The Bacterial *Methylorubrum extorquens* Product TS201 Induces Multiple Modes of Action to Reduce Insect Feeding Damage on Maize

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ABSTRACT

Pink-pigmented facultative methylotrophs (PPFMs) are known to promote plant growth and enhance pathogen resistance, but their role in insect defense remains largely unexplored. Here, we report that TS201, a U.S. EPA-registered bioinsecticide composed of *Methylorubrum extorquens*, enhances maize defense against corn rootworms (CRW) and improves yield through plant-mediated mechanisms. Over seven years of small-plot trials at 22 U.S. Midwest locations, TS201 increased yield by 220 kg/ha under moderate to high CRW pressure. Additional trials demonstrated rooting and lodging benefits. Large-scale trials across 81 sites in eight U.S. states (2023–2024) validated its agronomic benefit. Transcriptomic and metabolomic analyses revealed activation of anthranilate production and increased accumulation of methyl anthranilate, a volatile CRW-repellent compound. Insect assays indicated significant larval avoidance of TS201-treated roots. These findings reveal a novel mode of action for PPFM-induced plant defense and demonstrate TS201 as a strategic microbial tool for integration into broad-spectrum integrated pest management programs.

Keywords: Pink-pigmented facultative methylotrophs, corn rootworm, pests, microbial product, methyl anthranilate, repellent, IPM.

INTRODUCTION

Plant-associated microbiomes host diverse microflora that often establish symbiotic relationships with their host plants, thereby conferring health benefits and

protective effects[1]. Bacteria within the *Methylobacterium* and *Methylorubrum* genera constitute a significant component of the above-ground microbial community in the phyllosphere and are also prevalent in the rhizosphere, the soil region surrounding plant roots[2-5]. They are characterized as aerobic, Gram-negative, α-proteobacteria. These rod-shaped organisms primarily metabolize one-carbon compounds such as methanol as their principal energy source for growth and propagation[6,7]. Many *Methylobacterium* and *Methylorubrum* species synthesize carotenoids, which impart a distinctive red to pink coloration, designating them as pink-pigmented facultative methylotrophs (PPFMs)[8]. These bacteria are ubiquitous in nature, having been isolated from diverse habitats including soil, freshwater, and various plant species[4,9].

Several PPFMs have been documented to promote plant growth by supplying essential nutrients and modulating phytohormone levels[3]. These beneficial bacteria have also been reported to offer protection against pathogens by inducing the plant defense response including induced systemic resistance (ISR) against fungal rot pathogens in a variety of crops[10-12]. ISR is characterized by a lag time between when the elicitor (in this case, PPFMs) is applied and when the plant defense response is primed. After priming occurs, the plant grows as normal. If a pest/pathogen attacks the plant later in the season, then a potentiated defense response is activated and rapidly expressed throughout the entire plant[13]. This increased response in both timing and magnitude results in reduced damage to the host plant, and helps the plant efficiently balance growth and defense. Additionally, ISR plant defense is considered to have low resistance capacity as part of an integrated pest management (IPM) strategy[14]. Nevertheless, the potential role of these bacteria in enhancing plants against insect herbivores remains largely unexplored,

despite evidence that many other microbial taxa can induce plant responses which reduce herbivore damage and improve yield[15].

Corn rootworms (CRW) mainly comprising western corn rootworm (WCRW, *Diabrotica virgifera virgifera*) and northern corn rootworm (NCRW, *Diabrotica barberi*) are devastating pests of maize (*Zea mays*). Within the CRW complex, WCRW is the most invasive and economically damaging species, with established populations across the Americas and parts of Europe, whereas NCRW remains restricted to North America[16,17]. These pests are responsible for annual losses exceeding 2 billion USD, stemming from both the cost of control measures and yield reductions due to larval root feeding damage[18-20]. Larval feeding injures the root system and disrupts the plant's ability to efficiently absorb water and nutrients from the soil, thereby compromising overall plant vigor and structural stability[17,21]. In the presence of environmental stressors such as wind and rainfall, even moderate levels of root damage can cause plants to lodge or topple in the field[21]. Lodged plants exhibit reduced light interception and diminished photosynthetic capacity, resulting in lower biomass accumulation and grain yield, while also complicating mechanical harvesting and contributing to further losses through unharvested grain[19]. Furthermore, adult feeding on silks, pollen, kernels and foliage can occasionally result in yield loss, particularly when populations reach high densities prior to anthesis[22].

Management of CRW in maize continues to be a challenge for current producers since nearly all management strategies developed for controlling WCRW and NCRW have faltered due to the adaptation of these pests. Resistance in WCRW and NCRW has been documented to almost every management practice currently available to growers including cultural control techniques (e.g., crop rotation)[23-25], chemical insecticides[26-29],

and transgenic maize hybrids expressing at least one of the four currently available insecticidal toxins from *Bacillus thuringiensis* (Bt)[30-35], as well as innate plant resistance factors[36] and double-stranded RNA (dsRNA) targeting WCRW essential genes[37,38]. Therefore, additional control tools need to be developed and implemented for sustainable management programs of CRW[39].

Novel biological products which are among the fastest growing categories being adopted by growers to date[40] provide additional management tools to mitigate corn rootworm damage. However, biological control agents will only be commercially viable if their cost and efficacy are comparable to commonly used chemical control strategies[41]. A variety of biological control agents including entomopathogenic fungi, bacteria and nematodes, as well as a parasitoid and a predator have been investigated for managing WCRW in the United States and Europe[42-47]. Additionally, several microbial plant biostimulants including bacteria, fungi, and algae have been evaluated for their potential to control WCRW[46,48]. Despite promising results in some laboratory and field trials, none of the examined agents have yet been proven to be economically practical for large-scale applications.

In this study, we report a PPFM isolate, *Methylorubrum extorquens* that mitigates CRW damage in maize and improves yield. Initially, various PPFMs were evaluated as biological inoculants (biostimulants) to enhance maize yield in contract research organization (CRO) field trials across the U.S. Midwest. During one such trial, a field experiencing high CRW pressure was subsequently impacted by a wind event, resulting in widespread lodging in control plots without PPFM treatments. Notably, plots treated with selective PPFM strains exhibited no lodging, leading to further investigation into their

potential activity for controlling CRW in maize. This observation initiated multiple years of field trials to determine the most effective PPFM strain capable of both enhancing yield and reducing CRW damage. Through this screening process, *M. extorquens* strain NLS0042 designated TS201 was selected and further developed into a commercial product. Due to its demonstrated biopesticidal properties, TS201 was registered as a biological insecticide (TS201™, EPA Reg. No 95699-2, Terrasym®, NewLeaf Symbiotics, St. Louis, MO) with the U.S. Environmental Protection Agency (EPA) in 2022, under the regulatory framework of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)[49].

Here, we evaluated the efficacy of TS201 in protecting and enhancing maize yield under low to high CRW pressure in small-plot field trials conducted at 22 locations across the U.S. Midwest over seven years. Subsequently, large-scale on-farm evaluations were conducted across 81 locations in eight U.S. states over two years to assess yield improvement under a diverse range of agronomic conditions. To elucidate the underlying mode of action of TS201-induced maize defense against CRW, root phenotyping, transcriptomic and GC-MS analyses combined with insect choice assays were performed.

RESULTS

Field trials demonstrated TS201 treatment increased root growth, reduced lodging, and increased yield

Extensive field trials were conducted to evaluate the efficacy of TS201 in mitigating CRW feeding damage and improving maize yield and overall agronomic performance.

Prior to its registration by the U.S. EPA in 2022, TS201 was restricted to small-scale evaluations, with testing restricted to a maximum of ~4.05 ha (10 acres) per year in accordance with EPA and USDA-APHIS guidelines. Following its registration, this acreage limitation was removed, enabling large-scale evaluations of TS201 in commercial scale field trials, i.e. over 4.05 ha per location, during the 2023 and 2024 growing seasons.

From 2016 to 2022, small-plot field trials conducted at 22 locations across the U.S. Midwest with low to high CRW pressure demonstrated that TS201 treatment significantly increased maize yield, with an average gain of 220 kg/ha (3.5 bushels/acre (bu/A)) (Table 1, Suppl. Tables 1-2) compared to the untreated control. These trials included locations where CRW pressure was confirmed and was greater than the economic injury threshold, as indicated by a minimum nodal injury score (NIS) of 0.25[50,51] in control plots, based on the Iowa State University 0–3 root damage rating scale[50]. Across all trial sites over seven years, TS201 treatment consistently and significantly reduced NIS by an average of 0.11, revealing improved root protection under CRW pressure (Table 1, Suppl. Table 1). This reduction in NIS was observed consistently in both corn hybrids with and without insecticidal traits targeting CRW such as Bt toxins and lethal dsRNA constructs (Suppl. Table 2).

Table 1. TS201 improves maize yield and root protection in small-plot field trials under corn rootworm pressure. Field trials were conducted over seven growing seasons (2016-2022) at 22 locations across the U.S. Midwest. Data represent phenotypic responses in TS201 treatment relative to untreated control. Values summarize key agronomic and root protection traits observed in replicated small-plot trials. NIS= Nodal injury score.

Metric	TS201 treatment compared to control	P-value	Trialing scale

Performance	Yield	+220 kg/ha 11,706 kg/ha (control) vs. 11,926 kg/ha (TS201)	0.0108	22 locations over 7 years
Root damage	Nodal injury score (NIS)	-0.11 NIS 1.34 (control) vs. 1.23 (TS201)	0.0007	22 locations over 7 years
	Lodging	-20% at moderate CRW pressure; -36% at high CRW pressure	NA*	2 locations over 1 year
Root enhancement (TS201 + biostimulant)	Root phenotyping	+25% increase in total root length in nodes 1–3	0.0376	1 location over 1 year

*The lodging experiment was conducted in nine replicates (locations) across distinct locations in four U.S states (i.e., Colorado, Illinois, Iowa, and Minnesota); however, only two sites exhibited larval root injury above the economic threshold (NIS > 0.25) and were subsequently included in lodging analysis, thereby precluding statistical analysis.

Lodging incidence was measured in two 0.1 ha (quarter-acre) on-farm strip field trials in 2020. Lodging is one of the most visible and economically consequential outcomes of CRW damage recognized by growers. At the site with moderate CRW pressure of 1.2 NIS, TS201 treatment resulted in a 20% reduction in lodging, from 59% lodging in control to 39% lodging in TS201-treated (Table 1). At the site under high CRW pressure of 2.7 NIS, lodging was reduced by 36%, from 65% in control to 29% in TS201-treated (Table 1).

Based on prior observations of reduced CRW feeding damage and plant lodging, a small-plot field trial was conducted in 2024 to evaluate the effects of TS201 on maize root system architecture when applied in combination with Terrasym 450, a maize biostimulant containing *Methylobacterium gregans*. The single location trial was conducted in low but economically relevant CRW pressure, with a mean NIS of 0.26, slightly above the economic threshold of 0.25[50,51]. Application of TS201 + Terrasym 450

resulted in consistent numerical increases in total root volume, surface area, cumulative root length, and root tip number relative to the untreated control, independent of visible feeding damage (Fig. 1, Table 1, Suppl. Tables 3 & 4). The most pronounced and statistically significant enhancements were observed in nodal positions 1 through 3, where total root length increased by 25%, primarily driven by a 39% increase in fine root length (≤0.5 mm diameter) (Suppl. Table 4).

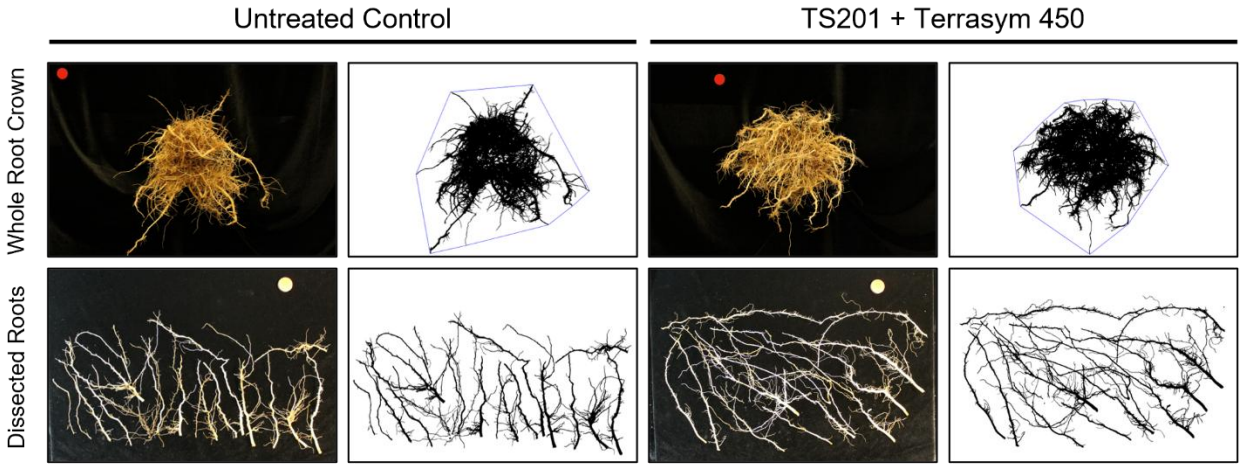

Figure 1. Enhanced maize root development observed under TS201 + Terrasym 450 treatment compared to untreated control. Root systems were collected from a small-plot field trial conducted in 2024 (10 plants per plot per replicate, four replicates per treatment). Following excavation and pressure washing, roots were imaged through a structured phenotyping workflow: intact root crowns imaged from below (top panel), then nodal segments were dissected and imaged (bottom panel). Images shown are representative of each treatment.

In 2023 and 2024, yield performance by TS201 was evaluated in large-scale on-farm evaluations in combination with Terrasym 450. Since these were commercial scale evaluations, they were tested with grower standard practice (GSP) including a range of corn hybrids, management practices, and insecticides as determined by the grower. These trials were conducted across 81 locations in eight U.S. states, representing a

diverse range of agronomic conditions (Fig. 2a). The combined treatment with GSP resulted in a significant yield increase of +351 kg/ha (5.6 bu/A, $P < 0.001$) and achieved a 72% win rate compared to GSP alone ($P < 0.001$, Fig. 2b). Importantly, these trials were included in the analysis regardless of actual CRW pressure to assess the agronomic performance and economic return of the TS201-biostimulant combination under real-world field conditions.

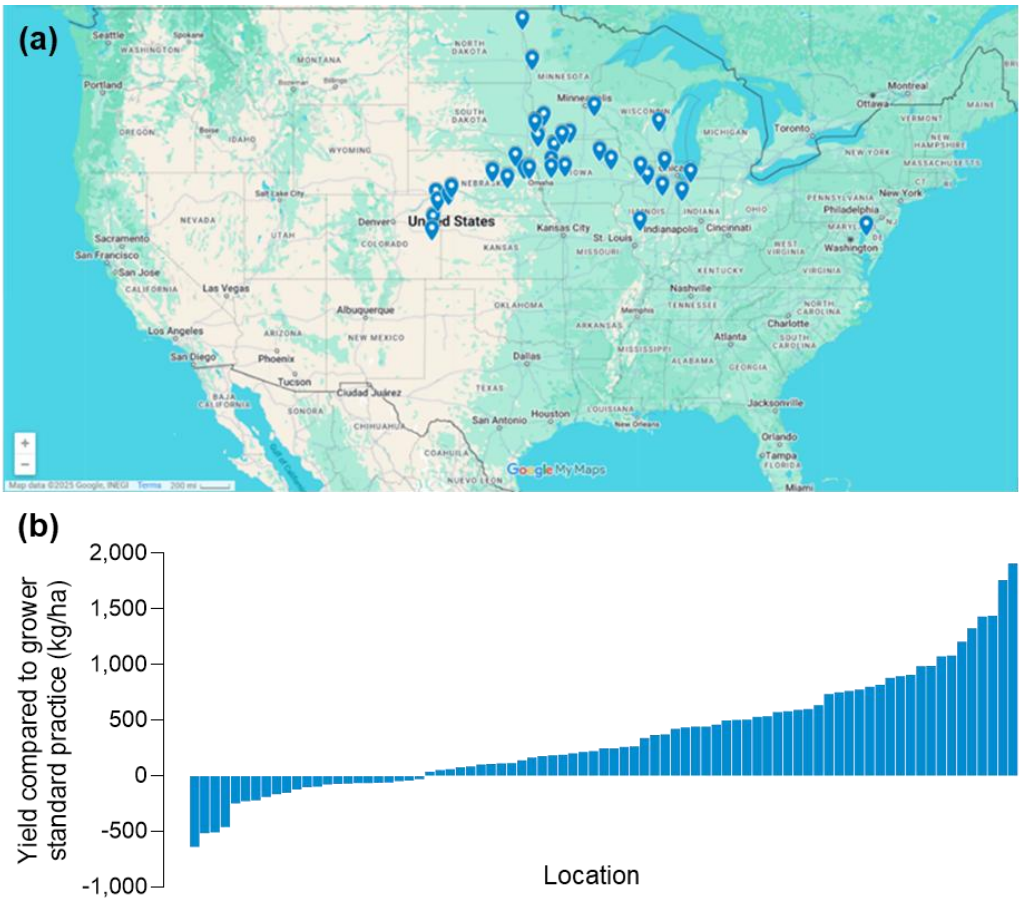

Figure 2. Large-scale on-farm evaluation of TS201 + Terrasym 450 in maize during 2023 and 2024 growing seasons across eight U.S. states. a) Geographic distribution of 81 commercial evaluation sites within eight U.S. states: Colorado (CO), Iowa (IA), Illinois (IL), Indiana (IN), Maryland (MD), Minnesota (MN), Nebraska (NE), and Wisconsin (WI). Trials were conducted regardless of corn rootworm pressure to assess real-world agronomic performance. Map

background adapted from Google Maps (Map data ©2025 Google) with markers indicating county-level trial locations. b) Piano chart showing yield improvement of TS201 + Terrasym 450 + grower standard practice (GSP) as compared to GSP alone across large-scale on-farm trials (ranging from 4.05 to 40.5 ha per site, n=81).

Transcriptomic analysis revealed the upregulation of defense-related genes associated with WCRW repellent in TS201-treated maize roots.

To elucidate the molecular mechanisms by which TS201 influences maize physiology under CRW pressure, an RNA-Seq analysis was conducted using root tissue collected from a field trial in Illinois. In this study, maize was planted and treated with TS201 in April, and root samples were harvested in mid-July, coinciding with root digs conducted to determine CRW feeding damage on maize roots. TS201-treated root samples collected from their corresponding plots that exhibited both a reduction in NIS and an increase in grain yield compared to UTC plots were utilized in the differential gene expression analysis (Suppl. Table 5).

Five genes were significantly upregulated at $P<0.05$ and four additional genes were upregulated at $P<0.1$ in TS201-treated maize roots compared to those roots from control plots (Table 2), with several annotated as being involved in stress response and/or defense mechanisms based on the B73 maize reference genome. An additional 18 genes were identified as downregulated and are shown in Supplementary Table 6. Notably, two of the upregulated genes were annotated as components of the anthranilate biosynthetic pathway. This result led to the hypothesis that TS201-treated roots may produce methyl anthranilate, a volatile compound previously demonstrated to repel WCRW[52].

Table 2. Up-regulated genes in TS201-treated maize roots relative to untreated control roots. TF= transcription factor, ROS=reactive oxygen species, ET=ethylene.

v4 Gene Model ID	Gene Symbol	Full Name	Putative Function	log2 Fold Change	Adjusted P-value
Zm00001d050455	pco096884	anthranilate N-benzoyltransferase protein 1	Anthranilate synthesis	1.63	0.0147
Zm00001d003395	ans1	anthranilate synthase homolog1	Anthranilate synthesis	1.55	0.0957
Zm00001d002799	hb66	Homeobox-transcription factor 66	TF in development	2.07	0.0995
Zm00001d049860	nactf75	NAC-transcription factor 75	TF in drought stress tolerance	1.73	0.0957
Zm00001d023617	lac23	laccase23	Enzyme in lignification/ defense	2.18	0.0957
Zm00001d024852	acco3	1-aminocyclopropane-1-carboxylate oxidase3	Enzyme in ET production	2.07	0.0408
Zm00001d002434	aox3	alternative oxidase3	Enzyme in ROS stress tolerance/ defense	1.85	0.0578
Zm00001d002052	NA	Probable isoaspartyl peptidase/ L-asparaginase 2	Nitrogen metabolism/ defense	2.48	<0.0001
Zm00001d011319	ZmABCG48	Probable ABCG Transporter 48	Transport	1.78	0.0408

Insect choice assays indicated that TS201-treated maize roots enhance the production of volatile compound(s) that repel WCRW larvae.

To determine whether TS201 induces the release of CRW-repellent volatiles, insect choice assays were conducted using a two-choice olfactometer system previously used for WCRW behavioral studies[53]. Western corn rootworm, the most destructive and economically significant species within the CRW complex, was selected as the insect model for this study. Initial assays conducted three days post-treatment showed no significant differences in larval behavior between TS201-treated and control roots (data not shown). However, when assays were performed 10 days after seed treatment,

TS201-treated roots exhibited significant repellency effects on WCRW larvae compared to the control.

Significantly fewer WCRW larvae were observed in all TS201-treated seed treatments, both with and without WCRW infestation compared to untreated controls and the infested arena (Figs. 3a, c–f). Notably, a significantly greater number of larvae were recovered from the TS201-treated seed without WCRW infestation than from the corresponding treatment with WCRW infestation (Fig. 3e), suggesting that prior herbivory feeding may modulate volatile production or impose fitness cost in maize. Similarly, a significantly higher proportion of larvae was found in the untreated seed with WCRW infestation compared to those without infestation (Fig. 3b).

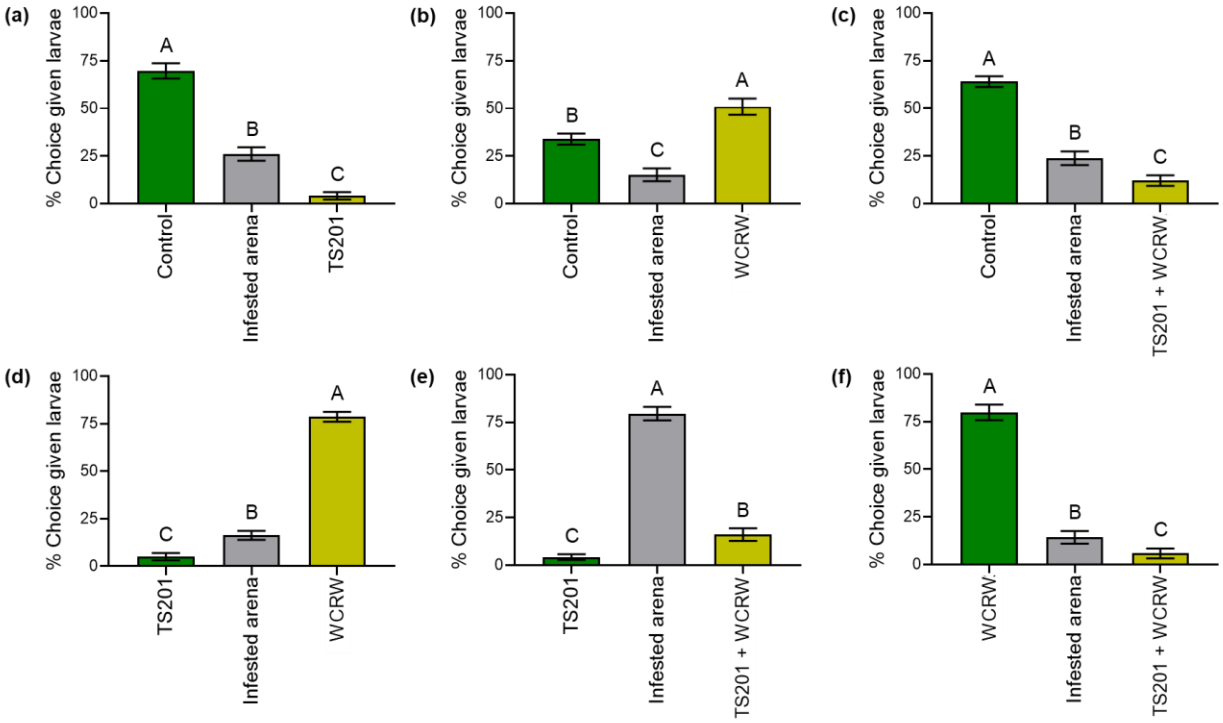

Figure 3. Percentage of larvae approaching experimental treatments in two-choice bioassays. Control: untreated seed without western corn rootworm (WCRW) infestation; TS201: TS201-treated seed without WCRW infestation; WCRW: untreated seed with WCRW infestation; TS201

+ WCRW: TS201-treated seed with WCRW infestation. Bars with different letters are significantly different ($P < 0.05$). Mean ± SEM (n=12 independent choice assays).

These results indicate that TS201 induces maize plants to increase the release of volatile compounds that are strongly repellent to WCRW larvae. This defense response, mediated by ISR[13], requires a characteristic lag period, typically 5 to 10 days, for priming to become effective. Interestingly, larval feeding was not required under the assay conditions to trigger the production of repellent volatiles, revealing that this effect was part of the initial ISR priming defense response induced by TS201.

Enhanced methyl anthranilate emission in maize was uniquely induced TS201

To identify WCRW-repellent volatiles, volatile organic compounds (VOCs) from TS201-treated maize plants and control plants were collected and analyzed using gas chromatography coupled to quadrupole time-of-flight mass spectrometry (GC-QTOF-MS). Among the 18 VOCs detected, methyl anthranilate was uniquely and markedly induced by TS201 treatment (Fig. 4). TS201-treated plants exhibited a significantly higher relative abundance of methyl anthranilate compared to untreated controls, whereas the remaining 17 VOCs showed smaller magnitude or non-significant differences between treatment groups (Fig. 4, Suppl. Tables 7 & 8). The elevated emission of methyl anthranilate was not attributed to differences in plant size, as VOC levels were normalized to plant biomass. Notably, the increase in methyl anthranilate production was independent of WCRW herbivory, with higher levels detected in TS201-treated plants not exposed to insect feeding. The emission pattern of this repellent compound corresponded with results from insect choice assays conducted on the same set of plants, in which

significantly fewer WCRW larvae were found in TS201-treated plants compared to controls (Suppl. Fig. 1).

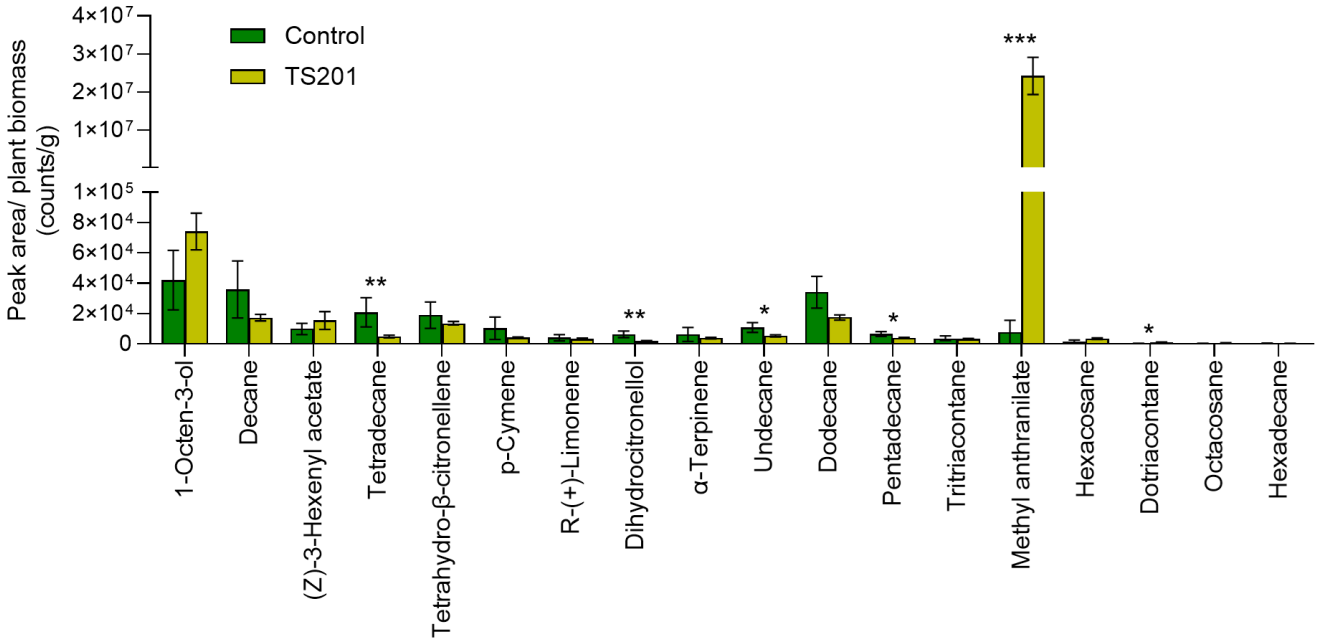

Figure 4. TS201 induces methyl anthranilate emission in maize. A list of all detected volatiles is available at Supplementary Table 7. Mean ± SEM (n ≥ 4). Untransformed data were presented, whereas analyses were performed with log10 transformed data. Signficant differences between the control and treated plants are indicated by *(P < 0.05), **(P<0.01), and ***(P<0.001).

Methyl anthranilate repels WCRW larvae

To validate the repellent effects of methyl anthranilate, insect choice assays were conducted using purified methyl anthranilate at varying concentrations. Across all assays, the percentage of WCRW larvae approaching methyl anthranilate treatments either alone or in combination with untreated maize plants was significantly lower than that observed for untreated plants and the infested arena (Fig. 5). Methyl anthranilate exhibited a strong,

dose-dependent repellent effect against WCRW larvae, with few or no larvae found in its treatments. Specifically, only 4.0 ± 2.5% of larvae approached the 0.1 µL methyl anthranilate treatment (Fig. 5d), which was significantly higher than the responses to the 1 µL (0.8 ± 0.8%) and 10 µL (0%) (Figs. 5b, c, e, and f). No significant difference was observed between untreated seed (40.9 ± 4.6%) and those with the solvent blank added (38.7 ± 3.4%) (Fig. 5a).

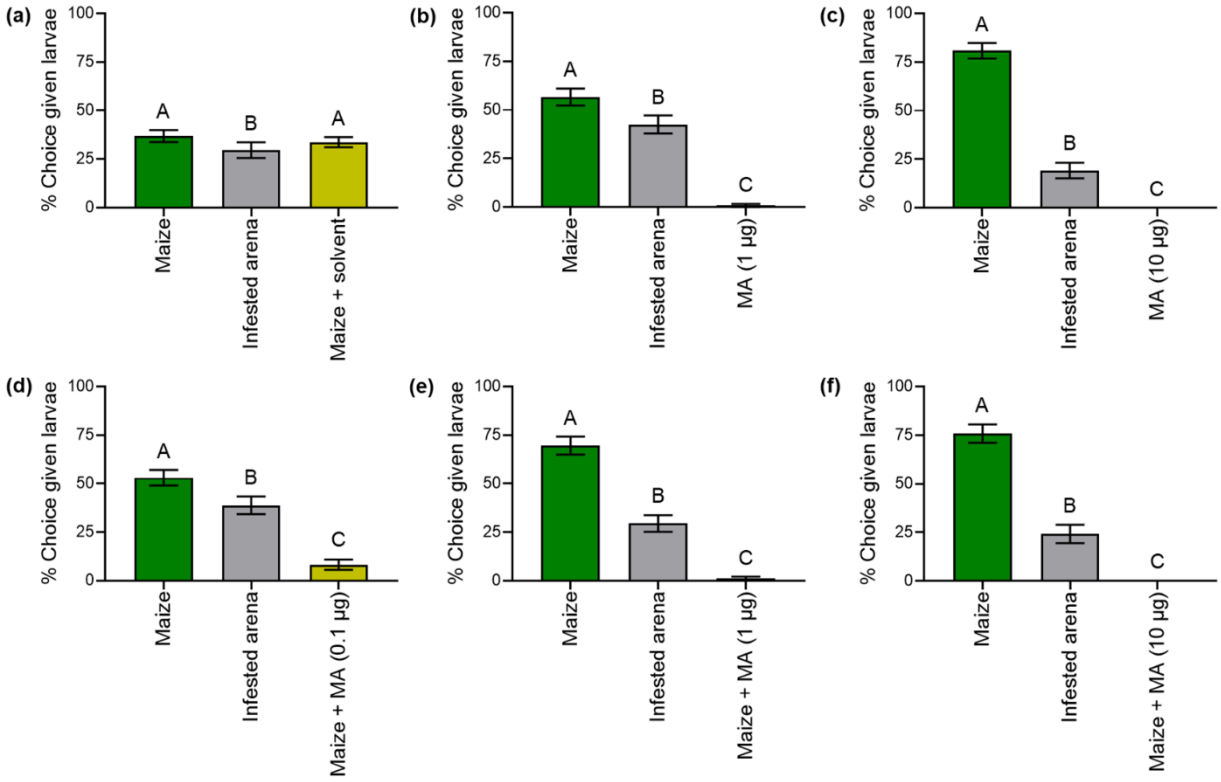

Figure 5. Percentage of larvae approaching experimental treatments in two-choice bioassays. Bars with different letters are significantly different ($P < 0.05$). Mean ± SEM (n=10 independent choice assays). MA: methyl anthranilate.

DISCUSSION

By bridging molecular mechanisms with ecological outcomes, this study provides a robust model for investigations into cross-kingdom signaling in agricultural ecosystems. Our study establishes that the PPFM species, *M. extorquens*, formulated as TS201, offers a novel and ecologically sound strategy for CRW management in maize. These findings advance the current understanding of plant-microbe-insect interactions by revealing how a naturally occurring rhizobacterium, originally isolated from the phyllosphere, can trigger endogenous plant defense pathways to mitigate belowground herbivory. To our knowledge, this is the first report demonstrating that a PPFM strain elicits ISR in plants against a subterranean insect pest, while simultaneously enhancing crop vigor and yield. Through an integrative approach combining multi-year field trials, root phenotyping, transcriptomic profiling, metabolomic analysis, and insect behavioral assays, this work presents a systems biology framework for harnessing microbially mediated plant defense responses toward effective and sustainable crop protection. Mechanistically, we show that TS201 modulates host transcriptional networks to activate volatile biosynthetic pathways, illustrating a functional convergence between microbial colonization, host gene expression, and volatile-mediated insect repellency (Fig. 6).

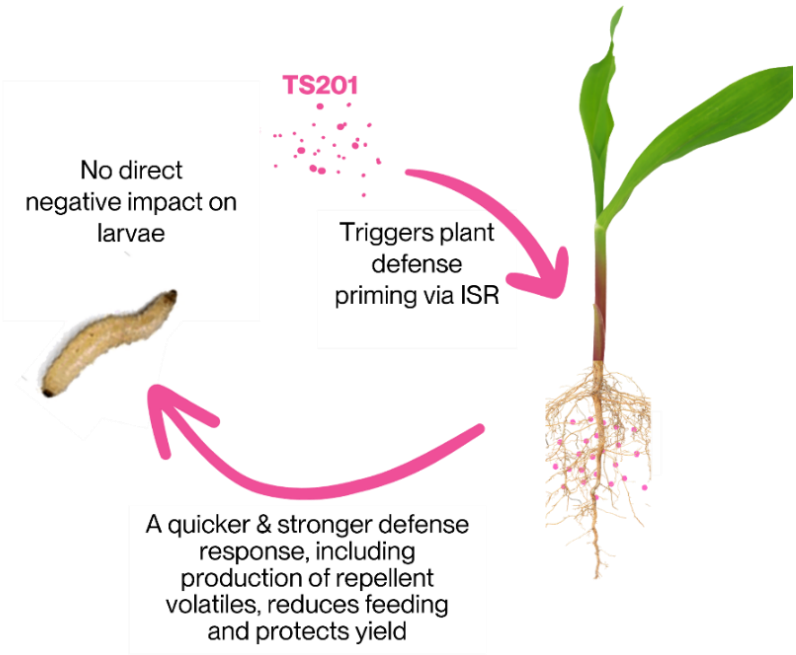

Figure 6. Schematic of TS201-induced enhancement of maize defense against western corn rootworm.

Our data provide compelling evidence that TS201 induces the biosynthesis of methyl anthranilate, a volatile compound previously documented to repel WCRW[52], through transcriptional activation of genes within the anthranilate biosynthetic pathway. Notably, this volatile-mediated repellency was elicited with and without herbivory, indicating that the TS201-induced systemic resistance mechanism was preemptively activated through microbial priming that requires a lag phase of more than three days post-application. ISR is characterized by the sensitization of the plant's defense machinery in the absence of immediate threat, enabling a faster and more robust activation of defensive responses upon biotic challenge. In contrast to systemic acquired resistance (SAR), which is primarily mediated by salicylic acid and is pathogen-specific, ISR typically engages jasmonic acid and ethylene signaling pathways and is triggered by beneficial rhizobacteria such as PPFMs[13,54,55]. Importantly, ISR does not constitutively activate defense genes but primes them for potentiated expression, conserving metabolic

resources while ensuring rapid deployment upon stress[56]. Furthermore, recent reports suggest that ISR can coordinate systemic metabolic reprogramming, hormone crosstalk, and oxidative signaling networks to strengthen plant resilience[57].

Across ecosystems, beneficial microbes are increasingly recognized as pivotal architects of plant resilience against biotic stressors. Our study provides experimental validation for the regulatory role of beneficial microbes in modulating plant-insect interactions. Shikano, et al. [15] reviewed how beneficial microbes can influence insect herbivores both directly and indirectly by altering plant defenses, nutritional quality, and volatile profiles, building upon earlier foundational concepts of microbe-mediated systemic resistance[13]. Here, we demonstrate that a naturally occurring PPFM species activates a primed defense state in maize, significantly enhancing the production of WCRW-repellent volatiles such as methyl anthranilate. Recent advances have expanded the understanding of microbe-induced volatile signaling in shaping herbivore behaviors both above- and below-ground[58,59]. Microbial modulation of root exudates has been shown to influence herbivore foraging patterns[58], while herbivore-induced restructuring of plant exudates can alter rhizosphere microbial communities with cascading effects on insect performance[60]. Furthermore, overlapping microbiome functions across root and shoot tissues have been shown to orchestrate systemic plant defenses against herbivores[59]. Beneficial microbes also serve as chemical brokers, modulating phytohormone signaling networks and orchestrating ISR-linked metabolic reprogramming under multi-stressor environments[61]. Collectively, our findings indicate TS201 as a vital integrator within this emerging paradigm, linking microbial application, volatile-mediated

defense priming, and pest deterrence, with direct translational relevance for advancing sustainable and resilient agricultural systems.

Our results reveal TS201 as a major advancement in IPM strategies for CRW, a pest complex that has evolved resistance to nearly all management tactics developed to its control[30,39]. Unlike conventional insecticides and Bt traits that impose direct lethal pressure and drive rapid resistance evolution, TS201 primes plant defenses through ISR, activating endogenous pathways without direct toxicity, thereby minimizing selection pressure and potentially preserving non-target organisms[14,62,63]. Whereas biological control agents such as *Heterorhabditis bacteriophora* and microbial biostimulants have demonstrated variable efficacy and inconsistent field performance[42,45,46,48], TS201 consistently enhances maize protection across diverse agroecosystems, independent of pest pressure or environmental variability. These findings position TS201 as an ecologically rational and needed addition to sustainable CRW management frameworks.

Beyond mitigating pest damage, TS201 significantly enhances agronomic performance by enhancing maize root development, reducing lodging, and improving yield under real-world production conditions. Over seven years of small-plot field trials conducted at 22 locations across the U.S. Midwest under low to high CRW pressure, TS201-treated maize consistently achieved a yield increase of 220 kg/ha relative to the untreated control. When applied in combination with a biostimulant product Terrasym 450, TS201 also improved maize root architecture, including increases in total root length and the number of root tips, which is likely associated with reduced lodging. The greatest effects were observed in the basal nodal zones, particularly among fine roots (0–0.5 mm diameter). Taken together, these findings suggest that TS201 may provide early

protection by preserving finer, actively growing roots that are preferentially targeted by CRW larvae[17,64]. Further studies at higher CRW pressure are needed to validate these hypotheses.

Following U.S. EPA registration, large-scale on-farm evaluations during the 2023 and 2024 seasons across 81 geographically and agronomically diverse environments further validated TS201's efficacy, with a yield gain of 351 kg/ha when applied in combination with Terrasym 450. These findings demonstrate that TS201 promotes scalable yield enhancement across diverse genotypes, soil microbiomes, and climatic conditions. The ability of TS201 to stably induce plant-mediated defenses and metabolic priming without reliance on pest pressure or chemical inputs aligns with emerging paradigms advocating for microbiome-assisted crop improvement[65,66]. Moreover, the integration of TS201 into production systems complements sustainable IPM frameworks, which emphasize resilience, nutrient use efficiency, and reduced ecological footprint[67]. By leveraging plant-microbe signaling networks to CRW, TS201 addresses a critical need in sustainable CRW management and promotes strategies for resilient agriculture[68].

This study highlights the transformative potential of harnessing beneficial microbes to reengineer plant resilience for sustainable agriculture. By activating ISR and promoting volatile-mediated defense, TS201 adds a fundamentally distinct mechanism to mitigate CRW damage. Unlike conventional methods challenged to CRW resistance evolution, TS201 strengthens innate plant defenses, while enhancing agronomic performance across diverse environmental conditions. These findings bridge molecular insights with ecological outcomes, demonstrating that microbe-induced priming strategies can achieve both pest management and yield enhancement. Additionally, ISR is effective in many

plants and against many pests/pathogens, suggesting that TS201 would be effective against other pests as well, an area that is under active investigation. As global agriculture faces escalating biotic and abiotic pressures, TS201 exemplifies a next-generation biopesticide that addresses the urgent need for resilient crop management strategies leveraging plant-microbe interactions, delivering scalable efficacy across geographies, ecological sustainability, and strategic compatibility with current IPM frameworks.

MATERIALS AND METHODS

Biological materials

The EPA registered biopesticide product TS201, containing *M. extorquens* strain NLS0042 (2.0%, 1 × 10^9 CFU/g) as an active ingredient, was provided by NewLeaf Symbiotics (St. Louis, MO). This bacterial strain was originally sourced from a soybean plant in Saint Louis County, MO. Maize hybrids (P1197, Pioneer, Johnston, IA) were purchased from local seed suppliers unless otherwise indicated.

Western corn rootworm neonates were obtained from a non-diapausing colony maintained at the Plant Genetics Research Unit-USDA-ARS in Columbia, MO[32,69]. The eggs were obtained in Petri dishes consisting of eggs and soil and were incubated at 25 ± 1 °C in darkness in an incubator (Percival, Perry, IA). Neonates that hatched within 24 h were used for insect assays.

Field experiments

From 2016 to 2022, a series of 22 small-plot field trials were conducted across multiple locations in the U.S. Midwest with documented histories of elevated WCRW and NCRW damage, capturing variability in climate, soil type, and pest pressure. TS201 was evaluated as a standalone treatment to establish proof of concept under diverse field environments. During this pre-registration phase, trials were conducted under USDA-APHIS jurisdiction, with testing limited to a maximum of 4.05 ha (10 acres) per year in compliance with U.S. federal guidelines while EPA registration was in process.

The small-plot field experiments were designed as a randomized complete block design (RCBD) with four row plots 12 m (40 ft) in length and 4 to 6 replicates per location. TS201 was applied via multiple delivery methods to evaluate its efficacy and consistency under agronomically relevant deployment applications, including in furrow (309 g/ha, 1×10^9 CFU/g wettable powder), liquid seed treatment (1×10^6 CFU/seed application rate, 1×10^9 CFU/g wettable powder) or planter box application (0.5 oz/ unit seed or 14 g/ 8×10^4 maize seed, 1×10^9 CFU/g powder with seed lubricity agent). In each trial, the cooperating contract researcher selected the maize hybrid and agronomic management practices with base insecticide treatments specifically excluded, and these plots served as the untreated control. TS201 application was the sole treatment variable distinguishing the treated plots from the untreated control. In a subset of these trials, the same maize hybrids were evaluated with and without belowground CRW protection traits such as Bt traits, with TS201 application was the only difference from the untreated control. Trials were conducted under continuous maize cultivation (corn-on-corn rotations) with select sites incorporating squash trap crops to facilitate consistent CRW feeding pressure. Root damage was evaluated in mid-July to early August, corresponding with the observed

emergence of adult CRW beetles on maize plants, using the Iowa State University NIS system as described previously[50]. To determine the efficacy of TS201 under CRW pressure, only field trials in which untreated control plots exhibited an NIS score greater than 0.25, a common economic damage threshold[50,51], were analyzed. Grain yield data were collected as bu/A and converted to kg/ha using the conversion of 1 bushel as 25.4 kg[70].

In 2020, an on-farm experiment was carried out to evaluate the impact of TS201 on maize lodging under CRW pressure. The trial included nine replicates in distinct locations in IA, IL, MN, and CO. At each site, 0.1 ha (¼-acre) strips were assigned to compare the untreated control with an in-furrow application of TS201 (309 g/ha, 1 x 10^9 CFU/g). TS201 was applied to a corn hybrid without CRW protection traits. Roots were sampled along two defined transects (37 m) in each treatment, 20 roots total per treatment, pressure washed and rated using the NIS system. Out of nine locations, two in IA exhibited larval root injury exceeding the economic threshold (NIS > 0.25) and were included in lodging analysis. Based on NIS values, trial sites were categorized into low (0.25–1), moderate (1–2), or high (2–3) levels of CRW pressure levels. Lodging was quantified as the percentage of lodged plants within two, 37 m predefined transects for each treatment.

In 2024, building on prior observations of reduced CRW feeding damage and plant lodging, a small-plot field trial was conducted to evaluate the impact of TS201 on maize root system architecture when applied in combination with Terrasym 450. The trial was designed as a RCBD with four replicates. To determine baseline CRW pressure, roots from untreated control plots were sampled, pressure washed, and scored using the NIS

system[50]. Three weeks later, 10 root crowns per replicate were collected from both TS201 and Terrasym 450 treated and control plots to assess root architecture under CRW pressure. Roots were imaged using a structured phenotyping workflow: intact crowns imaged from below in the transverse plane and dissected nodal segments imaged per individual crown to enable accurate imaging of total root length, root size classes, and numbers of root tips. Root phenotyping images were analyzed using RhizoVision Explorer v2.0.3[71]. All images included a size marker for normalization and to convert pixel dimensions to metric units (mm). Dissected roots were grouped by node: 1-3, 4, 5, and 6. No node 7 roots were present in this study. Yield data were not collected, as the trial employed destructive sampling for detailed root phenotyping.

Following EPA registration, field evaluations of TS201 shifted from small-plot studies to large-scale, on-farm evaluations under commercial production conditions during the 2023 and 2024 growing seasons. The large-scale field experiment included 81 replicates, each representing a maize field at different locations within eight U.S. states (Fig. 2a). Across 81 trials (≥ 4.05 ha each), conducted in collaboration with contract researchers and commercial growers, TS201 was applied in combination with Terrasym 450 as a planter box treatment (0.5 oz per unit of seed of each PPFM isolate at 1 x 10^9 CFU/g with seed lubricity agent) as an addition to the grower standard practice (GSP) and evaluated in comparison to GSP alone for CRW management. GSP reflected real-world production systems incorporating diverse IPM strategies, including combinations of belowground CRW protection traits (e.g., Bt, RNAi), in-furrow insecticides, crop rotation, and prior-season aerial applications targeting adult beetles. Trials were primarily conducted in corn-on-corn systems in regions with a reported history of CRW pressure.

Experimental designs included full-field side-by-sides, split-planter setups, and replicated strips ranging from 4.05 ha to over 40.5 ha. Win rate was determined by dividing the number of trials with a positive yield over GSP by the total number of trials and converting to a percentage. Yield data were collected using precision monitors or weigh wagons and converted from bu/A to kg/ha as described above. Data were expressed as differences relative to the GSP control.

Transcriptomic analysis

Root tissue samples from untreated and TS201-treated maize plants (one sample per plot; six replicates per treatment) were collected from different RCBD plots in a field trial. Total RNA was extracted using RNeasy Plant Mini Kits (74104, Qiagen, Germantown, MD), treated with DNase using the RNase-Free DNase Set (Catalog #15200, Qiagen), and assessed for RNA quality using an Agilent 2100 Bioanalyzer with RNA 6000 Nano Kits (5067-1511, Agilent Technologies, Santa Clara, CA).

Root samples from TS201-treated plots that exhibited both increased yield and reduced nodal injury scores (NIS) compared to control plots (three plots total) were used for gene expression profiling using QuantSeq FWD 3′ mRNA-Seq using 75bp reads with V2 Illumina chemistry (Lexogen, Vienna, Austria). QuantSeq is a tag-based sequencing method that captures and sequences the 3′ end of polyadenylated transcripts, allowing accurate gene expression quantification via read counting[72]. The B73 v.4 maize reference genome was used for read alignment. Differential gene expression analysis was performed using the BlueBee Genomics platform which utilizes DESeq2; genes were considered differentially expressed if the $\log_2$ (fold change) was greater than 1.5 and the

adjusted *P*-value was less than 0.1[73]. All raw sequencing data have been deposited in the NCBI Sequence Read Archive database with BioProject accession number PRJNA1260573.

Insect choice assays

Untreated maize seeds were rinsed and incubated in distilled water overnight in a Percival incubator maintained at 25 ± 1°C to promote germination. After incubation, seeds were dried out and treated with TS201 at a concentration of 1×10^6 CFU/seed, or with sterile distilled water (0.01 mL/seed, untreated seed). Subsequently, 3 kernels were planted per 50-mL conical tube and grown in the Percival incubator for 10 days. Plants were watered with 2 mL of distilled water every other day. The experiment included four treatment groups: untreated seed without WCRW infestation (control), serving as the control, TS201-treated seed without WCRW infestation, untreated seed with WCRW infestation, and TS201-treated seed with WCRW infestation. To allow for ISR priming to occur, seeds were allowed to germinate and grow for 7 days prior to insect infestation. Insect feeding was designed for 3-days to allow the potentiated ISR mediated-defense response to occur, if required. On day 7, 8 WCRW neonates (<24 h old) were transferred to plants assigned to infestation treatments. On day 10, plants were removed from the soil using a fine paintbrush and used for insect choice assays.

Insect choice assays were conducted using a modified olfactometer system adapted from Branson and Ortman[53]. The olfactometer consisted of three 50 × 15 mm Petri dishes connected in a linear arrangement via 12 mm segments of Teflon tubing (8 mm I.D. × 10 mm O.D.). The bioassays were designed as a completely randomized

experiment. Ten WCRW neonates were introduced into the central arena, while treatment and control plants were randomly assigned to the terminal arenas at either end of the olfactometer. The experiment was conducted in complete darkness in the Percival incubator, and larval positions were recorded after 30 minutes to assess behavioral responses. The experiment was run in three cohorts at different times, with each cohort consisting of four replicates, for a total of 12 replicates. Each replicate used unexposed larvae and freshly prepared plant seedlings.

Larval behavioral response was quantified by calculating the proportion of larvae that responded (PLR), using the formula: PLR = Nr / Nt, where *Nr* is the number of larvae found in each zone (treatment side, control side, or infested center) at the end of the assay (30 min), and *Nt* is the total number of larvae introduced[52].

GC-MS analysis

Volatile organic compounds from control and TS201-treated maize plants were collected using a modified headspace sampling system based on the method described previously[74]. Briefly, maize seeds were treated with TS201 or sterile water, and the treated seeds were planted without WCRW infestation in 50-mL conical tubes as previously described. At 10 days post-germination, seedlings were gently removed from the soil using a fine paintbrush. Maize seedlings were transferred into a 50-mL collection chamber (5 seedlings per chamber) fitted with Teflon tubing at both ends. One end of each chamber was connected to a flow meter (B07B6HQDDW, Wuhan, China), which was linked to a Whatman® carbon/HEPA filter capsule (WHA67041500, Sigma-Aldrich, St. Louis, MO) and a 6-arm air delivery pump. The opposite end was connected to a

Tenax® TA thermal desorption tube (35/60 mesh, 0.25 in. O.D. × 3.5 in. length, Tenax® TA, 29747-U, Sigma-Aldrich) for VOC trapping. Air was drawn through the chamber at a flow rate of 2 mL/min for 90 minutes to collect volatiles. VOCs from blank chambers containing sterile water and purified methyl anthranilate (≥98% purity, W268224, Sigma-Aldrich) were used as negative and positive controls, respectively. Adsorbed compounds were eluted from the Tenax tubes with 2 mL of methanol (Optima™ LC/MS Grade, A456, Fisher Chemical, Waltham, MA) for GC-MS analysis. The volatile assay was conducted as a completely randomized experiment with more than 4 replicates. Each replicate consisted of freshly prepared, unexposed maize seedlings randomly assigned to individual chambers within the headspace sampling system.

Headspace volatiles were analyzed using an Agilent 7890B gas chromatograph (GC, Agilent Technologies, Santa Clara, CA) coupled to an Agilent 7250 quadrupole time-of-flight mass spectrometer (QTOF-MS). Sample injection was performed using a Gerstel multipurpose sampler. The GC-MS operating conditions were as follows: helium was used as the carrier gas at a constant flow rate of 1 mL/min; injection volume was 1 µL with a split ratio of 5:1; and the inlet temperature was maintained at 280 °C. The oven temperature program began at 50 °C (held for 2 minutes), ramped at 6 °C/min to 215 °C, then increased at 5 °C/min to 315 °C, where it was held for 12 minutes before cooling back to 50 °C. Separation was performed using an Agilent DB-5MS capillary column (60 m × 250 µm i.d. × 0.25 µm film thickness). The MS transfer line was maintained at 250 °C, and electron ionization (EI) was conducted at 70 eV. Mass spectra were acquired in full-scan mode over a mass range of 40-650 m/z.

Data processing, including deconvolution, alignment, and compound identification, was performed using MS-DIAL software, with spectral matching against an in-house mass spectral library. The peak areas of identified compounds were integrated using Agilent MassHunter Qualitative Analysis 10.0 and normalized to plant biomass (mg) to determine their relative concentrations.

Insect choice assays with methyl anthranilate

To determine the behavioral response of WCRW larvae to methyl anthranilate (MA), insect choice assays were conducted using the olfactometer system described above. In each assay, purified methyl anthranilate (≥98% purity; Sigma-Aldrich) was applied to one side of the olfactometer arena at concentrations of 0 (solvent control, methanol), 0.1, 1, or 10 µg with and without untreated maize seedlings, while the opposite side contained untreated maize seedlings. Ten neonate WCRW larvae (<24 h old) were introduced into the central arena of the olfactometer, and larval distribution was recorded after 30 minutes in complete darkness. Each treatment was replicated at least five times. The proportion of larvae responding was calculated as described above to determine the repellent effect of methyl anthranilate at each concentration.

Statistical analysis

The VOC data were analyzed using Student's unpaired *t*-test (PROC TTEST, SAS 9.4, SAS Institute, Cary, NC). Compounds were considered significantly induced when $P < 0.05$ and $\log_{10}$(fold change) > 1. Other data (i.e., small-plot field trial, root phenotyping,

large-scale field trial, insect choice data) were analyzed using analysis of variance (ANOVA) followed by pairwise comparisons of least squares means (LSMEANs) or Fisher's least significant difference (LSD) for multiple comparisons. Analyses were conducted using a generalized linear mixed model in PROC GLIMMIX (SAS 9.4) or JMP v18.1.1 (JMP Statistical Discovery LLC, Cary, NC), with treatment specified as a fixed effect and year, location, block, or replication as random effects. For win rate analysis, trial-level outcomes were treated as binary responses (win = 1, loss = 0) and modeled using a binomial distribution. Normality and homogeneity of variance were evaluated using univariate analysis in PROC UNIVARIATE (SAS 9.4). Datasets that did not meet these assumptions were transformed prior to analysis: percentage variables were arcsine square-root transformed, and numeric variables were square-root transformed, to meet the corresponding distributional requirements.

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AUTHOR CONTRUBUTIONS

M.P.H. and N.W.B. wrote the first draft of the manuscript. M.P.H., B.E.H., and N.W.B. designed and supervised the project. M.P.H., K-V.H., A.Z.P., A.F.E., A.J., and N.W.B. performed the experiments. M.P.H., K.V.H., A.Z.P., Z.L., A.J., and N.W.B. performed the analyses and provided the visualization. D.C.L., B.E.H., D.F., and N.W.B. provided materials required for the experiments. All authors read and approved the final version.

DATA AVAILABILITY

All raw transcriptomic sequencing data has been deposited in the Sequence Read Archive database on NCBI with BioSample Accessions SAMN48399254, SAMN48399255, SAMN48399256, SAMN48399257, SAMN48399258, SAMN48399259 under BioProject accession number PRJNA1260573. All other pertinent data are found in the figures and tables. Request for data and additional information should be submitted to the corresponding author.

COMPETING INTEREST

Anne Phillips, Allison Jack and Natalie Breakfield are employees of NewLeaf Symbiotics, St. Louis, MO, USA. All other authors declare no competing interests.

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