± 0.17 10$^5$</td>
<td></td>
</tr>
<tr>
<td>aCO$_2$-Xy</td>
<td>8.46 ± 0.24 10$^{11}$; 4.48 ± 0.26 10$^5$</td>
<td>8.40 ± 0.28 10$^{11}$; 4.44 ± 0.11 10$^5$</td>
<td></td>
</tr>
<tr>
<td>eCO$_2$-Bt</td>
<td>8.25 ± 0.26 10$^{11}$; 4.21 ± 0.08 10$^5$</td>
<td>8.69 ± 0.23 10$^{11}$; 4.56 ± 0.22 10$^5$</td>
<td></td>
</tr>
<tr>
<td>eCO$_2$-Xy</td>
<td>8.36 ± 0.19 10$^{11}$; 4.43 ± 0.15 10$^5$</td>
<td>8.59 ± 0.21 10$^{11}$; 4.47 ± 0.17 10$^5$</td>
<td></td>
</tr>
<tr>
<td>Sampled soil at the maize seedling after 14 days (AB; AC copies/g)</td>
<td></td>
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</tr>
<tr>
<td>aCO$_2$-Xy</td>
<td>5.73 ± 0.24 10$^5$; 7.29 ± 0.17 10$^5$</td>
<td>5.36 ± 0.22 10$^5$; 7.66 ± 0.25 10$^5$</td>
<td></td>
</tr>
<tr>
<td>eCO$_2$-Bt</td>
<td>5.62 ± 0.30 10$^5$; 7.71 ± 0.15 10$^5$</td>
<td>5.13 ± 0.04 10$^5$; 7.32 ± 0.13 10$^5$</td>
<td></td>
</tr>
<tr>
<td>eCO$_2$-Xy</td>
<td>5.46 ± 0.28 10$^5$; 7.59 ± 0.17 10$^5$</td>
<td>5.42 ± 0.13 10$^5$; 7.57 ± 0.22 10$^5$</td>
<td></td>
</tr>
<tr>
<td>Sampled soil at the maize harvest (AB; AC copies/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCO$_2$-Bt</td>
<td>5.50 ± 0.29 10$^5$; 4.24 ± 0.15 10$^5$</td>
<td>5.33 ± 0.18 10$^5$; 4.31 ± 0.13 10$^5$</td>
<td></td>
</tr>
<tr>
<td>aCO$_2$-Xy</td>
<td>5.46 ± 0.08 10$^5$; 4.26 ± 0.18 10$^5$</td>
<td>5.62 ± 0.31 10$^5$; 4.48 ± 0.21 10$^5$</td>
<td></td>
</tr>
<tr>
<td>eCO$_2$-Bt</td>
<td>5.46 ± 0.18 10$^5$; 4.76 ± 0.23 10$^5$</td>
<td>5.47 ± 0.17 10$^5$; 4.21 ± 0.09 10$^5$</td>
<td></td>
</tr>
<tr>
<td>eCO$_2$-Xy</td>
<td>5.57 ± 0.31 10$^5$; 7.27 ± 0.26 10$^{11}$b; 4.65 ± 0.21 10$^5$b; 4.01 ± 0.26 10$^5$b</td>
<td>8.39 ± 0.26 10$^{11}$b; 4.01 ± 0.26 10$^5$b</td>
<td></td>
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</tbody>
</table>
| eCO$_2$-Bt | 8.44 ± 0.15 10$^{11}$b; 4.11 ± 0.23 10$^5$ | 8.65 ± 0.19 10$^{11}$b; 4.30 ± 0.18 10$^5$ |}

Note: Azoto infections: *A. brasilense* (AB) and *A. chroococcum* (AC) vs. the control buffer solution (CK). CO$_2$ levels: ambient CO$_2$ (aCO$_2$) and elevated CO$_2$ (eCO$_2$). Transgenic treatment: *Bt* maize (Bt) and non-*Bt* maize (Xy). Data in table are average ± SE. Different lowercase and uppercase letters indicate significantly different between ambient CO$_2$ and elevated CO$_2$ for same maize cultivar in same year, and between *Bt* maize and non-*Bt* maize under same CO$_2$ level in same year by the Duncan test at $P<0.05$, respectively.

587
Table 2 Four-way ANOVA on the rhizosphere soil densities of *Azospirillum brasilense* (AB) and *Azotobacter chroococcum* (AC) inoculated in the potted soil of *Bt* maize and its parental line of non-*Bt* maize treatments grown under ambient and elevated CO₂ in 2016 and 2017 (F/P values)

<table>
<thead>
<tr>
<th>Impact factors</th>
<th>Sampled soil at the maize seedling after 14 days</th>
<th>Sampled soil at the maize harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AB</td>
<td>AC</td>
</tr>
<tr>
<td>Y^a</td>
<td>0.00/0.99</td>
<td>0.002/0.96</td>
</tr>
<tr>
<td>Cv^b</td>
<td>0.32/0.574</td>
<td>0.36/0.55</td>
</tr>
<tr>
<td>CO₂^c</td>
<td>0.19/0.89</td>
<td>0.80/0.38</td>
</tr>
<tr>
<td>Azoto^d</td>
<td>2555.00/&lt;0.001***</td>
<td>1380.37/&lt;0.001***</td>
</tr>
<tr>
<td>Y × Cv.</td>
<td>0.62/0.44</td>
<td>1.96/0.17</td>
</tr>
<tr>
<td>Y × CO₂</td>
<td>1.21/0.28</td>
<td>2.50/0.12</td>
</tr>
<tr>
<td>Y × Azoto</td>
<td>0.00/1.00</td>
<td>0.02/0.99</td>
</tr>
<tr>
<td>Cv. × CO₂</td>
<td>0.35/0.55</td>
<td>0.010/0.92</td>
</tr>
<tr>
<td>Cv. × Azoto</td>
<td>0.32/0.73</td>
<td>0.36/0.70</td>
</tr>
<tr>
<td>CO₂ × Azoto</td>
<td>0.019/0.98</td>
<td>0.80/0.45</td>
</tr>
<tr>
<td>Y × Cv. × CO₂</td>
<td>5.99/0.018*</td>
<td>0.06/0.94</td>
</tr>
<tr>
<td>Y × Cv. × Azoto</td>
<td>0.62/0.54</td>
<td>1.96/0.15</td>
</tr>
<tr>
<td>Y × CO₂ × Azoto</td>
<td>1.21/0.31</td>
<td>2.50/0.093</td>
</tr>
<tr>
<td>Cv. × CO₂ × Azoto</td>
<td>0.35/0.70</td>
<td>0.10/0.99</td>
</tr>
<tr>
<td>Y × Cv. × CO₂ × Azoto</td>
<td>5.99/0.05</td>
<td>0.006/0.99</td>
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</table>

**Note:** *P*<0.05, **P**<0.01, ***P***<0.001; ^a: Year (2016 vs. 2017); ^b: Transgenic treatment (*Bt* maize vs. non-*Bt* maize); ^c: CO₂ levels (elevated CO₂ vs. ambient CO₂); ^d: Azoto infection (*A.brasilese* and *A. chroococcum* vs. the buffer control).
<table>
<thead>
<tr>
<th>Impact factors</th>
<th>Larval life-span (day (n=828))</th>
<th>Pupation rate (% (n=828))</th>
<th>Pupal weight (g (n=663))</th>
<th>Pupal duration (day (n=663))</th>
<th>Adult longevity (day (n=576))</th>
<th>Fecundity (eggs per female (n=198))</th>
<th>The 3rd-6th instar larvae (n=828)</th>
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<tbody>
<tr>
<td></td>
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<td>FGR (mg g⁻¹day⁻¹)</td>
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<td>Covariateæ</td>
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<td>1.81/0.11</td>
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<tr>
<td>Y×</td>
<td>1.62/0.32</td>
<td>0.94/0.71</td>
<td>1.21/0.62</td>
<td>0.11/0.90</td>
<td>1.65/0.33</td>
<td>2.99/0.10</td>
<td>1.19/0.31</td>
</tr>
<tr>
<td>Cv.ª</td>
<td>1662.03/0.001* 329.16/0.001* 275.04/0.001* 229.78/0.001* 450.27/0.001* 2032.99/0.001* 1545.53/0.001* 302.67/0.001* 185.62/0.001* 716.17/0.001* 1038.95/0.001* 1038.95/0.001*</td>
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<tr>
<td>CO₂¢</td>
<td>62.13/0.001*** 55.53/0.001*** 12.44/0.001*** 19.02/0.001*** 41.62/0.001*** 278.70/0.001*** 67.09/0.001*** 27.98/0.001*** 9.69/0.003*** 35.90/0.001*** 57.98/0.001***</td>
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<tr>
<td>Azotoδ</td>
<td>3.64/0.034* 2.53/0.077 1.20/0.63 7.04/0.011* 0.35/0.70 27.54/0.001*** 12.26/0.001*** 26.84/0.001*** 35.28/0.001*** 13.64/0.001*** 7.22/0.002***</td>
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<tr>
<td>Y× Cv.</td>
<td>9.22/0.004** 2.22/0.14 2.22/0.14 1.98/0.32 3.10/0.054 0.102/0.75 4.27/0.049* 5.69/0.021* 1.86/0.18 19.21/0.001*** 15.64/0.001***</td>
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<tr>
<td>Y× CO₂</td>
<td>5.33/0.025* 2.16/0.20 0.067/0.80 0.17/0.85 13.53/0.001*** 2.74/0.10 6.90/0.012* 4.82/0.033* 6.73/0.013* 6.22/0.016* 3.41/0.071</td>
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<tr>
<td>Y× Azoto</td>
<td>3.63/0.034* 1.69/0.058 0.63/0.57 0.48/0.71 0.59/0.64 1.87/0.053 5.04/0.010* 0.17/0.84 0.27/0.77 0.43/0.65 0.25/0.78</td>
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<tr>
<td>Cv.× CO₂</td>
<td>19.11/0.001*** 6.62/0.001*** 2.68/0.008** 14.11/0.001*** 8.26/0.006** 5.86/0.049* 30.44/0.001*** 7.39/0.009** 1.40/0.043* 1.80/0.017* 1.61/0.011*</td>
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<tr>
<td>Cv.× Azoto</td>
<td>224.53/0.001*** 73.67/0.001*** 30.41/0.001*** 42.06/0.001*** 26.38/0.001*** 195.08/0.001*** 213.46/0.001*** 48.31/0.001*** 73.42/0.001*** 132.82/0.001*** 144.91/0.001***</td>
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<tr>
<td>CO₂× Azoto</td>
<td>12.81/0.001*** 12.22/0.004** 11.63/0.005** 14.98/0.001*** 4.24/0.02* 22.02/0.001*** 13.25/0.001*** 6.70/0.003* 9.78/0.001*** 13.01/0.001*** 12.63/0.001***</td>
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<tr>
<td>Y× Cv.× CO₂</td>
<td>0.01/0.92 1.07/0.55 0.10/0.75 1.16/0.52 0.006/0.94 4.25/0.085 0.220/0.64 1.83/0.18 4.16/0.047* 0.743/0.39 0.56/0.46</td>
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<tr>
<td>Y× Cv.× Azoto</td>
<td>1.55/0.33 1.53/0.17 0.06/0.94 2.02/0.23 0.46/0.68 8.87/0.071 3.27/0.047* 0.55/0.58 2.23/0.12 4.01/0.025* 1.41/0.25</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Y× CO₂× Azoto</td>
<td>0.063/0.69 0.51/0.76 0.48/0.62 1.72/0.45 1.78/0.18 14.61/0.045* 0.72/0.49 1.88/0.16 1.33/0.28 2.66/0.080 2.47/0.095</td>
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<td></td>
</tr>
<tr>
<td>Cv.× CO₂× Azoto</td>
<td>8.88/0.006** 12.61/0.003** 7.41/0.011* 5.66/0.005** 13.55/0.002** 24.04/0.000*** 0.62/0.043* 13.24/0.001*** 9.84/0.001*** 9.59/0.001*** 9.92/0.001***</td>
<td></td>
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</tr>
<tr>
<td>Y× Cv.× CO₂× Azoto</td>
<td>0.19/0.83 0.33/0.71 0.14/0.87 0.32/0.72 1.73/0.30 0.36/0.70 0.48/0.62 0.087/0.92 0.98/0.38 0.59/0.56 0.06/0.94</td>
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</tbody>
</table>

Note: æ Initial weight as a covariate for FGR and RGR, and food consumption as a covariate for AD and ECI, and food assimilated as a covariate for ECD.
Table 4 The growth, development, reproduction and food utilization of armyworm, *M. separata* fed on the detached leaves of *Bt* maize (i.e., Bt) and its parental line of non-*Bt* maize (Xy) during the heading stage, infected with azotobacters (Azoto), *A. brasiliense* (i.e., AB) and *A. chroococcum* (AC) as well buffer solution as the control (CK) under ambient and elevated CO₂ in 2016 and 2017

<table>
<thead>
<tr>
<th>Impact factors</th>
<th>Factor levels</th>
<th>Larval life-span (day)</th>
<th>Pupation rate (%)</th>
<th>Pupal weight (g)</th>
<th>Pupal duration (day)</th>
<th>Adult longevity (day)</th>
<th>Fecundity (eggs per female)</th>
<th>The 3rd–6th instar larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Bt</td>
<td>24.19±0.16a</td>
<td>40.98±2.45b</td>
<td>0.192±0.007b</td>
<td>10.98±0.18a</td>
<td>6.63±0.14b</td>
<td>171.50±13.27b</td>
<td>82.79±6.43b</td>
</tr>
<tr>
<td></td>
<td>Xy</td>
<td>21.28±0.17b</td>
<td>71.94±2.38a</td>
<td>0.218±0.006a</td>
<td>10.21±0.19b</td>
<td>7.32±0.09a</td>
<td>300.92±28.64a</td>
<td>94.26±7.16a</td>
</tr>
<tr>
<td>CO₂</td>
<td>Elevated</td>
<td>23.42±0.20a</td>
<td>51.78±2.69b</td>
<td>0.197±0.007b</td>
<td>10.78±0.18a</td>
<td>6.77±0.11b</td>
<td>212.25±19.13b</td>
<td>84.33±8.67b</td>
</tr>
<tr>
<td></td>
<td>Ambient</td>
<td>22.05±0.17b</td>
<td>61.14±2.68a</td>
<td>0.213±0.005a</td>
<td>10.41±0.19b</td>
<td>7.18±0.13a</td>
<td>260.17±21.87a</td>
<td>92.72±6.59a</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>22.53±0.16b</td>
<td>56.06±2.24</td>
<td>0.205±0.004</td>
<td>10.54±0.09b</td>
<td>6.97±0.24</td>
<td>228.00±15.27b</td>
<td>89.64±5.52a</td>
</tr>
<tr>
<td>Azoto infection</td>
<td>AC</td>
<td>22.42±0.15b</td>
<td>55.53±2.01</td>
<td>0.204±0.004</td>
<td>10.53±0.08b</td>
<td>6.96±0.24</td>
<td>229.38±17.46b</td>
<td>90.34±5.52a</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>23.25±0.16a</td>
<td>57.79±2.58</td>
<td>0.206±0.005</td>
<td>10.72±0.13a</td>
<td>6.99±0.18</td>
<td>251.25±23.21a</td>
<td>85.58±6.14b</td>
</tr>
</tbody>
</table>

Note: Data in table are average ± SE. Different lowercase letters indicate significant difference between treatments by the Duncan’s test at *P*<0.05.
Fig.1 Effects of the bi-interactions between transgenic treatment and CO$_2$ level (A–F), between transgenic treatment and azoto infection (G–L) and between CO$_2$ level and azoto infection (M–R) on the growth, development and reproduction of armyworm, *Mythimna separata* fed on the detached leaves of *Bt* maize (i.e., Bt) and its parental line of non-*Bt* maize (Xy) during the heading stage, infected with *Azospirillum brasilense* (i.e., AB) and *Azotobacter chroococcum* (AC) as well buffer solution as the control (CK) under ambient and elevated CO$_2$ in 2016 and 2017.

Larval life-span–A, G, M; Pupation rate–B, H, N; Pupal weight–C, I, O; Pupal duration–D, J, P; Adult longevity–E, K, Q; Fecundity–F, L, R; Each value represents the average (±SE). Different lowercase letters indicate significant differences treatments by the Duncan test at $P<0.05$.

Fig.2 Impacts of the tri-interactions among CO$_2$ level, transgenic treatment and azoto infection on the growth, development and reproduction of *M. separata* fed on the detached leaves of *Bt* maize (i.e., Bt) and non-*Bt* maize (Xy) during the heading stage, infected with *A. brasilense* (i.e., AB) and *A. chroococcum* (AC) as well buffer solution as the control (CK) under ambient and elevated CO$_2$ in 2016 (A–F) and 2017 (G–L). Each value represents the average (+SE).

Different lowercase and uppercase letters, and * indicated significant difference among three types of azoto infection for same type of maize under same CO$_2$ level, between *Bt* maize and non-*Bt* maize for same type of azoto infection under same CO$_2$ level, and between ambient and elevated CO$_2$ for same type of maize and azoto infection by the Duncan test at $P<0.05$ respectively.

Fig.3 Effects of the bi-interactions between transgenic treatment and CO$_2$ level (A–E), between transgenic treatment and azoto infection (F–J) and between CO$_2$ level and azoto infection (K–O) on the food utilization of armyworm, *Mythimna separata* from the 3rd to the 6th instar larvae fed on the detached leaves of *Bt* maize (i.e., Bt) and its parental line of non-*Bt* maize (Xy) during the heading stage, infected with *Azospirillum brasilense* (i.e., AB) and *Azotobacter chroococcum* (AC) as well buffer solution as the control (CK) under ambient and elevated CO$_2$ in 2016 and 2017. RGR–A, F, K; RCR–B, G, L; AD–C, H, M; ECD–D, I, N; ECI–E, J, O; Each value represents the average (±SE).

Different lowercase letters indicate significant differences treatments by the Duncan test at $P<0.05$.

Fig.4 Impacts of the tri-interactions among CO$_2$ level, transgenic treatment and azoto infection on the food utilization of *M. separata* from the 3rd to the 6th instar larvae fed on the detached leaves of *Bt* maize (i.e., Bt) and non-*Bt* maize (Xy) during the heading stage, infected with *A. brasilense* (i.e., AB) and *A. chroococcum* (AC) as well buffer solution as the control (CK) under ambient and elevated CO$_2$ in 2016 (A–E) and 2017 (F–J). Each value represents the average (+SE). Different lowercase and uppercase letters, and * indicated significant difference among three types of azoto infection for same type of maize under same CO$_2$ level, between *Bt* maize and non-*Bt* maize for same type of azoto infection under same CO$_2$ level, and between ambient and elevated CO$_2$ for same type of maize and azoto infection by the Duncan test at $P<0.05$ respectively.
Figure 1
Figure 2
Figure 3
Figure 4