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Insect frass fertilizer upregulates maize defence genes and resistance against an invasive herbivore pest

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The black soldier fly frass fertilizer (BSFFF) has gained global attention as a multipurpose input for soil fertilization and pest and disease management. However, there are limited studies that have examined its effects on insect pest resistance and the underlying mechanisms. We investigated the impact of amending soil with BSFFF on maize growth, defense gene expression and resistance to a polyphagous insect herbivore, *Spodoptera frugiperda* (Lepidoptera: Noctuidae) through larval feeding assay. Maize growth was evaluated by measuring plant height, chlorophyll concentration, and biomass accumulation in soils amended with BSFFF, synthetic fertilizers (Di-ammonium phosphate and Calcium ammonium nitrate) and unfertilized soils at various growth stages. Larval feeding assays were conducted using leaf discs from maize plants grown in different amended soils. The expression level of three maize defense genes: pathogenesis related protein 5 (*pr-5*), maize proteinase inhibitors (*mpi*), and lipoxygenase 3 (*lox-3*) were analyzed using quantitative polymerase chain reaction (qPCR) while yield was assessed through a field trial over two cropping seasons. Maize plants grown in BSFFF amended soils showed 30% more growth, higher chlorophyll, 0.93–2.86 t ha^{-1} higher yield, and 48% better nitrogen use efficiency than from those in synthetic or unfertilized soils. Moreover, *S. frugiperda* larvae consumed significantly less leaf tissue from maize plants grown in BSFFF amended soils than synthetically fertilized and non-fertilized soils. Maize defense genes *pr-5*, *mpi*, and *lox-3* were highly expressed both constitutively and inductively in maize planted in BSFFF amended soils compared to those grown in synthetically fertilized and non-fertilized soils. We observed a significant negative correlation between *mpi* gene expression and larval feeding, suggesting its role in maize resistance. Our results show that soil amendment with BSFFF strengthens plant defense systems and positively impacts plant growth and yield, contributing to increased agricultural productivity and sustainability.

Keywords Black soldier fly frass fertilizer, Maize, Insect resistance, *Spodoptera frugiperda*, Plant performance, Plant defense genes

The intensification of agriculture to meet the needs of a rapidly growing population is a continuous process that demands multi-dimensional approaches to improve crop yield, soil fertility, and resistance to insect pests. Soil amendments using organic and inorganic fertilizers are common strategies for replenishing depleted soil nutrients, thus improving soil fertility, plant growth and crop production[1–3]. However, the effectiveness of these amendments varies depending on the types and application strategies. For instance, revitalizing soil with inorganic fertilizers temporarily improves soil fertility, requiring multiple applications during growing seasons making it costly[4]. Additionally, continuous use of synthetic fertilizers has adverse impacts on human and environmental health, and can increase plant susceptibility to pathogens and herbivore pests[5–9]. For example, Culliney & Pimentel[10] observed that plants grown in soils highly fertilized with inorganic nitrogen had increased populations of mites and aphids. This implies that soil amendment practices that improve soil fertility and plant growth do not necessarily increase plants' capacity to resist herbivore pests.

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Unlike inorganic fertilization, organic amendments maintain long-term enhancement of soil fertility[1]. In addition to its cost-effectiveness, organic fertilizers improve soil chemical properties by increasing availability and uptake of essential nutrients for crop growth and production[4,11,12]. Organic soil amendments also improve soil structure, reduce erosion while strengthening its water holding capacity[13]. They further enhance soil biological quality by promoting beneficial macro- and micro-biotic organisms such as nematodes, springtails, earthworms, ground beetles, bacteria, fungi, and protozoa[14–16]. This underscores the ability of organic soil amendments to restore overall soil health by improving its physical, chemical, and biological properties. Given that plant defenses are intrinsically linked to soil health, enhancing these properties through organic amendments may positively impact soil and plant health[1,9,17–19]. Indeed, soil amendment with organic fertilizer has been shown to improve plant resistance to herbivore pests[8,20]. For instance, European corn borer, *Ostrinia nubilalis* Hubner (Lepidoptera: Crambidae) female moths oviposited more on plants grown in chemically fertilized soils than those organically fertilized[8]. These studies suggest that not all soil amendments improve plant defense against herbivore pests[21,22].

The use of black soldier fly (*Hermetia illucens*, L., Diptera: Stratiomyidae) frass fertilizer (BSFFF) as a novel approach for organic soil amendment is gaining global traction due to its pronounced agroecological benefits. It is generated by treating biodegradable waste with black soldier fly larvae which converts organic waste into safe and enriched organic fertilizer[23,24]. Like many other organic fertilizers, BSFFF positively impacts soil chemical properties by enhancing nutrient abundance and adsorption in the soil[25]. In addition, BSFFF strengthens soil biological quality by increasing populations of beneficial soil microbiota and improves soil physical properties by increasing organic matter and porosity[25,26]. Apart from being naturally derived and cost-effective, BSFFF has been shown to interfere with plant-pathogen interactions by reducing the spread of soil-borne fungal pathogens such as *Fusarium oxysporum* and *Rhizoctonia solani*[27,28]and aboveground and soil-dwelling pests[29,30]. However, its impact in plant-insect interactions, particularly invasive herbivore pests like fall armyworm (*Spodoptera frugiperda* (Lepidoptera: Noctuidae)), remains largely unexplored.

Spodoptera frugiperda is a highly destructive phytophagous pest, native to Americas but invasive in Africa[31]Asia[32] and Australia[33]. It represents one of the major global threats to food security, attacking over 80 different agricultural crops including cereal grains (maize, rice, sorghum, groundnut, soybean, millet and cotton), vegetables (cabbage, tomato and potato) and legumes, thus threatening the livelihood of farmers[34–37]. The use of agrochemical strategies to control *S. frugiperda* has proven ineffective due to ecological hazards and the rapid development of pest resistance[38–40]. Ecologically sound pest management strategies that involve less synthetic chemical inputs and emphasize nature-based approaches, including the exploitation of plant resistance, offer a promising solution for mitigating the economic impacts of this pest[20].

Generally, plants employ a diverse array of defense genes that are upregulated or downregulated depending on attacking insect species and nutrient availability[41,42]. Nitrogen, a key soil nutrient, plays a central role in plant growth and defense expression. Apparently, an increase in inorganic nitrogen negatively impacts plant physical and chemical defenses[43–45]. Lu et al.[46] demonstrated how the quality and quantity of nitrogen fertilizers significantly affected gene expression under organic and inorganic fertilization. Similarly, Kavroulakis et al.[47] and Tenea et al.[48] found that plants grown in organically amended soils expressed more defense genes than those grown in conventionally amended soils. Since plant defense response and soil management are intrinsically dependent[49]soil amendments that influence plant physiological and biochemical components play a critical role in enhancing innate plant defenses against herbivore pests. Therefore, given that BSFFF stimulates soil quality and beneficial microorganisms[26]it has the potential to enhance both acquired and induced systemic plant resistance.

Numerous studies have investigated the impact of soil amendments with BSFFF on nutrient availability, plant growth, and resistance to pests[26,29,30,46]. However, little is known about the effects of soil amendment with BSFFF on maize resistance to the invasive *S. frugiperda*. This study, therefore aimed to investigate how BSFFF amended, synthetically fertilized and unfertilized soils affect the expression of plant defense-related genes. In addition, we evaluated direct plant resistance through larval feeding assays. To capture agronomic outcomes at the field level, we compared maize yields across the three soil treatments. We hypothesized that BSFFF amended soils enhance expression of plant defense genes, thereby increasing resistance to herbivore pests while promoting plant growth and yield.

Materials and methods

Plants and fertilizers

Maize seeds (SC Duma 43) were obtained from Simlaw seeds Ltd, Nairobi, Kenya. The experiments were carried out using two types of fertilizers: organic fertilizer (BSFFF) and synthetic fertilizers, specifically Calcium ammonium nitrate (CAN) and Di-ammonium phosphate (DAP). The BSFFF was obtained from a BSF colony at the Animal Rearing and Containment Unit (ARCU) at *icipe*, while the synthetic fertilizers were obtained from Kenya Farmers Association Ltd, Nairobi, Kenya. The BSFFF was produced upon feeding black soldier fly larvae on brewery spent grain sourced from Kenya Breweries Ltd, Nairobi, Kenya, and composted following the procedure outlined by Beesigamukama et al.[26].

Insects

Spodoptera frugiperda moths were reared in 80 × 60 × 120 cm oviposition cages at the ARCU of *icipe*, Nairobi, Kenya. The cages were provided with three-week-old maize plants for adult moths to oviposit on. After two days, eggs were collected and transferred to rearing jars (1000 ml with steel-infused lids to allow airflow), where they were incubated until hatching. For all experiments that required neonates, one-day-old neonates were used. Rearing conditions were maintained at 25 ± 2 °C, 72 ± 5% relative humidity (RH), and a 12:12-hour light-

dark photoperiod. Second-generation insects were used in all experiments and were mixed with field-collected colonies every two months to maintain their biological characteristics and prevent genetic degradation[17].

Laboratory and greenhouse experiments

Laboratory and greenhouse experiments were conducted at Duduville Campus (1.2921° S, 36.8219° E; 1616 m above sea level), of the International Centre of Insect Physiology and Ecology (*icipe*) located at Nairobi, Kenya. Maize seeds were individually sown in 5 L plastic pots in a greenhouse under optimum conditions (25 ± 2 °C, 72 ± 5% RH; 12 L:12D photoperiod). For soil amendments, BSFFF was mixed with soil at a 1:3 ratio. Five days after planting, 10 g of DAP was applied to each pot with synthetic fertilizer treatment. As a top dressing, 10 g of CAN was added to each pot of synthetic fertilizer treatment 7 days after germination. Soil with no amendments was used as the control. Pots used for the experiment were thoroughly cleaned using 70% ethanol and household bleach (*Jik*) to prevent cross-contamination. Each 5 L pot was half-filled with fresh soil mixed with the different treatments and arranged in a randomized complete block design with 10 replicates per treatment. Maize seedlings were placed 70 cm apart and watered once a day with 0.2 L until they were four weeks old for use in the experiments.

Maize plant growth parameters were assessed by continuously measuring plant height and chlorophyll concentration weekly for four weeks after germination. Plant height was measured by positioning a tape measure from the soil surface to the arch of the uppermost leaf that had at least halfway emerged from the whorl region of the shoot. Maize chlorophyll concentration was recorded by measuring the average of the three newly and fully formed leaf using SPAD-502 chlorophyll meter (Konica Minolta corporation, Ltd, Osaka, Japan)[50].

At the end of the experiment (four weeks), biomass accumulation was assessed by measuring dry shoot and root weight through destructive sampling. Maize plants were cut above the soil level, oven-dried at 80 °C for 48 h, and weighed afterwards for shoot weight. Similarly, below-ground parts of the maize plants were cut, cleaned with water to remove attached soil particles, dried in an oven at 80 °C for 48 h, and then weighed to determine root weight.

Gene expression

To determine the effects of soil amendments on gene expression, the pathogenesis related protein 5 (*pr-5*), maize proteinase inhibitors (*mpi*), and lipoxygenase 3 (*lox-3*) genes were quantified using qRT-PCR. For constitutive defense genes, 1 g of undamaged, newly developed leaf tissue was cut from maize plants grown in soil amended with BSFFF, synthetic fertilizer and non-fertilized soil. The leaf tissue was then placed into 2.0 mL Eppendorf tubes, freeze-dried using liquid nitrogen, and stored at -80 °C for later RNA extraction. To further understand the effect of soil amendments on induced defense genes, another set of maize plants were exposed to ten *S. frugiperda* neonates for 24 h. Afterwards, 1 g of the newly developed leaf tissue of herbivore damaged maize plant from each of the soil treatments was cut, placed into 2.0 mL Eppendorf tubes, freeze-dried in liquid nitrogen, and stored at -80 °C for later RNA extraction.

RNA extraction and cDNA synthesis

For sample processing, 100 mg of freeze-dried leaf tissue from each treatment was cut and placed into 2 mL Epperdorf tubes with glass beads (BioSpec Products Inc., Bartlesville, Oklahoma, USA) and mechanically homogenized using a Tissue Lyser II (Qiagen Retsch GmbH, Hannover, Germany). Total RNA was extracted using ISOLATE II RNA Mini Kit (Meridian Bioscience, UK) following the manufacturer's instructions. DNAse treatment was done before elution to clear all DNA contaminants and the resultant RNA concentration and purity was determined using a Nanodrop 2000/2000c spectrophotometer (Thermo Fischer Scientific, Wilmington, USA), and samples were stored at -80 °C for downstream processes. Complementary DNA (cDNA) synthesis was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham, USA) following manufacturer's instructions. Into a microcentrifuge tube, a 20 µL reaction mix consisting of 10X RT buffer, 8 mM dNTP mix, 0.5 pmol μL^{-1} RT randoms primers, 45 ng μL^{-1} of total RNA, 5 U/µL MultiScribe™ Reverse Transcriptase and nuclease free water were added. The reactions were set up in a Nexus Mastercycler gradient (Eppendorf, Hamburg, Germany), under the following thermal cycling conditions: initial activation for 10 min at 25 °C, cDNA synthesis for 120 min at 37 °C, the Reverse Transcriptase inactivation at 85 °C for 5 min, then a final holding step at 4°C[47,51],.

qPCR analyses

The specific maize defense primers (*pr-5*, *mpi*, and *lox-3*) were selected based on those previously reported by Stratton et al.[42]. The specific primers were designed using Primer 3.0 software hosted by NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table 1). Quantitative PCR (qPCR) was performed using a QuantStudio 5 Real-Time System (Thermo Fisher Scientific, Waltham, USA). The reactions were all in triplicates and a minimum of 4 biological replicates. These were set up in 10 µL final reaction volume consisting of 5 µL of SensiFAST SYBR® Hi-ROX Kit (Meridian Bioscience, London, UK), 0.5 pmol $\mu L^{[-}$ [1] of each primer, 2.5 µL of cDNA template, and 1.5 µL of nuclease free water. The qPCR cycling conditions consisted of initial activation of 95 °C for 2 min; followed by continuous 40 cycles at 95 °C for 15 s, annealing for 45 s, and extension and plate reading at 72 °C for 30 s. Beta tubulin was used as the reference gene to normalize the expression of target genes of interest. Relative gene expression was determined using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method[52].

Larval feeding

To evaluate the influence of soil amendments on insect feeding, larval feeding assays were conducted on undamaged and herbivore-damaged maize plants. One leaf disc of 2.0 cm diameter was cut from a newly formed leaf of maize plants grown in soil amended with BSFFF, synthetic fertilizer and non-fertilized soil. The newly

Primer	Sequence	Target gene	Accession No.
pr5-F	GCACCAACAATGGCCGC	pathogenesis related protein 5	U82201.1
pr5-R	TAGCCGTCGATGACCGAGAT	pathogenesis related protein 5	
mpi-F	TGGTGACCTACACGCGAAC	maize proteinase inhibitor	X78988
mpi-R	GCCATTAGCTAGGATCGGCAT	maize proteinase inhibitor	
lox-3-R	ATCACCGCGTGCTTTTCAG	lipoxygenase 3	AF149803.1
lox-3-F	CACCATCACGGCGGAGACAT	lipoxygenase 3	
β-tub-F	CTACCTCACGGCATCTGCTATGT	beta tubulin	NM001111987.1
β-tub-R	GTCACACACACTCGACTTCACG	beta tubulin	

Table 1. Primers of defense-related genes for maize plant and internal reference gene.

formed leaves are known to contain high levels of defense compounds in maize[53]. The leaf discs were then placed in 30 mL clear plastic cups containing agar medium (Technical Agar #3) to maintain the physiological state. Ten naïve neonates of *S. frugiperda* were introduced into the 30 mL small cup containing a maize leaf disc from each treatment group. Afterwards, the small cups were gently sealed with parafilm paper to prevent neonates from escaping, and tiny holes were made using a thin needle at the cup lid to allow air circulation. The neonates were allowed to feed on the leaf discs for 24 h.

To determine the relationship between herbivore-damaged responses and soil amendments, maize plants were exposed to 10 *S. frugiperda* neonates for 24 h. Afterwards, 2.0 cm diameter leaf discs were cut from newly formed leaf of the exposed maize plants grown in all soil treatments as described in the above constitutive larval feeding assay. Six unique treatments were established, involving *S. frugiperda* neonates feeding on leaf discs from plants grown in soil amended with BSFFF, synthetic fertilizer and non-fertilized soils, for both constitutive and induced feeding responses. Each treatment was replicated nine times. Images of the leaf discs were taken, and the area fed on each leaf disc calculated using ImageJ software[54]– [55].

Gene expression and larval feeding

To examine the relationship between the expression of anti-chewing gene (*mpi*) and larval feeding[56]Pearson's correlation analysis was performed, and a scatter plot was constructed. The average feeding by *S. frugiperda* neonates on both damaged and undamaged plants was correlated with the expression levels of induced and constitutive *mpi* defense genes, respectively. Each treatment included four biological replicates.

Field experiments

Field experiments were conducted for two growing seasons; April to September 2023 and October 2023 to March 2024 at the Kenyatta University Teaching and Demonstration Farm (1°10′59″ S, 36°55′34″ E; 1580 m above sea level) in Kiambu County, Kenya. This region experiences bimodal rainfall, with an average annual precipitation of approximately 925 mm and mean monthly temperatures ranging from 21 to 28 °C (www.meteo.go.ke). The short rainy season often starts from March to June, while the long rainy season begins in October and extends to January. During the experimental periods, cumulative rainfall totals were 246 mm and 278 mm for the short and long rainy seasons, respectively. The treatments included: (i) BSFFF amended soil, (ii) synthetic fertilizer applied at a rate equivalent to 60 kg N ha^{-1}, and (iii) an unfertilized control soil. To eliminate nutrient limitations from either organic or synthetic fertilizer, phosphorus (P) [supplied as triple super phosphate – TSP (46% P_2O_5)] and potassium (K) [supplied as muriate of potash (60% K_2O)] were obtained from Kenya Farmers' Association and applied at uniform rates of 60 kg P ha^{-1} and 50 kg K ha^{-1}, respectively[57]. For organic fertilizers treatment, additional inorganic P and K were applied as top up to the nutrients content already present in the dry matter, used to supply the required N, ensuring equivalent nutrient supply across treatments[57].

The maize variety SC Duma 43 was used as the test crop. The experiment followed a randomized complete block design (RCBD) with three replicates. Each plot measured 4 × 4 m (m) with border widths of 0.5 m and 1 m between the plots and blocks, respectively[29]. The TSP fertilizer was applied at planting, while urea and muriate of potash were applied in two equal splits: 50% at 4 weeks after planting and another 50% at 7 weeks after planting. Weeding was conducted three times using a hand hoe, and all plots were managed following standard agronomic practices up to crop maturity. Grain yield data was collected at the harvesting period from each plot area after all the ears had fully dried. Maize plants in the harvested area were cut at ground level and their ears threshed to determine grain and residue weights using a weighing scale. Grain samples were taken to the laboratory and air-dried to 12.5% moisture content for determination of grain yields per plot and on a hectare basis (t ha^{-1}). Agronomic nitrogen use efficiency was determined following the method of Baligar et al.[58].

Data analyses

Prior to statistical analyses, data were tested for normality and homogeneity of variance using Shapiro-Wilk test and Levene test, respectively. One way analysis of variance (ANOVA) was used to test whether soil amendments had effects on maize growth parameters (plant height, chlorophyll concentration, and shoot and root weight) and yield. Larval feeding and defense gene expression data were analyzed using generalized linear model (GLM) with a quasi-Poisson distribution. Means were compared and separated using Student-Newman-Keuls (SNK) test and Tukey *post hoc* test ($P < 0.05$). Two-sample student's *t*-test (independent) was used to determine if there were differences between constitutive and induced larval feeding as well as differences between constitutive and

induced expression of maize defense genes and agronomic nitrogen use efficiencies. The principal component analysis (PCA) was conducted to explore the relationship between soil amendments and growth parameters, as well as interactions between soil amendments, larval feeding, and expression of maize defense genes. Pearson's correlation coefficient was performed to investigate the linear relationship between expression of anti-feeding gene (*mpi*) and larval feeding. All statistical analyses were performed using R software packages (v4.1.2)[59]with α set at 0.05.

Results

Growth parameters

We found differences regarding plant height, chlorophyll concentration, and biomass accumulation among maize plants grown in BSFFF amended soil, synthetically fertilized, and non-fertilized soils. Maize grew faster in BSFFF amended soil compared to those grown in synthetically fertilized and non-fertilized soils at 14, 21, and 28 days after germination ($F_{2,57}$ = 22.38, P < 0.001, $F_{2,57}$ = 33.78, P < 0.001, $F_{2,57}$ = 75.95, P < 0.001, respectively, Fig. 1A). However, this difference was not observed in the first week after germination ($F_{2,57}$ = 5.24, P = 0.080, Fig. 1A). In addition, we observed higher chlorophyll concentrations in maize plants grown in BSFFF amended soil in comparison to those grown in synthetically fertilized and non-fertilized soils at 7, 14, 21, and 28 days after germination, ($F_{2,57}$ = 19.15, P < 0.001, $F_{2,57}$ = 81.25, P < 0.001, $F_{2,57}$ = 85.47, P < 0.001, $F_{2,57}$ = 112.70, P = < 0.001, respectively, Fig. 1B). Chlorophyll concentration for maize plants grown in BSFFF amended soil and synthetically fertilized soil were comparable in first, second, and third week after germination (P = 0.140,

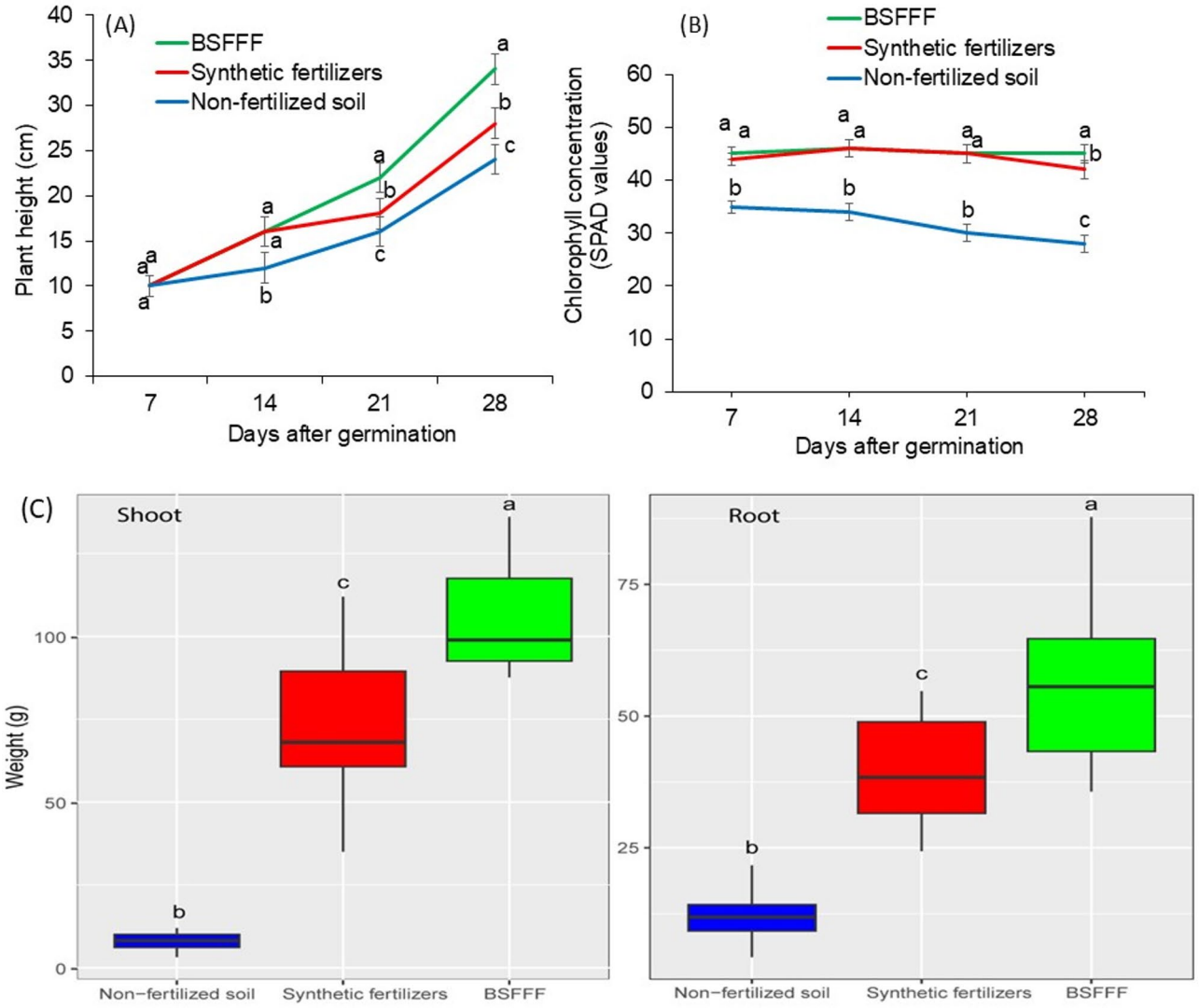

Fig. 1. (**A**) maize plant height, (**B**) chlorophyll concentration at one, two, three and four weeks after germination, and (**C**) box plots representing maize root and shoot weight after four weeks of growth in soil amended with black soldier fly frass fertilizer (BSFFF) and synthetic fertilizers and non-fertilized soil. Different small letters above the error bars and upper whisker indicate a significant difference between the means of the treatments (P < 0.05).

$P = 0.410$ and $P = 0.460$, respectively, Fig. 1B). We observed higher shoot and root dry weight in the maize plants grown in BSFFF amended soil compared to those grown in synthetically fertilized and non-fertilized soils ($F_{2,27} = 81.18$, $P < 0.001$, $F_{2,27} = 81.18$, $P < 0.001$ respectively, Fig. 1C).

The PCA indicated distinct variations in root and shoot weight, chlorophyll concentration, and plant height based on soil treatment (Fig. 2). For growth parameters, PC1 accounted for 84.3% of the total data variability, while PC2 explained 8.9%, for a combined total of 93.2% (Fig. 2). All measured growth parameters (root and shoot weight, chlorophyll concentration, and plant height) were highest in soil amended with BSFFF compared to the other soil treatments and showed a positive correlation with each other.

Gene expression

Undamaged maize plant grown in BSFFF amended soil constitutively expressed significantly higher *mpi* and *lox-3* defense genes than those grown in soil amended with synthetic fertilizers and non-fertilized soils ($F_{2,9} = 13.59$, $P < 0.001$; $F_{2,9} = 23.92$, $P < 0.001$, Fig. 3A). However, these differences were not observed in constitutive expression of *pr-5* defense gene among the different soil treatments ($F_{2,9} = 0.39$, $P = 0.680$, Fig. 3A). We noted significant high expression of induced defense genes (*pr-5* and *mpi*) in maize plants grown in soil amended with BSFFF than those grown in synthetic fertilizers and non-fertilized soils ($F_{2,9} = 3.82$, $P = 0.020$, $F_{2,9} = 6.65$, $P = 0.004$, Fig. 3B). These differences were not apparent in induced expression of *lox-3* gene in undamaged maize plant grown in BSFFF, synthetic fertilizer and non-fertilized soils ($F_{2,9} = 0.78$, $P = 0.480$, Fig. 3B).

Spodoptera frugiperda-damaged maize plants grown in soil amended with BSFFF inductively expressed significantly higher *pr5*, *mpi*, and *lox-3* defense genes compared to undamaged maize plants grown in soil amended with BSFFF ($P = 0.003$, $P = 0.010$, $P = 0.050$, respectively, Fig. 3C). However, there were no differences observed between constitutive and induced expression of *pr5*, *mpi*, and *lox-3* in maize plant grown in synthetically

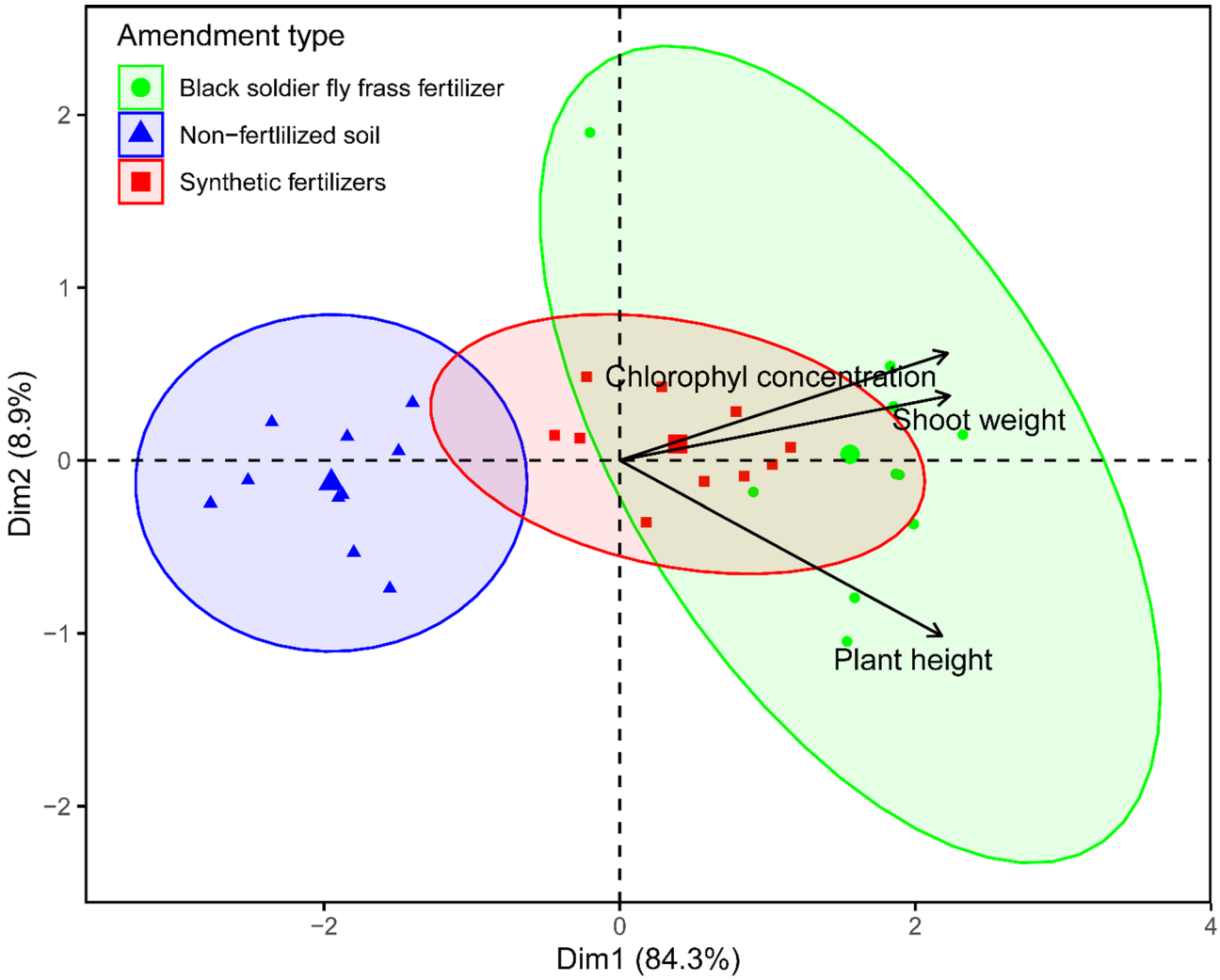

Fig. 2. Principal component analysis (PCA) of growth parameters on soil amendments with black soldier fly frass fertilizer (BSFFF), synthetic fertilizers, and non-fertilized soils. PC1 = principal component 1; PC2 = principal component 2.

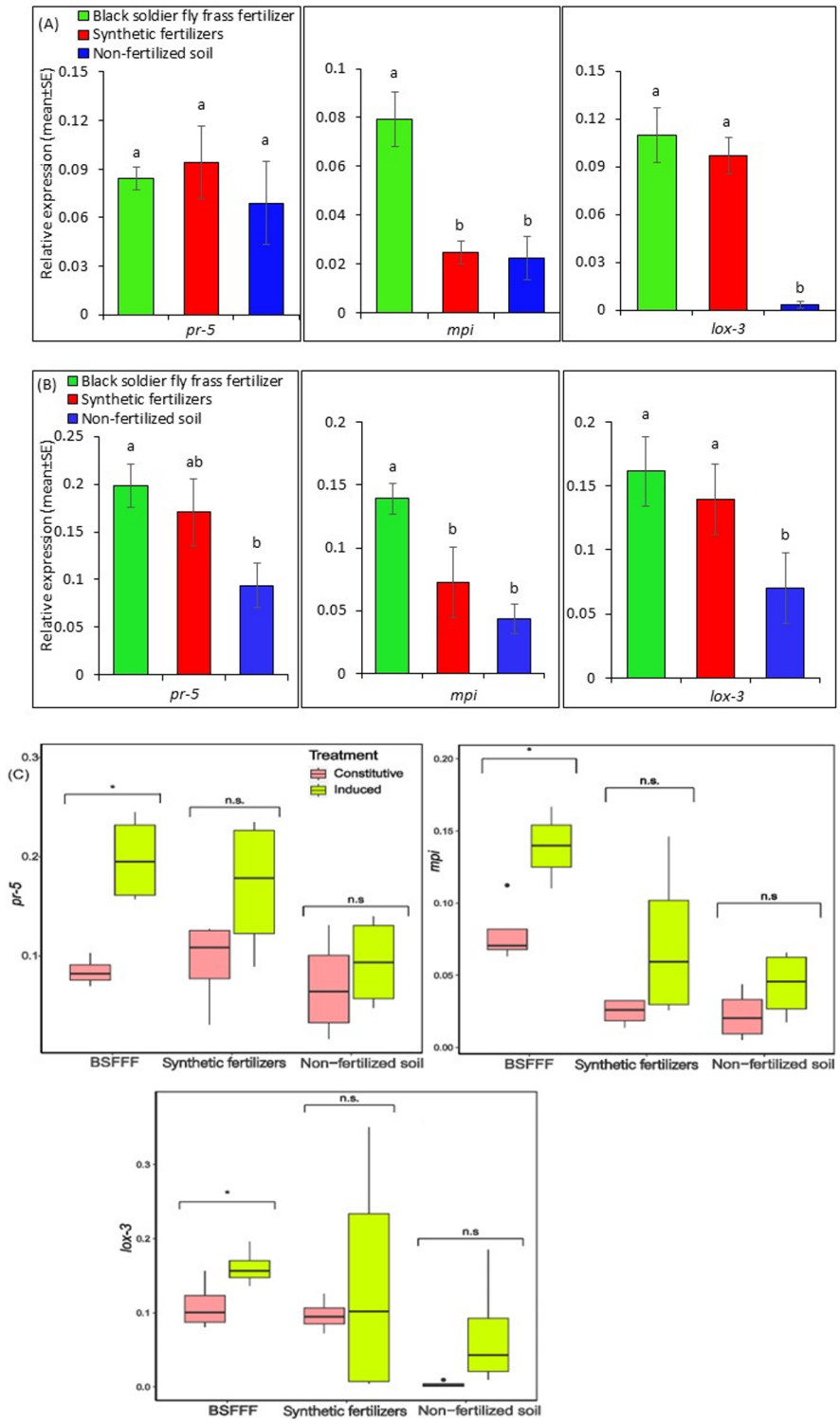

Fig. 3. Relative quantification of pathogenesis related protein 5 (*pr-5*), maize proteinase inhibitor (*mpi*) and lipoxygenase 3 (*lox-3*) maize defense genes on, (**A**) undamaged maize plants, (**B**) *S. frugipreda*-damaged maize plants, and (**C**) constitutive and induced expression of defense genes in maize plants grown in soil amended with black soldier fly frass fertilizer (BSFFF), synthetic fertilizers and non-fertilized soils. Different letters above the error bars and upper whisker indicate a significant difference between the means of the treatments ($P < 0.05$) for A and B. * indicate significant difference, while n.s indicates no significant difference (**C**).

fertilized soil ($P = 0.110$, $P = 0.150$, and $P = 0.630$, respectively, Fig. 3C) as well as those planted in non-fertilized soils ($P = 0.500$, $P = 0.200$, and $P = 0.150$, respectively, Fig. 3C).

Larval feeding

Spodoptera frugiperda larvae fed significantly less leaf tissue from undamaged maize plants grown in soil amended with BSFFF in comparison to leaf tissue from maize plants grown in synthetically fertilized and non-fertilized soils ($F_{2,42} = 62.73$, $P < 0.001$, Fig. 4A). Similarly, *S. frugiperda* larvae consumed significantly less maize leaf tissue in initially damaged maize plants grown in BSFFF amended soil in comparison to those grown in soil amended with synthetically fertilized and non-fertilized soils ($F_{2,42} = 103$, $P < 0.001$, Fig. 4B). There was a significant difference in leaf area fed by *S. frugiperda* between undamaged and damaged maize plants in soil amended with BSFFF ($P = 0.020$, Fig. 4C). However, there were no significant differences in larval feeding between damaged and undamaged maize plants grown in synthetically fertilized ($P = 0.140$) and non-fertilized soils ($P = 0.250$, Fig. 4C).

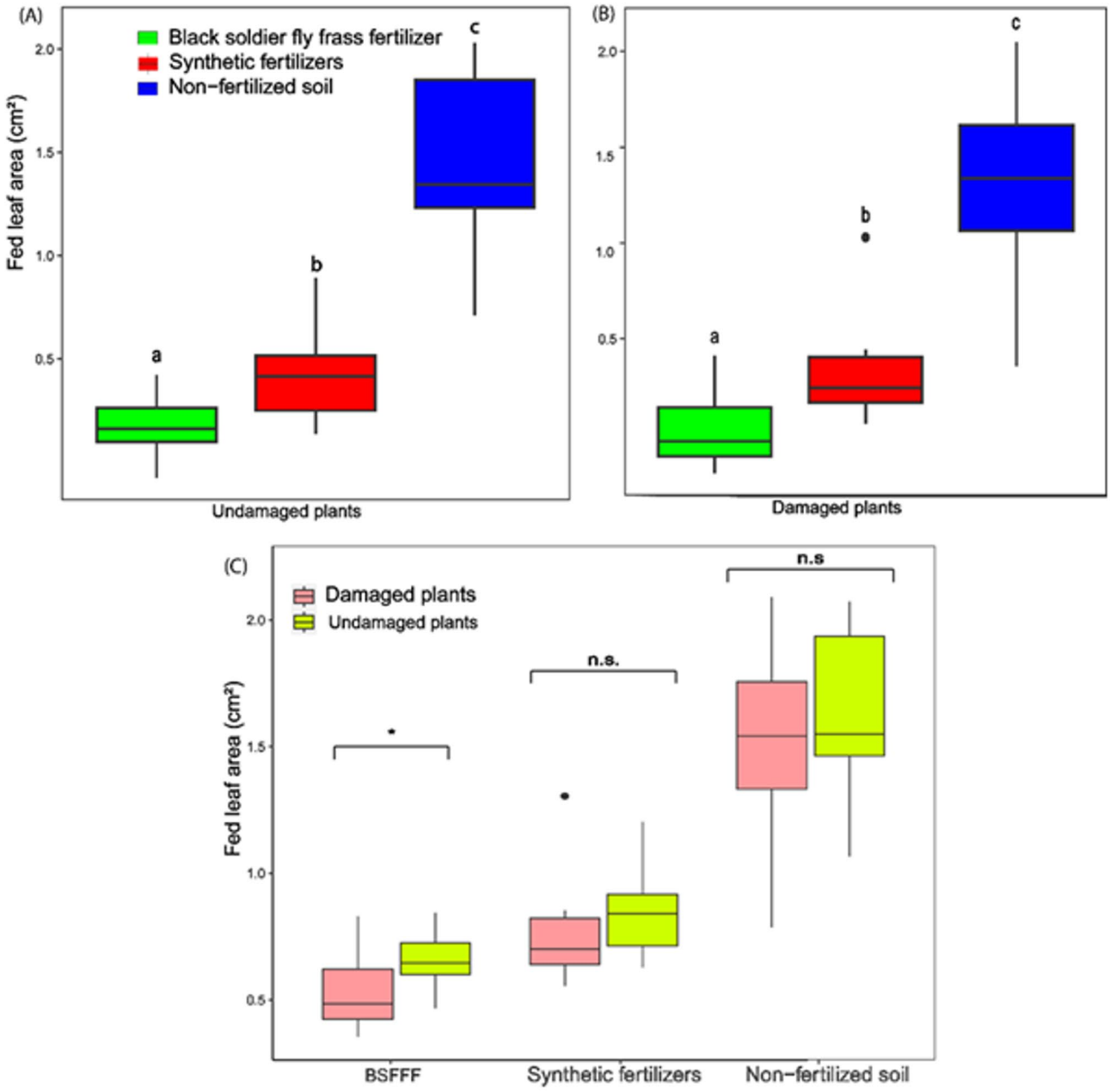

Fig. 4. Box plot representing mean leaf area consumed by *Spodoptera frugiperda* naïve neonates in a no-choice experiment on (**A**) undamaged maize plant, (**B**) damaged maize plant, and (**C**) comparison between undamaged and damaged maize plant grown in soil amended with black soldier fly frass fertilizer (BSFFF), synthetic fertilizers and non-fertilized soils.

Gene expression and larval feeding

A strong and significant negative correlation was observed between larval feeding and the relative expression of the *mpi* gene in maize plants grown in soil amended with BSFFF (R = -0.850, P<0.010, Fig. 5). On the other hand, maize plants grown in soil amended with synthetically fertilized and non-fertilized soils showed no significant correlation and only a weak negative correlation between larval feeding and expression of *mpi* gene (R = -0.460, P=0.250; and R = -0.160, P=0.700, respectively, Fig. 5). Larval feeding and gene expression were explained by PC1 that accounted for 66.1% of the total data variability, and PC2 explained 17.5%, for a total of 83.6% (Fig. 6). Larval feeding was highest in non-fertilized soil while *mpi* and *lox-3* gene expression were highest in soil amended with BSFFF, and *pr-5* was highest in soil amended with synthetic fertilizer treatments. Expression of maize defense genes positively correlated with each other but negatively correlated with larval feeding (Fig. 6).

Impact of fertilizer treatments on maize yield and nitrogen use efficiency

The different fertilizer treatments had a significant effect on maize yield ($F_{2,6}$ = 26.60, P=0.001), but not on agronomic N use efficiency (T=1.24, df=3.99, P=0.282). Soil amendment with BSFFF and synthetic fertilizer increased maize yield by 2.86 t ha^{-1} (105%) and 1.93 t ha^{-1} (71%), respectively, compared to the unfertilized control soil (Fig. 7A). Moreover, maize grown in BSFFF amended soil exhibited 0.93 t ha^{-1} (20%) higher yield and 48% greater N use efficiency than those grown with synthetic fertilizer (Fig. 7B).

Discussion

The current study provides empirical evidence that soil amendment with BSFFF not only improves maize plant growth but also reduces larval feeding of an invasive insect pest, *S. frugiperda* by upregulating maize defense genes. Our findings suggest that BSFFF amended soil mediated alterations in plant defense genes that positively affect direct plant defense traits. To the best of our knowledge, this is the first study to show enhancement of plant defense genes through soil amendment using an insect frass fertilizer while simultaneously deterring insect foliar feeding.

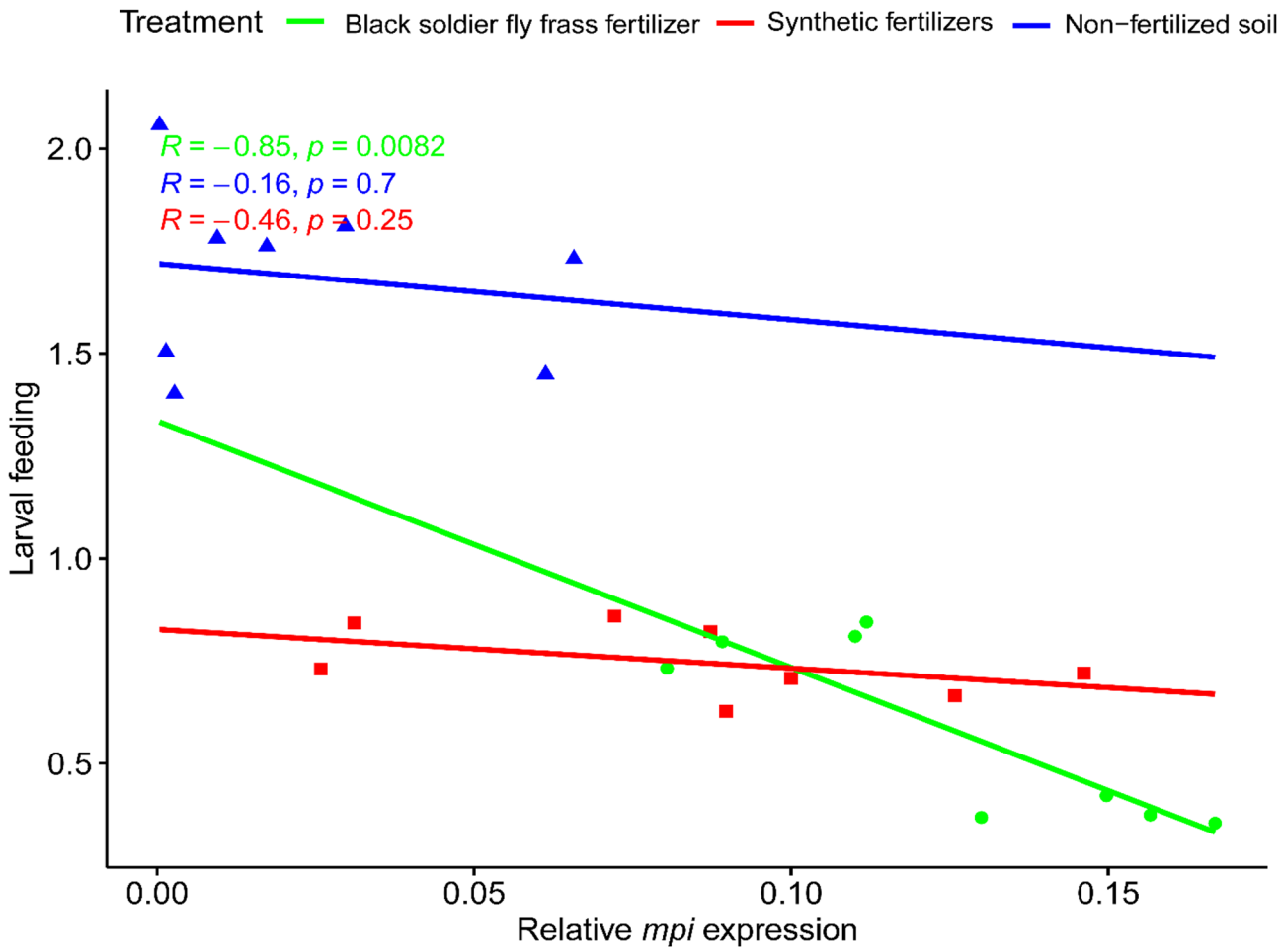

Fig. 5. Scatter plot of Pearson correlation between larval feeding of *Spodoptera frugiperda* naïve neonates and expression of anti-chewing defense gene (maize proteinase inhibitor (*mpi*)).

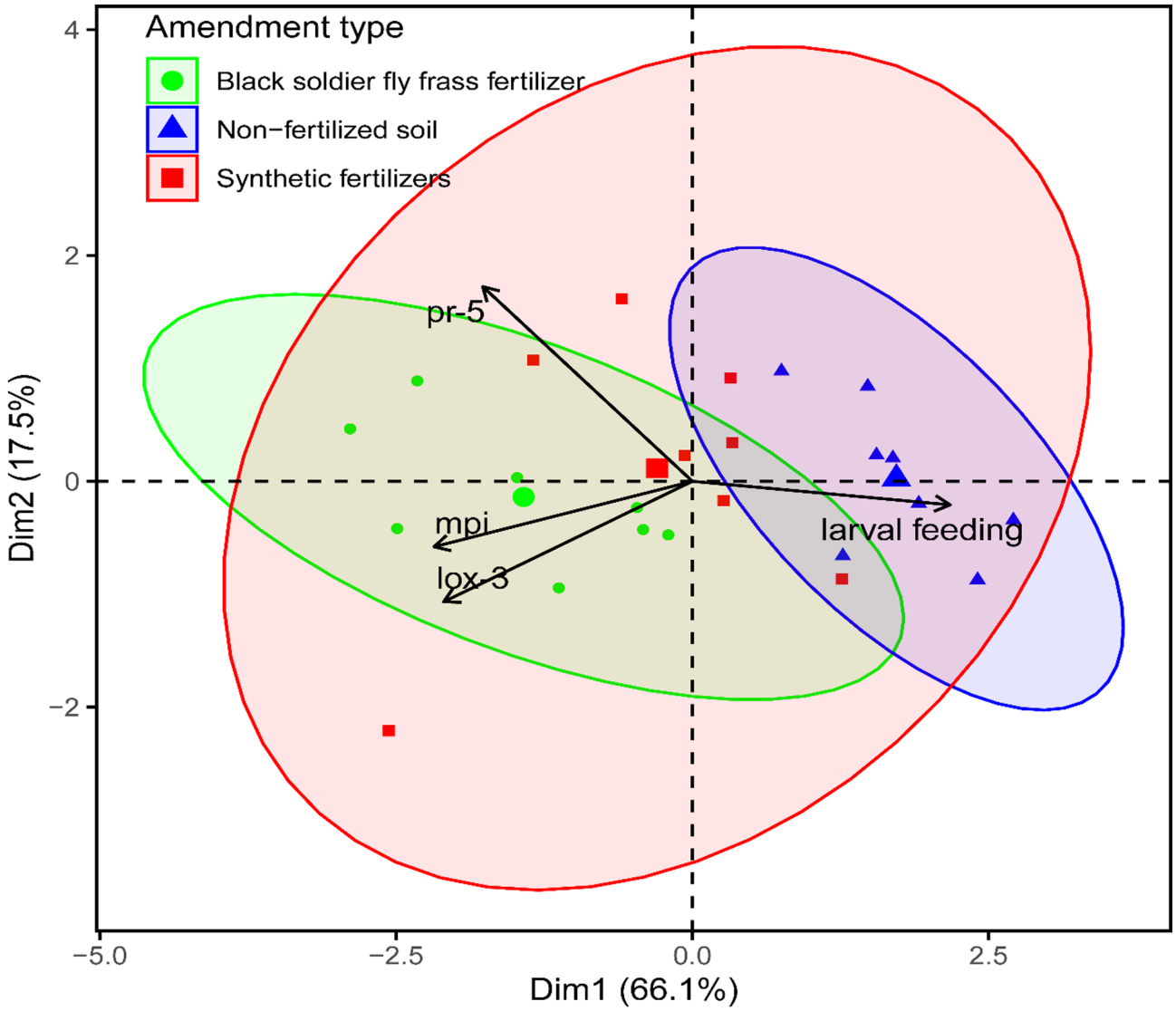

Fig. 6. Principal component analysis (PCA) of larval feeding and gene expression in soil amendments with black soldier fly frass fertilizer (BSFFF), synthetic fertilizers and unfertilized soils. PC1 = principal component 1; PC2 = principal component 2.

Plant height, chlorophyll concentration, and biomass accumulation on soils amended with BSFFF increased considerably compared to those grown in synthetically fertilized and non-fertilized soils. Soil amendments that improve nitrogen, potassium, and phosphorous availability restore cell growth and promote plant growth and development[60]. Both organic and inorganic fertilizers enrich soil with essential nutrients that stimulate plant growth[61]. Possibly, the increased plant growth regardless of the amendment regime was associated with increased nutrient availability. Studies by Beesigamukama et al.[25] and Tanga et al.[62] have also reported higher plant growth of maize grown in soils amendment with BSFFF than unamended soils. Interestingly, plant height and chlorophyll concentration varied over time after maize germination. Notably, there were no differences in the early days after germination, likely due to slow release and uptake of nutrients in BSFFF amended soils[63] resulting in initially low nutrient availability. Thus, the similar early growth rates across all treatments could be due to equal nutrient availability at the start of germination. These results concur with those obtained by Bashir et al.[64]who showed that maize plants grown in organic, inorganic, and non-fertilized soils attained similar growth rates within the first two weeks of germination. However, at later days of growth stages, plant height and chlorophyll concentration in maize plants grown in soil amended with BSFFF surpassed maize plants grown in synthetically fertilized and non-fertilized soils. This can be linked to the fact that nitrogen release from BSFFF or organically amended soils exhibit a slow pace during the initial stages of plant growth but sustained adequate levels for prolonged soil quality and plant growth[25,63,65,66]. On the other hand, synthetic fertilizers release nitrogen faster but only for a short period of time[67]thus, lower growth rate at later stages of plant growth. The increased plant growth at late stages of maize growth in soil amended with BSFFF is likely due to enhanced

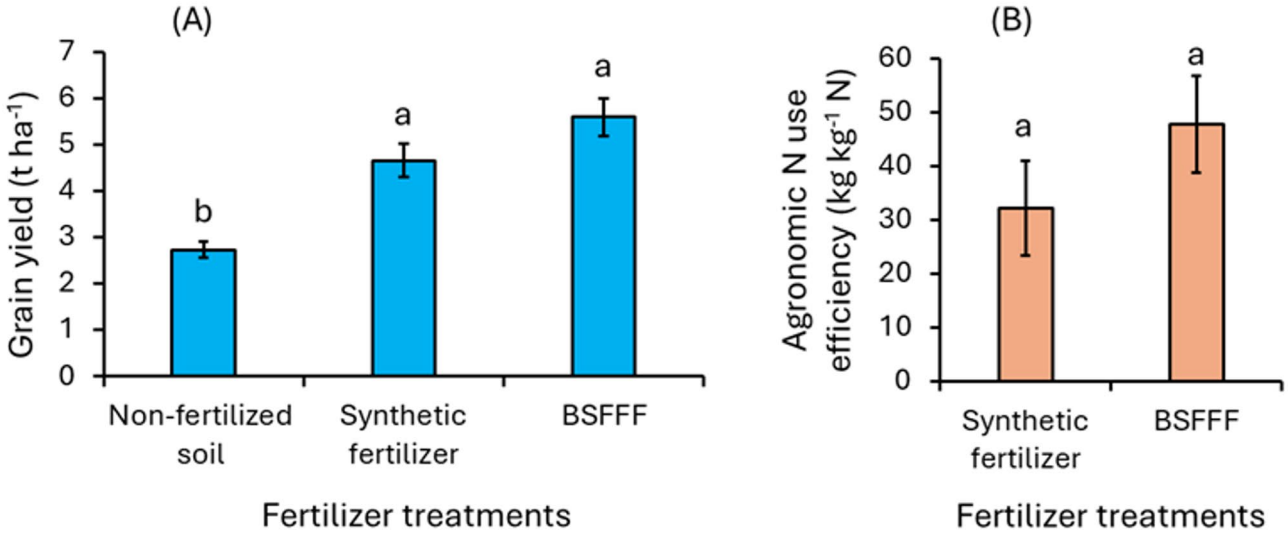

Fig. 7. Grain yield (**A**) and agronomic nitrogen use efficiency (**B**) of maize grown in soil amended with black soldier fly frass fertilizer (BSFFF) and synthetic fertilizer. Different letters above the error bars indicate a significant difference at $P < 0.05$.

and sustained nutrient mineralization and adsorption[68]which not only supports plant growth but also promotes biomass accumulation.

Our study revealed significantly higher maize grain yields in fertilizer treated plots compared to the unfertilized control, highlighting the crucial role of fertilizers in enhancing crop productivity, particularly in the degraded soils of Kenya[69]. Increased maize yield and agronomic nitrogen use efficiency achieved using BSFFF treated plots, compared to plots treated with mineral fertilizers has been previously reported[25,62]. These improvements may be attributed to the more effective nutrient supply and availability from the newly introduced frass fertilizer[65]. Additionally, the high nutrient release associated with the rapid mineralisation rate of BSFFF has been reported to enhance plant growth and productivity[26,68]. Beyond nutrient supply, BSFFF may also contribute to improved crop performance by enhancing drought and salt stress tolerance, suppressing pests and diseases, and boosting plant defense mechanisms[70]. Generally, plant defense theory projects a trade-off between plant growth and defense, across species and genotypes[51]. It further predicts resource allocation in response to biotic and abiotic stresses with rapidly-growing plants typically exhibiting poor defense mechanisms[51]. Here, we demonstrated that maize grown in soil amended with BSFFF not only had superior growth but also exhibited upregulated maize defense genes, leading to reduced herbivore feeding. This increased direct resistance to *S. frugiperda* in BSFFF amended soil is both economically and ecologically important, since leaf tissue removal adversely affects photosynthetic activity and reduces yield[17,71]. Furthermore, the extent of herbivore damage can be associated with pathogen infections, further threatening plant health[72–74].

What drives this increased resistance to herbivores in maize planted in BSFFF amended soil? In Poaceae species, including maize, plant defense genes such as proteinase inhibitors (PIs) are usually present in seeds and other plant parts where they are synthesized and stored[75]. These molecules have been identified as potent precursors that mediate resistance against pathogens and herbivore pests[53,75]. Plant defense genes are often activated by defense signaling pathways such as jasmonic acid, ethylene and salicylic acid to confer anti-chewing and anti-digestive properties against insect attack[58,71,78]. In this study, maize defense genes including *pr-5*, *lox-3*, and *mpi* were elevated in maize seedlings grown in soil amended with BSFFF. This elevated synthesis of these known defense genes especially the *mpi* in maize planted in BSFFF amended soil strongly correlated with reduced *S. frugiperda* larval feeding observed in our results. This reduction in herbivore leaf consumption in otherwise better growing plants in BSFFF amended soil constitutes a paradox. Generally, insect herbivores tend to feed poorly on nutrient-deficient plants and better on well fertilized plants like those grown in soil amended with BSFFF[17,76]. For example, *S. exigua* fed more on nitrogen-fertilized plants compared to those with low nutrient availability[77]. The observed differences are correlated with reduced production of defense molecules in plants fertilized with synthetic fertilizers, making them more susceptible to herbivore attack[47,48]. Here, we suggest that constitutive expression of higher defense especially the *mpi* in maize plants grown in BSFFF amended soil could also have higher defense response against the lepidopteran herbivores. Indeed, there is strong evidence that PIs play a key role in plant defense response against insect herbivores through inhibition of proteolytic enzymes such as elastase and chymotrypsin in insect herbivore midguts[75]. This enzymatic inhibition reduces food digestibility, leading to reduced insect feeding rates[54]. Our results indicate that BSFFF amended soil increases maize resistance to *S. frugiperda* by enhancing PIs synthesis, thus limiting insect feeding.

Moreover, exposure of maize plant to *S. frugiperda* larval feeding further increased expression of defense genes relative to undamaged maize plants in BSFFF amended soil. Consequently, herbivore feeding between insect damaged and undamaged maize plants was found to be significantly reduced in insect damaged maize plants grown in BSFFF amended soil as opposed to other soil treatments. Herbivore feeding not only causes

physical damage to plants but also releases oral cues (saliva, regurgitant, and frass) which trigger defense mechanisms[53], [78,79], . The observed increment in induction of defense on damaged plants than undamaged plants could therefore be associated with insect-derived elicitors produced by *S. frugiperda* larvae during feeding which further upregulated maize defense genes in maize grown in BSFFF amended soil. Indeed, plant damage by *S. exigua* and *S. frugiperda* has been shown to stimulate accumulation of *mpi* in plant tissue adjacent to the herbivore damaged parts[53,75]. What is interesting in the current results is that this accumulation of PIs following insect herbivore damage was only significantly higher in maize plants grown in soil amended with BSFFF as opposed to amendments with synthetically fertilized and non-fertilized soils.

What components in BSFFF-amended soil drive the upregulation of defense genes and subsequent reduction in herbivore feeding? Plants employ comprehensive defense mechanisms and their activation to initiate resistance to herbivore insects are often controlled by quantity and quality of soil nutrients among other factors[44]. Therefore, nutritional deficiency negatively impact plants' ability to protect themselves against insect attack through expression of plant defense genes[41,42]. In addition, plant resistance to herbivore pests is intrinsically linked to not only soil physicochemical properties but mainly soil biological properties[9,20]. Mattoo & Abdul-Baki[80] noted that plant genetic responses are likely influenced by soil microbial communities, shaping plant resistance traits. Given that soil amendment with BSFFF improves soil biological quality[25,65,67] the heightened induction of defense genes in BSFFF amended soils could be explained by the effects of the amendment on soil health. Conversely, synthetic fertilizers adversely affect soil microbial diversity[81] describing the decreased expression of defense genes. These results align with previous studies which demonstrated that plants grown in soil amended with organic fertilizers induced higher defense gene expression than those grown in inorganic fertilizers[47,48].

Conclusions

This study aimed at assessing the impact of amending soil with BSFFF on plant growth, defense genes expression, herbivore pest resistance, agronomic nitrogen use efficiency and yield. The study demonstrates that soil amendment with BSFFF improves maize plant growth and upregulates defense genes, contributing to increased resistance against *S. frugiperda*, high nutrient use efficiency and grain yield. The elevated expression of maize defense genes plays an important role in plant-insect interactions, effectively reducing *S. frugiperda* larval feeding. The link between BSFFF soil amendment and the associated expression of plant defense genes demonstrates a new mechanism through which insect frass fertilizer can reduce plant damage by invasive *S. frugiperda.* This study provides empirical evidence that soil amendments can influence plant defense traits, offering a promising strategy for sustainable pest management and crop protection. Due to the rapid development of insect farming, further studies on the impact of bioactive compounds of BSFFF on soil microbiome need to be systematically explored with particular attention to above-and below-ground microbial shifts. Additionally, future studies should investigate the role of soil amendment with BSFFF on plant phytochemistry and subsequent attraction of insects' natural enemies and repellence of herbivore pests. A deeper understanding of these mechanisms will provide valuable insights into how soil amendments shape plant growth, defense responses, and herbivore resistance, ultimately contributing to more sustainable agricultural practices and enhanced agroecosystem productivity.

Data availability

The data that support the findings of this study are currently available from the corresponding author upon reasonable request.

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Author contributions
DMM, JMM, and AAJ conceived the idea and designed the study; JMM collected data; DMM, AAJ, DB, and JMM analyzed data; and led the drafting of the manuscript; AK, DB, SS, TD, SE, and CMT read and reviewed the manuscript. DMM supervised the work. SS and CMT provided resources. All authors critically reviewed and approved the final.

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Declarations

Competing interests
The authors declare no competing interests.

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