EFFECTS OF DOMESTICATION, SPREAD, AND BREEDING ON MAIZE DEFENSES AGAINST WESTERN CORN ROOTWORM

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2019

Major Subject: Entomology

ABSTRACT

This study addressed whether maize (*Zea mays mays* L.) defenses against Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte, were mediated by domestication, spread, and modern breeding, three transitions shaping the crop's evolution. Trends of decreasing resistance to WCR with maize domestication, spread, and breeding, and of increasing tolerance with decreasing resistance were expected. Concomitant variation was expected in maize's constitutive and induced phytohormone profiles, constitutive root volatile profiles, and recruitment of WCR larvae. To test these expectations, assays compared between four *Zea* plant types encompassing the three transitions: Balsas teosintes (*Zea mays* L. ssp. *parviglumis*), Mexican maize landraces, US maize landraces, and Mid-western US maize inbred lines. Specifically, the expected trends were tested by comparing between pairs of consecutive plant types: (i) resistance and tolerance to WCR; (ii) profiles of constitutive and induced levels of biochemical defenses, and; (iii) recruitment of WCR and profiles of constitutive root volatiles. The results suggested that domestication and spread decreased both maize resistance to WCR as well as accumulation of biochemical compounds relevant to resistance to WCR, and increased recruitment of WCR and diversity of constitutive root volatiles, as expected. However, these trends were reversed with breeding, contrary to expected. The results also showed that maize resistance and tolerance to WCR are negatively correlated, as expected. Overall, my results suggested that evolution of defense strategies in maize, from the crop's wild ancestor to modern Mid-western cultivars, is predicted by ecological-evolutionary

hypotheses explaining defense strategy evolution in plants generally. I discussed my results in the contexts of plant resistance-productivity trade-offs, plant tolerance-resistance trade-offs, and varying resource availability in relation to physiological stress and herbivory pressure.

DEDICATION

I dedicate this work to:

My lovely husband, Jorge H. Montaño Nanetti.

My adorable son Leonardo Montaño Fontes, who I hope one day will understand why mommy could not put him into bed for so many nights. This work is yours, so you one day find inspiration to establish your own goals, keep fighting until you accomplish them, or be wise enough to create new ones. You are important to me, no matter what. I love you so much 'Chiquitino mino.'

And the memory of my beloved Father, José Luis Fontes Martínez (1948-2017).

ACKNOWLEDGEMENTS

I want to thank my committee chair, Dr. Julio S. Bernal, and my committee members, Dr. Mike Kolomiets, Dr. Raúl F. Medina, and Dr. Keyan Zhu Salzman, for their patience, guidance, and support throughout this research.

Thanks to Dr. Anjel Helms and Dr. Lupita Corella for your help, support, guidance, and laying me a hand when I most needed it the most.

Thanks to Dr. Kolomiets' lab for their help and guidance.

Thanks to the department faculty and staff for making my stay at Texas A&M University a pleasant experience.

Thanks to my friends and colleagues (worldwide) who took a moment to ask me how I was or help me when I needed it. I hope to return the favor someday.

Thanks to my mother, my brother, and his family for their encouragement. Even when we were separated geographically, I always felt your love and support. Thank you for that.

Thanks to my husband and son for always believing in me and being supportive in the most critical moments of this project. I love you 'Moniti' and 'Chiquitino mino.' My two-year-old son, who one day will understand why mommy could not put him into bed for so many nights.

Lastly, but certainly not least, thank you, Dad, for becoming a strong reason for not giving up!

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Julio S. Bernal (advisor), Dr. Raul F. Medina and Dr. Keyan Zhu Salzman of the Department of Entomology and Dr. Mike Kolomiets of the Department of Plant Pathology and Microbiology.

All other work conducted for the dissertation was completed by the student independently.

Funding Sources

Graduate study and this work were made possible in part by support from CONACyT-Mexico (CONACyT scholarship #382690), INIFAP-Mexico, and Department of Entomology Teaching Assistantships (Fall 2018, Spring, Summer, and Fall 2019). Partial research support was provided by TAMU-CONACyT Special Research Project “Characterization of Resistance to Root- And Foliage-Feeding Insects in Maize Breeding Lines, Landraces and Wild Ancestors” (Project #2014-024(S)).

The contents of this work are solely responsibility of the authors and not necessarily represent the views from TAMU, CONACyT or INIFAP.

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CHAPTER I

INTRODUCTION

Domestication, spread, and breeding are processes that mediate crop evolution, including herbivore defense evolution. Accordingly, domestication modified interactions between crops and insects so that they differ substantially from those between crop wild ancestors and their herbivores (Macfadyen and Bohan, 2010; Chen et al., 2015a; Wang et al., 2018). As crops spread, they commonly face novel environmental variables (e.g., diverging climatic conditions, less competition, genetic drift associated with dispersal, among other variables) which may reshape plant-insect interactions through changes in herbivory resistance, among other changes (Baker, 1972; Rasmann et al., 2005; Zangerl and Berenbaum, 2005; Erb et al., 2011; Agrawal et al., 2012; Meyer et al., 2012; Züst et al., 2012; Chen, 2016; Turcotte et al., 2017). Systematic breeding, along with geographical spread, and agriculture intensification, also affects crop traits, including herbivore defenses (Bellota et al., 2013; Davila-Flores et al., 2013; de Lange et al., 2014; Maag et al., 2015b; Chinchilla-Ramírez et al., 2017).

Enhanced plant growth – such as in crop plants in agricultural settings – in the face of novel herbivory pressure may lead to tolerance evolution, as posited under the resource availability hypothesis, which predicts that fast-growing plants in resource-rich environments may be selected to favor herbivory tolerance over resistance (Rosenthal and Dirzo, 1997; Zou et al., 2007; Agrawal et al., 2010). Trade-offs between productivity (growth and reproduction) and herbivore resistance, and between herbivore resistance and

tolerance are at the base of hypotheses positing that with plant domestication and improvement for yield, a crop's resistance will suffer compared to that of its wild ancestor, and that tolerance increases as resistance decreases (Hahn and Maron, 2016).

Maize (*Zea mays mays* L.) underwent successive bouts of artificial and natural selection as it was domesticated and gradually spread in the Americas and beyond, and more recently underwent systematic breeding, which enhanced yield and shaped the ways the crop responds to environmental challenges, including herbivory and disease (Rosenthal and Dirzo, 1997; Bellota et al., 2013; Davila-Flores et al., 2013; de Lange et al., 2014; Maag et al., 2015a; Chinchilla-Ramírez et al., 2017). Maize resistance (e.g., antibiosis) and tolerance (e.g., compensatory growth, enhanced photosynthesis) to the key pest Western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte depends to large extent on the triggering of signaling cues, which prepare the plant for the insect's attack (Varsani et al., 2016). Resistance may imply the synthesis of secondary metabolites, whereas tolerance may depend in part on rapid or compensatory growth, and both processes depend on hormonal responses (Vlot et al., 2009; Zhao, 2014; Borrego and Kolomiets, 2016). Thus, constitutive and herbivore-induced biochemical compound profiles relevant to maize resistance and tolerance to WCR, and other herbivores, may have been mediated by the crop's domestication, spread, and breeding (Wright et al 2005, Fontes-Puebla and Bernal, 2019). Similarly, the diversity of constitutive or induced signaling chemicals (i.e. semiochemicals) emitted by maize plants and the responses that these may trigger in WCR may have been altered with domestication, spread, and breeding, as shown for other crops (Rodriguez-Saona et al., 2011; Bellota et al., 2013;

Szczepaniec et al., 2013; Turcotte et al., 2014; Bernal et al., 2015; Chen et al., 2015b; Maag et al., 2015a). Therefore, overall it is expected that plants selected for yield over defense against herbivory will vary in their biochemical and volatile profiles from those of their wild ancestors, resulting in less resistant plants, and that parallel to increases in productivity, crop plants may increases their tolerance to herbivory.

In this study, the main goal was to test whether maize defenses against WCR were mediated by the crop's domestication, spread, and breeding processes. To that end, Chapter II, addresses whether resistance and tolerance to WCR among four *Zea* plant types spanning those processes were affected by domestication, spread, and breeding. The *Zea* plant types are Balsas teosinte (*Zea mays* L. spp. *parviglumis* Iltis and Doebley), Mexican maize landraces, USA maize landraces, and USA maize breeding lines. Each *Zea* plant type was represented by three accessions. Chapter III, addresses whether those domestication, spread, and breeding mediated the profiles of constitutive and induced maize phytohormones relevant to WCR resistance and tolerance. Chapter IV addresses whether recruitment of WCR larvae by *Zea* plants, as well as constitutive root volatile profiles, were affected by domestication, spread, and breeding. Finally, Chapter V provides a general conclusion for the study. Overall, my results were discussed in the contexts of ecological-evolutionary hypotheses seeking to explain defense strategy evolution in plants generally, within the contexts of plant resistance-productivity trade-offs, plant tolerance-resistance trade-offs, and varying resource availability in relation to physiological stress and herbivory pressure. Broadly, my results suggested that defense

strategy evolution in maize, from domestication to the present, is predicted by those ecological-evolutionary hypotheses.

CHAPTER II

RESISTANCE AND TOLERANCE TO ROOT HERBIVORY IN MAIZE WERE MEDIATED BY DOMESTICATION, SPREAD, AND BREEDING[1]

Introduction

Though sessile, plants are not helpless organisms incapable of avoiding their enemies through various defensive means. When directed against herbivory, such defensive means include physical and chemical defenses, an ability to manipulate primary metabolite allocation to reduce herbivore fitness, and tolerance, which are important mediators of plant reproductive success (Zhou et al., 2015; Zust and Agrawal, 2017). Broadly, plant defensive strategies include resistance and tolerance. Resistance relies on direct (physical and chemical) and indirect (e.g., natural enemies, phenology) defenses, while tolerance involves compensatory growth, increased photosynthesis, and other responses that allow plants to reproduce without selecting for herbivore resistance and at no net metabolic cost (Painter, 1951; Strauss and Agrawal, 1999; Boege and Marquis, 2005; Schoonhoven et al., 2005; Stout, 2013). Generally, plant investment in defense seems to be mediated by resource availability, herbivory pressure, and genetic diversity (Hahn and Maron, 2016; Zust and Agrawal, 2017).

[1] The content of this chapter was previously published as a pre-print titled "Resistance and Tolerance to Root Herbivory in Maize were Mediated by Domestication, Spread, and Breeding" by Fontes-Puebla, A.A. and Bernal, J.S., 2019. *BioRxiv*. doi: 10.1101/751982 and has not been certified by peer review.

Whether below- or aboveground, defense against herbivores may be costly to both wild and cultivated plants. Generally, limited metabolic resources are distributed among multiple, competing processes, including defense (e.g., resistance) and productivity, (i.e. growth and reproduction). Defenses against herbivores may be constitutive, which are continuously present, or induced, which are summoned in response to herbivory. Subjected to herbivory, plants may allocate resources to defense responses accordingly, while other processes, such as reproduction (e.g., production of flowers, fruits, seeds), may be allocated fewer resources (Bazzaz et al., 1987; Rodriguez-Saona et al., 2011; Zust and Agrawal, 2017). However, in cultivated plants more resources tend to be allocated toward productivity than defense. For example, breeding enhanced productivity and quality in cranberries, but compromised herbivore defenses, so that constitutive and induced defenses were weakened and herbivore performance was enhanced compared to wild cranberries (Rodriguez-Saona et al. (2011). Similarly, studies on *Zea* L. plants showed that modern maize (*Zea mays mays* L.) cultivars were highly productive but poorly defended, compared to teosinte wild relatives (*Zea diploperennis* Iltis, Doebley & Guzman, *Zea mays* L. spp. *parviglumis* Iltis and Doebley) (Rosenthal and Dirzo, 1997). Interestingly, landrace maize, a form intermediate between teosintes and modern maize, showed intermediate defense and productivity. Overall, those study's results supported a hypothesis positing that herbivore resistance in maize decreased with domestication and improvement for yield (Rosenthal and Dirzo, 1997).

Domestication, spread, and breeding are processes that can mediate crop evolution, including herbivore defense evolution. Accordingly, domestication modified interactions

between crops and insects so that they differ substantially from those between crop wild ancestors and their herbivores (Macfadyen and Bohan, 2010; Chen et al., 2015a; Wang et al., 2018). For instance, following the initial domestication of maize ca. 9000 years before present (YBP) (Matsuoka et al., 2002), the sap-sucking herbivore *Dalbulus maidis* (Delong and Wolcott) became a pest as the crop's defenses were weakened and as its distribution expanded from the Mexican subtropical lowlands to the temperate highlands and beyond (Nault, 1990; Medina et al., 2012; Bernal et al., 2017). As crops spread, they commonly face novel environmental variables, which may reshape their interactions with both associated and newly-acquired herbivorous insects (Baker, 1972; Erb et al., 2011; Meyer et al., 2012; Chen, 2016; Turcotte et al., 2017). Indeed, diverging climatic conditions, reduced competition, genetic drift associated with dispersal, among other variables, have been shown to produce changes in herbivory resistance in a variety of plants and crops (Rasmann et al., 2005; Zangerl and Berenbaum, 2005; Agrawal et al., 2012; Züst et al., 2012). Systematic breeding, along with geographical spread, also affects crop traits, including herbivore defenses. For example, maize underwent natural and artificial selection as it spread into new environments following its domestication (van Heerwaarden et al., 2012; Swarts et al., 2017; Kistler et al., 2018), and was subjected to systematic artificial selection (i.e. breeding) mainly for yield as agriculture was intensified in the 20th century (Troyer, 1999; Whitehead et al., 2017). Such selection shaped maize's herbivore defenses (Bellota et al., 2013; Davila-Flores et al., 2013; de Lange et al., 2014; Maag et al., 2015b; Chinchilla-Ramírez et al., 2017). Moreover, enhanced plant growth in the face of novel herbivory pressure may lead to tolerance evolution, as posited under the

resource availability hypothesis, which predicts that fast-growing plants in resource-rich environments, such as crop plants, may be selected to favor herbivory tolerance, at the expense of resistance (Rosenthal and Dirzo, 1997; Zou et al., 2007; Agrawal et al., 2010).

Crop plants can become hosts for herbivores as a consequence of domestication, spread to new environments, and breeding for high yield, as noted previously (Chen et al., 2015a; Chen, 2016; Chen and Schoville, 2018). After maize's spread from the central Mexican highlands to North America, the oligophagous, root-chewing insect Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* Le Conte) shifted to maize from an unknown ancestral Poeaceae host to later become a pest (Lombaert et al., 2017). WCR likely spread with maize from northern Mexico to southwestern United States as maize became a significant crop and part of the Native American diet ca. 500 years ago (Merrill et al., 2009; da Fonseca et al., 2015; Lombaert et al., 2017; Smith et al., 2017). WCR prefers maize over other hosts, which may be due to the crop plant's comparatively weakened resistance against herbivory and greater nutritional value (de Lange et al., 2014; Bernal and Medina, 2018). Additionally, maize tolerance to WCR may have evolved as the crop faced less competition and non-native herbivory after its spread, and was grown in increasingly rich environments (Buckler and Stevens, 2006; Hahn and Maron, 2016; Robert et al., 2017). Currently, WCR distribution includes northern Mexico, USA, and Europe, where it recently became an invasive pest (Branson and Krysan, 1981; Gerdes et al., 1993; Gray et al., 2009). The economic damage that this herbivore can cause varies, though is substantial, e.g., economic losses attributed to WCR may exceed US$1B yearly

in the USA (Gray et al., 2009), while in Europe they are estimated at €472 million per year (Wesseler and Fall, 2010).

Trade-offs between productivity (growth and reproduction) and herbivore resistance, and between herbivore resistance and tolerance are the bases of hypotheses positing that with plant domestication and improvement for yield a crop's resistance will suffer compared to that of its wild ancestor, and that tolerance will increase as resistance decreases (Hahn and Maron, 2016). Indeed, prior studies comparing the defense responses of maize wild ancestors and maize exposed to different herbivores showed resistance de-escalations with domestication, spread, and breeding (Bellota et al., 2013; Szczepaniec et al., 2013; Bernal et al., 2015; Maag et al., 2015b; Chinchilla-Ramírez et al., 2017), as well as increasing tolerance with spread (Zou et al., 2007). In this study, we tested whether maize defense against WCR was mediated by the crop's domestication, spread, and breeding. To that end, we compared resistance and tolerance among four *Zea* plant types spanning those processes: Balsas teosinte (*Z. mays parviglumis*), Mexican maize landraces, USA maize landraces, and USA maize breeding lines. Each *Zea* plant type was represented by three accessions. The effects of domestication were assessed by comparing resistance and tolerance levels between Balsas teosintes and Mexican maize landraces; the effects of northward spread were assessed by comparing between Mexican landraces and US landraces, and; the effects of breeding were assessed by comparing between US landraces and US inbred lines. Specifically, we measured (i) performance of WCR larvae as a proxy for resistance, and (ii) plant growth as affected by WCR feeding as a proxy for tolerance. Overall, we expected to find decreasing resistance to WCR with maize

domestication, spread and breeding, and increasing tolerance with decreasing resistance. We discussed our results in the context of plant resistance and tolerance evolution, as mediated by artificial and natural selection, geographical spread, and systematic breeding. Specifically, we discussed our findings in relation to ecological-evolutionary hypotheses seeking to explain defense strategy evolution in the contexts of plant resistance-productivity trade-offs, plant tolerance-resistance trade-offs, and varying resource availability vis-à-vis plant physiological stress and herbivory pressure.

Materials and Methods

Plants and Insects

Four plant types belonging to the *Zea* genus were tested: Balsas teosinte, Mexican landraces, US landraces and US inbred lines (Table 1). These plant types were selected to represent the evolution of maize from its wild ancestor through the processes of domestication, spread, and breeding (Troyer, 1999; Matsuoka et al., 2002; Labate et al., 2003; Lombaert et al., 2017). Specifically, (i) Balsas teosinte is the immediate ancestor of maize, thus represented maize in its wild state, prior to domestication; (ii) Mexican landraces were included as descendants of Balsas teosinte, and served to assess the effects of domestication and the crop's early upland spread; (iii) US landraces were included as descendants of Mexican landraces, and used to assess the effects of the crop's spread to North America, and; (iv) US inbred lines were included as descendants of US landraces, and used to assess the effects of modern breeding. Three accessions were chosen as

Table 1. Plant types, accessions, their geographic origins, and reference numbers. From top to bottom, the plant types and their locations of origin span the domestication, spread, and breeding processes of maize from Mexico to the US Corn Belt.

PLANT TYPE	ACCESSION	ORIGIN	REFERENCE[4]
		Jalisco state, Mexico:	
Balsas teosintes[1]	El Cuyotomate	Ejutla, Ejutla (19°58'N, 104°04'W)	—
	Talpitita	Talpitita, Villa Purificación (19°42'N, 104°48'W)	—
	El Rodeo	El Rodeo, Tolimán (19°33'N, 104°03'W)	—
		Mexico state, Mexico:	
Mexican landraces[2]	Palomero Toluqueño	Toluca Valley, Toluca	NSL 2824
	Chalqueño	San Mateo Atenco, San Mateo Atenco	PI 629215
	Cacahuacintle	Toluca Valley, Toluca	NSL 2823
		United States:	
US landraces[2]	Lancaster Sure Crop	Ohio	PI 280061
	Reid Yellow Dent	Indiana	PI 213698
	Gourdseed	Ennis, Texas	PI 414179
		United States:	
US inbred lines	Mo17[2]	Missouri	PI 558532
	B73[3]	Iowa	PI 550473
	W438[3]	Wisconsin	AMES 29447

[1]Collected by JSB; [2]Provided by USDA, ARS Germplasm Resources Information Network (GRIN); [3]Provided by M. J. Kolomiets, Texas A&M University, College Station; [4]USDA, ARS GRIN reference number.

representatives of each of the plant types: “El Cuyotomate,” “Talpitita,” and “El Rodeo” for Balsas teosinte; Palomero Toluqueño, Chalqueño, and Cacahuacintle for Mexican landraces; Lancaster Sure Crop, Reid Yellow Dent, and Gourdseed for US landraces, and; Mo17, B73, and W438 for US inbred lines (Table 1). The teosinte seeds were collected from subtropical lowland locations in Jalisco state, Mexico, whereas the Mexican landraces are grown in the central Mexican highlands. These landraces are ancestral to the selected US landraces through northern Mexican and southwestern US landraces (Merrill et al., 2009; Sánchez, 2011). The US landraces selected for this study are early, parental landraces (Northern Flint and Southern Dent) used to create the early, US Corn Belt inbreds and hybrids (Troyer, 1999; Labate et al., 2003; van Heerwaarden et al., 2012).

Seeds of each accession were germinated in disposable Petri dishes (150×15mm) within moistened paper towels for 3 d. Teosinte seeds were initiated 1 d before maize seeds because they required more time to germinate, and were removed from their fruitcases with a nail clipper. Preliminary germination assays showed no need for seed surface sterilization. After germination, individual seedlings were transplanted to cone-tainers (4×25 cm diameter × length) (Stuewe & Sons, Tangent, OR, USA) and grown for additional 10-12 d; water was provided as needed. The cone-tainers were modified with chiffon mesh covering the bottom to prevent escape of Western corn rootworm larvae (preliminary assays not shown here). Growing conditions were 25 $\pm$ 2°C, 50% RH, and 12:12 photoperiod (L:D). The soil used was Baccto® premium potting soil (Michigan Peat Co., Houston, TX, USA), and was sifted (60 mesh strainer) to facilitate subsequent washing of roots (see below). The number of biological replicates per treatment

(=seedlings)used for all assays were as follow: Balsas teosinte, n = 25 (8 = El Cuyotomate; 9 = Talpitita; 8 = El Rodeo), Mexican landraces, n = 21 (7 = Palomero Toluqueño; 8 = Chalqueño; 6 = Cacahuacintle), US landraces, n = 23 (7 = Lancaster Sure Crop; 8 = Reid Yellow Dent; 8 = Gourdseed), and US inbred lines, n = 23 (7 = Mo17; 8 = B73; 8 = W438).

WCR eggs (diapause strain) were provided by USDA-ARS-North Central Agricultural Research Laboratory (Brookings, SD, USA). Eggs were incubated in Petri dishes at 25 $\pm$ 2°C, ~ 80% RH for 12 $\pm$ 1 d over moistened absorbing paper. Neonate 1st-instar larvae (< 24 h after eclosion) were used in all assays.

Host Plant Resistance and Tolerance Assays

Plant Resistance

The aim of this assay was to assess plant resistance through insect and plant performance variables, and compare between pairs of plant types representing the domestication, spread, and breeding transitions in maize. We expected to find decreasing resistance from Balsas teosinte to US inbred lines, manifested as both enhanced WCR larval performance and increased seedling growth.

To assess WCR performance, 10 neonate WCR larvae were placed in each cone-tainer holding a ~15 d-old seedling, and allowed to feed for 10 d (Robert et al., 2012c); each seedling was paired with a control seedling of similar size and equal number of leaves in order to estimate seedling growth ratios, as explained below. After 10 d, the cone-tainer soil was carefully examined and WCR larvae were recovered, counted and stored in 75%

EtOH. Subsequently, each larva's head capsule width was measured to record whether they were in their 1st, 2nd, or 3rd instar (Hammack et al., 2003). These measurements were made with a dissecting stereoscope at 75× magnification, and equipped with an eyepiece reticle ruler with 100 subdivisions within 10 mm, which had been previously calibrated with a micrometer. Following these measurements, larvae from each cone-tainer were placed in a vial, dried to constant weight (≥ 2 days at 65 °C), and weighed to obtain average weight per larva per each cone-tainer. Each cone-tainer represented a replicated sample for a plant type.

To assess plant performance, true-leaves 2 and 3 (from the bottom, exclusive of cotyledon) were excised from each seedling, and scanned to measure their surface area using ImageJ® software (Rasband, 2017). After this, the seedling was cut at the base of its stem, placed in a paper envelope (together with the corresponding excised leaves) and dried to constant weight (≥ 2 days at 65 °C) (Becker and Meinke, 2008). Seedling roots were rinsed under running water while gently rubbed to remove soil particles, and also dried to constant weight. Stem diameter for each seedling was measured before infestation with WCR, and again prior to harvesting of seedlings, using a digital micrometer (Pittsburgh®, Harbor Freight Tools, Camarillo, CA, USA). These measurements were used to assess seedling growth rate and lost seedling growth under WCR herbivory, as explained below.

A multivariate analysis of variance (MANOVA) was applied to evaluate whether resistance differed among the four plant types, indicating effects of domestication, spread, and breeding. The independent variables were 'plant type' (Balsas teosintes, Mexican

landraces, US landraces, US inbred lines), and 'accessions' (three per plant as described above in *Plants*) which were nested within plant type in the MANOVA model. The dependent variables were foliar weight (leaves and stem), leaf surface area, root weight, larval survivorship (number of recovered larvae/10 initial larvae), and average larval weight (per cone-tainer); additionally, growth rate (= the ratio between seedling stem diameter at days 0 and 10), and lost growth (= the ratio between seedling stem diameter of WCR-infested and -noninfested seedlings at day 10 of the assay) were estimated, and included in the analyses. These growth ratios were used to account for known differences in seedling size among plant types (Chinchilla-Ramírez et al., 2017). All data were transformed to ln(x) prior to analyses; prior to ln(x)-transformation, surface area data were converted to square-root values, and weight data to cubic-root values. *A priori* contrasts were used for paired comparisons between Balsas teosintes and Mexican landraces, Mexican landraces and US landraces, and US landraces and US inbred lines, using a Sidak-adjusted significance level of $P \leq 0.017$ (Abdi, 2007). Pearson correlations of canonical scores with dependent variables were used to determine the contributions of each dependent variable to the total variation in the canonical axes of MANOVA's centroid plots; Pearson's r values $\geq |0.50|$, and $P \leq 0.05$ were considered significant.

Analysis of variance (ANOVA) was performed for each dependent variable ($P < 0.05$), except for the frequencies of WCR larval instars per plant type. Ratios of plant dependent variables (WCR-infested/noninfested) were used to avoid bias due to phenotypic differences between plant types, as explained above. ANOVA was followed by *a priori* contrasts to compare between pairs of plant types, as described above. *G*-tests

were performed ($P \leq 0.017$, per Sidak's correction) to test whether the frequency distributions of WCR larval instars varied between pairs of plant types (Abdi, 2007). Additionally, the proportions of 3rd-instar larvae were calculated for each plant type, and used as a proxy for WCR developmental speed; comparisons between plant types were made using *a priori* contrasts ($P \leq 0.017$). All statistical analyses were performed using JMP software (SAS Institute Inc., 2018).

Plant Tolerance

The aim of this assay was to compare plant tolerance between plant types by measuring plant growth in presence and absence of WCR larvae. As before, the comparisons between plant types sought to assess the effects of domestication, spread, and breeding, as described above for *Plant resistance*. We expected to find increasing tolerance from Balsas teosintes to US inbred lines, manifested as compensation for tissue loss due to feeding by WCR larvae.

The methodology used to assess plant tolerance followed that of an earlier study, with appropriate modifications (Chinchilla-Ramírez et al., 2017). The plant variables measured for plant resistance (foliar weight, leaf surface area, final stem diameter, and root weight; see above) were measured in treated (with 10 WCR larvae) and control (without WCR larvae) seedlings. Control seedlings were plants similar in size and number of leaves to treated seedlings, so that each treated seedling had a paired, control seedling. MANOVA and Pearson correlations of canonical scores were conducted as described above under *Plant Resistance*, with some exceptions. Independent variables included

'plant type' (Balsas teosintes, Mexican landraces, US landraces, US inbred lines), 'herbivory' (with and without WCR larvae), 'accessions' (three per plant as described above in plants) nested within plant type, initial stem diameter (at 0 days) (as covariate), and the interaction term 'herbivory × plant type;' initial stem diameter was included to account for anticipated size different across plant types and accessions (Chinchilla-Ramírez et al., 2017). The dependent variables included were final stem diameter (at 10 days of the assay), foliar weight, leaf surface area, and root weight. Following MANOVA, *a priori* contrasts between plant types were used to separate multivariate means between pairs of plant types (critical $P \leq 0.017$, per Sidak's correction), as described above. To examine whether seedlings compensated tissue lost to herbivory by WCR, we calculated the mean ratios (= weight of infested seedlings/weight of non-infested seedlings) for each dependent variable, and applied one-sample *t*-tests with the null hypothesis that ratios would not differ from 1 (i.e. $H_0 = 1$, no loss nor gain of tissue with WCR herbivory); the critical significance level was set to $P \leq 0.012$, per Sidak's correction for four tests (Abdi, 2007). We considered ratio values < 1 as indicative of under-compensation, values = 1 of compensation, and > 1 of over-compensation. Data for these comparisons were transformed to cubic root(x) values for analyses. All statistical analyses were performed using JMP software (SAS Institute Inc., 2018).

Plant Resistance-Plant Tolerance Trade-off

To address the hypothesis that plant resistance trades off with plant tolerance (i.e. are negatively correlated) we conducted correlation analysis of data obtained in the *Plant*

Resistance and *Plant Tolerance* assays described above. Specifically, we estimated the per-plant accession means for WCR larva weight from the *Plan resistance* assay, and the per-plant accession mean differences in foliar weight between infested (with WCR larvae) and control (without WCR larvae) seedlings in the *Plant tolerance* assay. We considered larva weight as a proxy for resistance, and the difference in foliar weight as a proxy for tolerance; the difference in foliar weight, rather than the difference in root weight, was used as a tolerance proxy to preclude the effect of lost root tissue due to WCR feeding on any gain of root tissue due to compensation. Mean larva weights were converted to cube-root(x) values, and differences in foliar weight to ln(x) values to comply with the expectation of normality. Our null hypothesis was that Pearson's correlation coefficient, r, was larger than -0.5, i.e. $r > [-0.5, 1]$ at $P \leq 0.05$, indicating the absence of a negative correlation.

Results

Plant Resistance

Through MANOVA we assessed whether insect and plant performances were affected by plant type (Figure 1). The analysis revealed a significant multivariate effect on both plant type (Wilks' $\lambda = 0.365$, $P < 0.001$) and accession nested within plant type ($\lambda = 0.361$, $P = 0.037$). *A priori* contrasts between plant types showed significant differences between Balsas teosintes and Mexican landraces ($F_{7, 69} = 4.489$, $P < 0.001$) (i.e. a domestication effect) as well as for Mexican landraces and US landraces ($F_{7, 69} = 2.643$, $P = 0.017$) (i.e.

a geographical spread effect), but not between US landraces and US inbred lines ($F_{7, 69}$ = 1.894, P = 0.083) (i.e. a non-significant breeding effect). The vertical axis in the canonical plot explained 82% of the variation, with root (r = 0.814, $P < 0.001$) and foliar (r = 0.766, $P < 0.001$) weights as the variables that contributed the most to the separation between plant types, whereas the horizontal axis explained 12% of the variation between plant types, with foliar weight (r = 0.526, $P < 0.001$) and plant growth (r = 0.519, $P < 0.001$) as the variables separating plant types (Figure 1).

Analysis of variance on each dependent variable revealed significant plant type effects on foliar ratio, root ratio, and larval weight, growth rate, and lost growth ($P \leq 0.026$), but no effect on leaf surface area and larval survivorship (Table 2). *A priori* contrasts between plant types were applied to each significant dependent variable to assess domestication, spread, and breeding effects. These contrasts revealed significant differences between Balsas teosintes and Mexican landraces in foliar and root ratios ($P \leq 0.005$); between Mexican landraces and US landraces in foliar ratio ($P = 0.001$), and; between US landraces and US inbred lines in foliar ratio, and larval weight ($P \leq 0.008$) (Figure 2).

The distributions of larval instar frequencies varied among plant types (G = 40.43, 6 d.f., $P < 0.001$), (Figure 3A). Pairwise comparisons of frequency distributions showed significant differences between Balsas teosintes and Mexican landraces (G = 17.82, 2 d.f., $P < 0.001$), US landraces and US inbred lines (G = 17.32, 2 d.f., $P < 0.001$), but not between Mexican landraces and US landraces (G = 2.34, 2 d.f., P = 0.309), i.e. significant domestication and breeding effects, but not spread effects (Figure 3A). The development

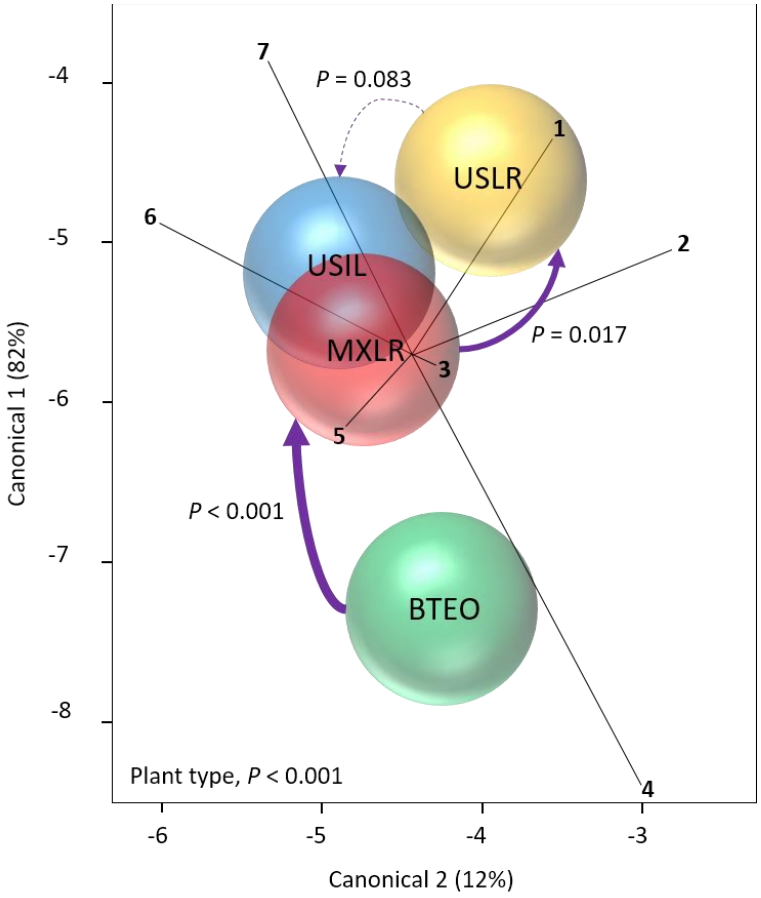

Figure 1. Canonical centroid plot from a Multivariate Analysis of Variance (MANOVA) for plant and Western corn rootworm variables associated with plant resistance. Wilks' $\lambda = 0.365$, $P < 0.001$. Circles represent 95% confidence intervals around multivariate means for each plant type. The model included the independent variables 'plant type' (Balsas teosintes, Mexican maize landraces, US maize landraces, US inbred maize lines), and 'accessions' nested within plant type (three accessions per plant type, not shown here), and the dependent variables larval weight (ray 1), foliar weight (2), leaf surface area (3), plant growth (4), larval survivorship (5), root weight (6), and lost plant growth (7). Significant pair-wise comparisons between plant types (*a priori* contrasts with critical *P* of 0.017, per Sidak correction) are indicated by solid arrows (width is proportional to the confidence level); dashed arrow indicates a non-significant difference. The pair-wise comparisons are between plant types representing the domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLR vs. USIL) transitions evident in maize. BTEO = Balsas teosintes; MXLR = Mexican landraces; USLR = US landraces; USIL = US inbred lines.

Table 2. ANOVA statistics for variables associated to plant resistance. The independent variables 'plant type' (Balsas teosintes, Mexican maize landraces, US maize landraces, and US inbred maize lines) and seven plant and Western corn rootworm dependent variables associated with plant resistance are listed below. *P* values for variables significantly affected by plant type are shown in bold ($P \leq 0.05$).

VARIABLE	df	SS	F	P
Western corn rootworm				
Survivorship	3	379.4	0.363	0.780
Larval weight	3	15.8	6.926	**< 0.001**
Plant type				
Foliar ratio	3	2.3	11.462	**< 0.001**
Growth rate	3	0.5	2.039	**0.001**
Lost growth	3	0.4	3.235	**0.026**
Leaf surface area	3	0.9	2.170	0.098
Root ratio	3	4.9	7.060	**< 0.001**

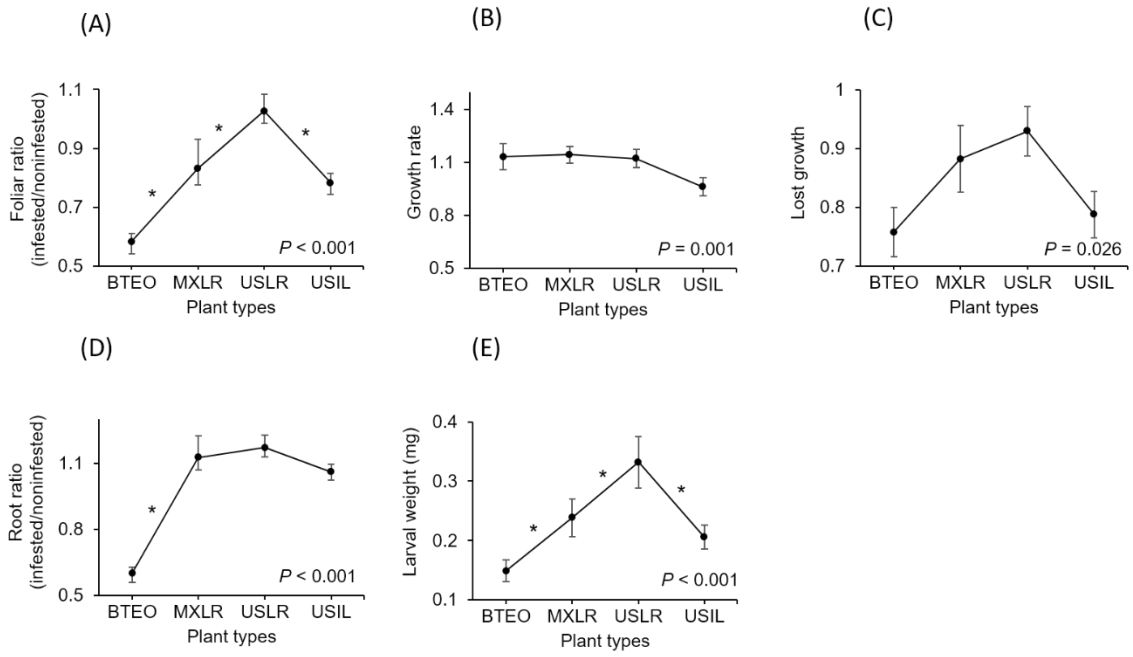

Figure 2. Analysis of variance (ANOVA) between plant types of *Zea* and WCR variables. Paired comparisons between per-plant type means (± SE) of plant and Western corn rootworm (WCR) variables associated with plant resistance. Plant types are ordered left to right from most ancestral to most derived: Balsas teosintes (BTEO), Mexican maize landraces (MXLR), US maize landraces (USLR), and US maize inbred lines (USIL). Asterisks indicate significant difference (*a priori* contrasts with critical $P \leq 0.017$, per Bonferroni correction) between means of contiguous plant types representing the domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLR vs. USIL) transitions in maize; univariate analysis of variance (ANOVA) *P* statistics are inset in each plot (see Table 2 for complete statistics). **(A)** Foliar ratio (= above-ground weights after 10 d, WCR-infested plants/noninfested plants); **(B)** Growth rate (= ratio between WCR infested seedling stem diameter at days 0 and 10 of the assay); **(C)** Lost growth (= stem diameter ratio after 10 d of WCR-infested plants/noninfested plants); **(D)** Root ratio (= belowground weights after 10 d of WCR-infested plants/noninfested plants). **(E)** Larval weight (= weights of WCR larvae after 10 d).

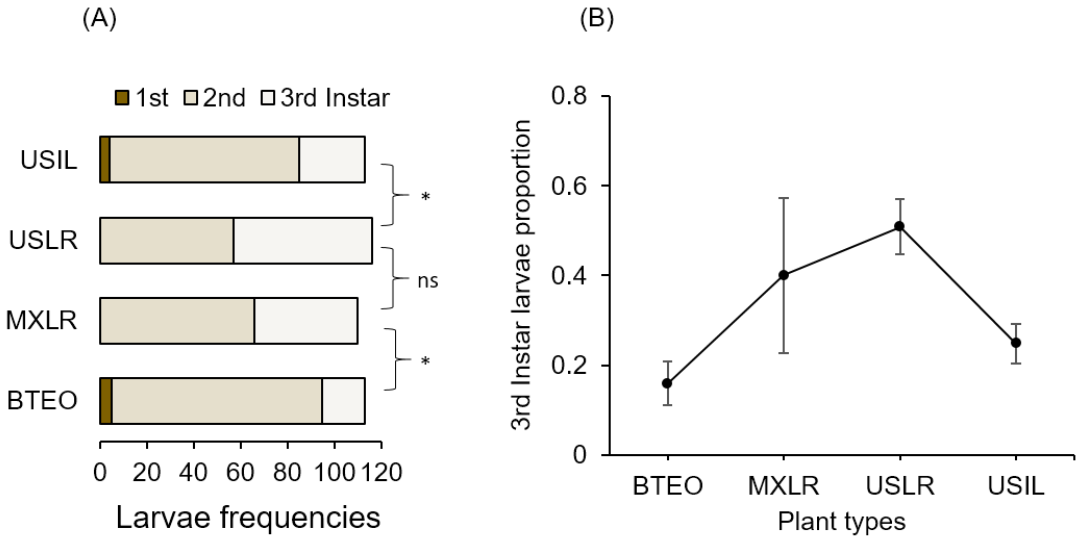

Figure 3. Frequency distribution and developmental speed of WCR. (A) Frequency distributions of 1st-, 2nd-, and 3rd-instar larvae, and (B) development speed (= proportion of larvae reaching 3rd-instar) of larvae of Western corn rootworm in trials concluding 10 d after neonates were allowed to feed on one of four plant types. Plant types were Balsas teosintes (BTEO), Mexican maize landraces (MXLR), US maize landraces (USLR), and US inbred maize lines (USIL), and are ordered from most ancestral to most derived. *A priori*, pair-wise comparisons between frequency distributions representing the domestication (BTEO vs. MXLR) and breeding (USLR vs. USIL) transitions were significant (* = $G \geq 17.25$, $P < 0.001$), while the comparison representing the spread transition (MXLR vs. USLR) was not significant ($G = 2.34$, $P = 0.309$); the critical P value for these comparisons was set at $P \leq 0.017$, per Sidak's correction. Univariate analysis of variance (ANOVA) indicated that development speed did not vary across plant types ($F_{3, 8} = 2.33$, $P = 0.150$).

speed of WCR larvae did not differ significantly among plant types ($F_{3, 8} = 2.33$, $P = 0.150$) (Figure 3B).

Overall, these results suggested that *Zea* resistance to WCR decreased with domestication and spread, and was partially recovered by breeding. Balsas teosintes appeared as the most resistant plant type, US landraces as the least resistant, and Mexican landraces and US inbred lines as intermediately resistant.

Plant Tolerance

MANOVA (overall Wilk's $\lambda = 0.142$, $P < 0.001$) revealed significant effects of herbivory ($F_{4, 156} = 16.555$, $P < 0.001$), plant type ($\lambda = 0.622$, $P < 0.001$), and herbivory × plant type interaction ($\lambda = 0.869$, $P = 0.025$) on seedling tolerance levels to WCR feeding (Figure 4). *A priori* contrasts within plant types revealed significant differences between WCR-infested and -noninfested Balsas teosinte ($F_{4, 164} = 9.922$, $P < 0.001$), Mexican landrace maize ($F_{4, 164} = 4.115$, $P = 0.003$), and US inbred maize ($F_{4, 164} = 4.684$, $P = 0.001$), but not within US landrace maize ($F_{4, 164} = 2.253$ $P = 0.065$) (Figure 4), suggesting that only US landraces displayed broad tolerance to WCR feeding. Correlation analysis of canonical scores showed that the vertical axis of the centroid plot explained 77% of the variation, with final stem diameter ($r = 0.67$, $P < 0.001$), foliar weight ($r = 0.91$, $P < 0.001$), and root weight ($r = 0.90$, $P < 0.001$) as the variables that most contributed to the separation between infested and non-infested plant types. The same analysis showed that the horizontal axis explained 19% of the variation between infested and noninfested plant

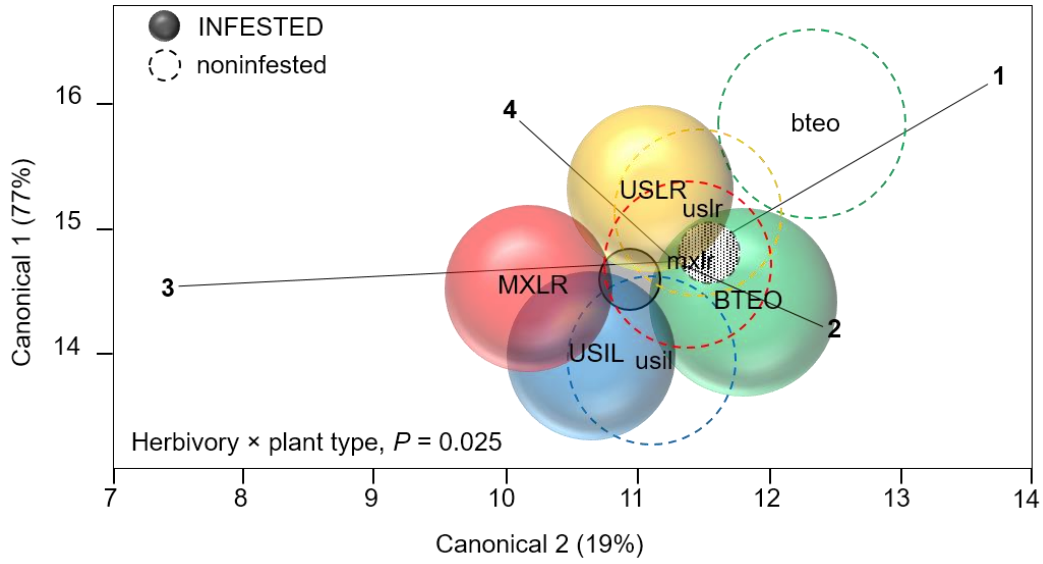

Figure 4. Canonical centroid plot from MANOVA on plant variables associated with plant tolerance to feeding by Western corn rootworm. Circles represent 95% confidence intervals around multivariate means for each plant type. The model includes the independent variables 'plant type' (Balsas teosintes, Mexican maize landraces, US maize landraces, US inbred maize lines), 'accessions' nested within plant type (three accessions per plant type, not shown here), herbivory (Western corn rootworm presence or absence) and the interaction term 'herbivory × plant type.' The dependent variables were foliar weight (ray 1), leaf surface area (2), final stem diameter (3), and root weight (4). The overall model (Wilks' $\lambda = 0.142$, $P < 0.001$) and main effects were significant: plant type ($\lambda = 0.622$, $P < 0.001$), herbivory ($F_{4, 164} = 16.555$ $P < 0.001$), and herbivory × plant type ($\lambda = 0.869$, $P = 0.025$). Pair-wise comparisons between Western corn rootworm-infested and -noninfested plants within plant types (depicted by continuous circles/upper-text and dashed circles/lower-case text) were significant for all plant types, except for US landraces; Balsas teosintes, $F_{4, 164} = 9.922$, $P < 0.001$; Mexican landraces, $F_{4, 164} = 4.115$, $P = 0.003$; US landraces. $F_{4, 164} = 2.253$, $P = 0.065$; US inbred lines, $F_{4, 164} = 4.684$, $P = 0.001$. Smallest circles (filled) near plot center represent overall Western corn rootworm-infested (solid line and filling) and -noninfested (dashed line, patterned filling) plants. BTEO, bteo = Balsas teosintes infested or noninfested, respectively, by Western corn rootworm; MXLR, mxlr = Mexican landraces; USLR, uslr = US landraces; USIL, usil = US inbred lines.

types, with leaf surface area as the main explanatory variable ($r = 0.53$, $P < 0.001$) (Figure 4).

Within each plant type, tissue losses, assessed as mean ratios (= WCR-infested seedlings/non-infested seedlings) of foliar weight, leaf surface area, final stem diameter, and root weight, were found to be undercompensated (i.e. ratio < 1.0, $P < 0.001$) in both Balsas teosintes and US inbred lines, with the exception of root tissue, which was compensated in US inbred lines (i.e. ratio > 1.0, $P = 0.780$) (Figure 5). Mexican landraces compensated foliar, final stem diameter and root tissue losses (i.e. ratio did not differ from 1.0, $P \geq 0.019$), and undercompensated leaf surface area losses ($P < 0.001$). Finally, US landraces compensated all tissue losses, foliar, leaf surface area, final stem diameter, and root tissue ($P \geq 0.020$). These results suggested that US landraces displayed tolerance to WCR as they consistently compensated tissue losses, Mexican landraces and US inbreds displayed partial tolerance, and Balsas teosintes did not display tolerance (Figure 5). Herbivory × plant type interaction effects are shown in Figure 6. Significant differences between infested and noninfested seedlings were found for foliar ($F_{3, 167} = 3.126$, $P = 0.027$) and root ($F_{3, 167} = 4.039$, $P = 0.008$) weights, but not for final stem diameter ($F_{3, 167} = 0.8140$, $P = 0.487$) nor leaf surface area ($F_{3, 167} = 0.471$, $P = 0.702$). *A priori* contrast comparisons between infested and noninfested seedlings ($P \leq 0.012$; Sidak corrected) revealed significant foliar tissue losses (i.e. undercompensation) in Balsas teosintes ($F_{1, 167} = 27.536$, $P < 0.001$) and Mexican landraces ($F_{1, 167} = 7.543$, $P = 0.007$), while US landraces ($F_{1, 167} = 0.890$, $P = 0.346$) and US inbred lines ($F_{1, 167} = 4.127$, $P = 0.041$) did not lose nor gain tissue (i.e. compensation) (Figure 6a). *A priori* contrast comparisons for

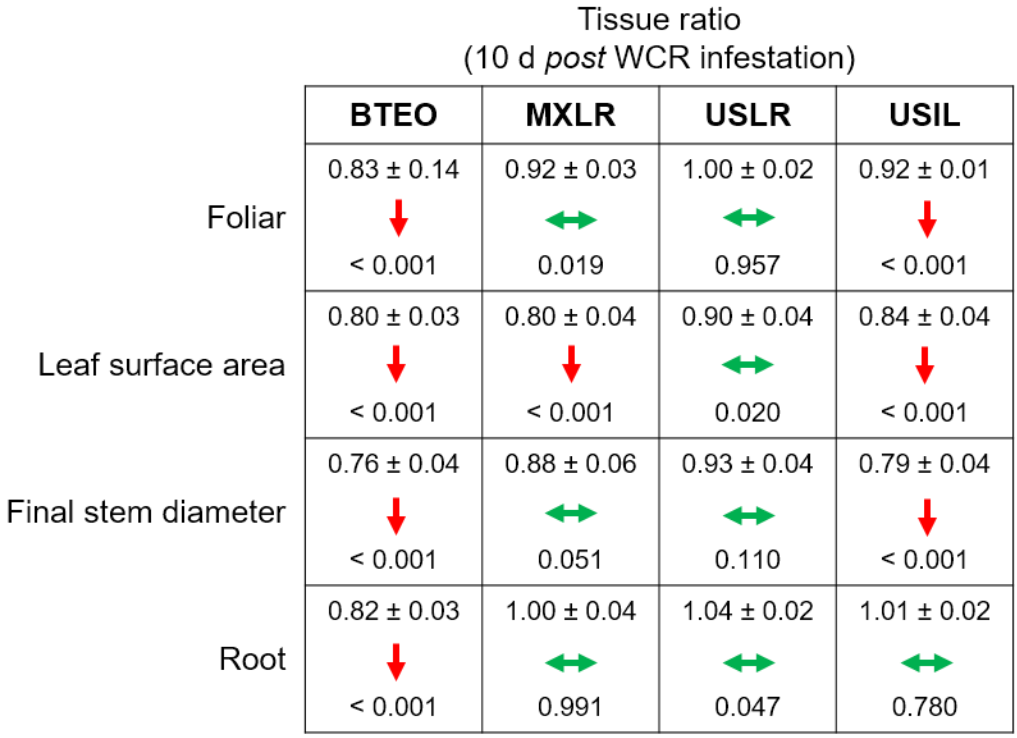

Figure 5. Effects of herbivory by Western corn rootworm on plant tolerance variables from the four plant types. The Plant types are ordered from most ancestral to most derived: Balsas teosintes (BTEO), Mexican maize landraces (MXLR), US maize landraces (USLR), and US inbred maize lines (USIL). The plant tolerance variables are mean ratios (= Western corn rootworm-infested plants/noninfested plants) of final stem diameters, foliar weights, leaf surface areas, and root weights. One-sample t-tests were used to compare mean ratios for each plant type against expected ratio of 1.0, which indicates tissue compensation (i.e. no tissue lost or gained in Western corn rootworm-infested plants relative to noninfested plants); the mean ratio (± SE) and P value are shown within each cell. Within each cell, double-pointed, horizontal green arrows indicate compensation (mean ratio does not differ from 1.0), and downward, red arrows indicate undercompensation (mean ratio < 1.0). Critical P for each t-test was set at $P \leq 0.012$, per Sidak's correction.

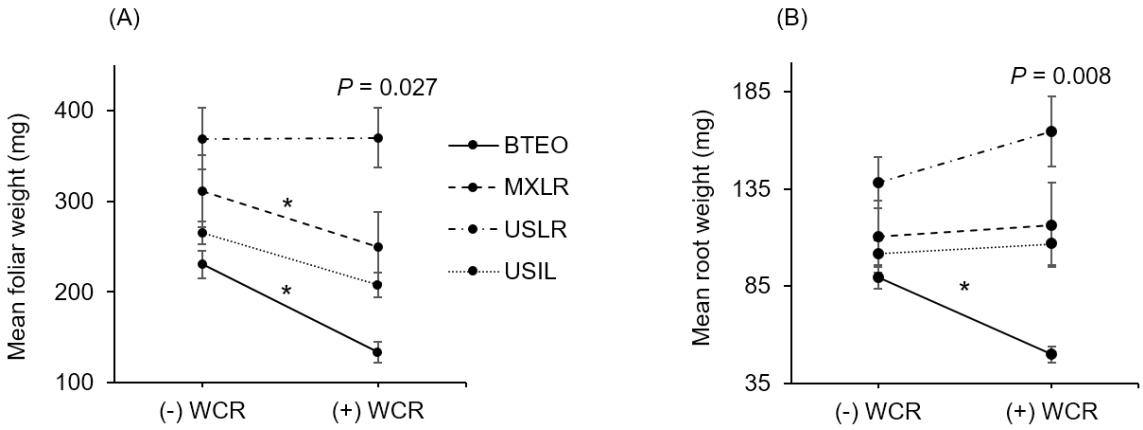

Figure 6. Tissue losses in four plant types. (A) Above- and **(B)** belowground tissue losses in four plant types, Balsas teosintes (BTEO), Mexican maize landraces (MXLR), US maize landraces (USLR), and US inbred maize lines (USIL), exposed to root herbivory by Western corn rootworm (WCR) larvae. Inset in each plot are the univariate analysis of variance (ANOVA) statistics for the herbivory (+WCR, -WCR) × plant type effect in foliar weight ($F_{3, 167} = 3.126$, $P = 0.027$) and root weight ($F_{3, 167} = 4.039$, $P = 0.008$). Comparisons between plant types exposed (+WCR) and unexposed (-WCR) to Western corn rootworm larvae were made via *a priori* contrasts, with a critical *P* value for each paired comparison set at $P \leq 0.012$, per Sidak's correction. Significant herbivory effects are indicated by an asterisk (*).

root weights revealed that Balsas teosintes lost tissue (i.e. undercompensation) ($F_{1, 167}$ = 13.576, $P < 0.001$), whereas Mexican landraces ($F_{1, 167} = 0.005$, $P = 0.942$), US landraces ($F_{1, 167} = 0.424$, $P = 0.515$), and US inbred lines ($F_{1, 167} = 0.158$, $P = 0.691$) did not lose nor gain tissue (i.e. compensation) (Figure 6b).

Overall, these results suggested that *Zea* tolerance to WCR was gained with domestication and reinforced by spread. However, it also suggested that breeding weakened tolerance to a point comparable to that evident in Mexican landraces. The tolerance levels, ordered from most to least tolerant plant type appeared to be US landraces, Mexican landraces, and US inbreds, while Balsas teosintes appeared to be intolerant.

Plant Resistance-Plant Tolerance Trade-off

Correlation analysis showed a significant negative correlation between per-plant accession larval weights and differences in foliar weights between WCR-infested and non-infested seedlings ($r = -0.646$, $P = 0.023$) (Figure 7). Consistent with the *Plant resistance* and *Plant tolerance* results, the analysis suggested that Balsas teosintes was the most resistant plant type, US landraces was the least resistant, and Mexican landraces and US Inbred lines were intermediately resistant. Conversely, it suggested that US landraces was the most tolerant plant type, Balsas teosintes was the least tolerant, and Mexican landraces and US Inbred lines were intermediately tolerant. Overall, these results suggested that resistance declines with increasing tolerance in *Zea*.

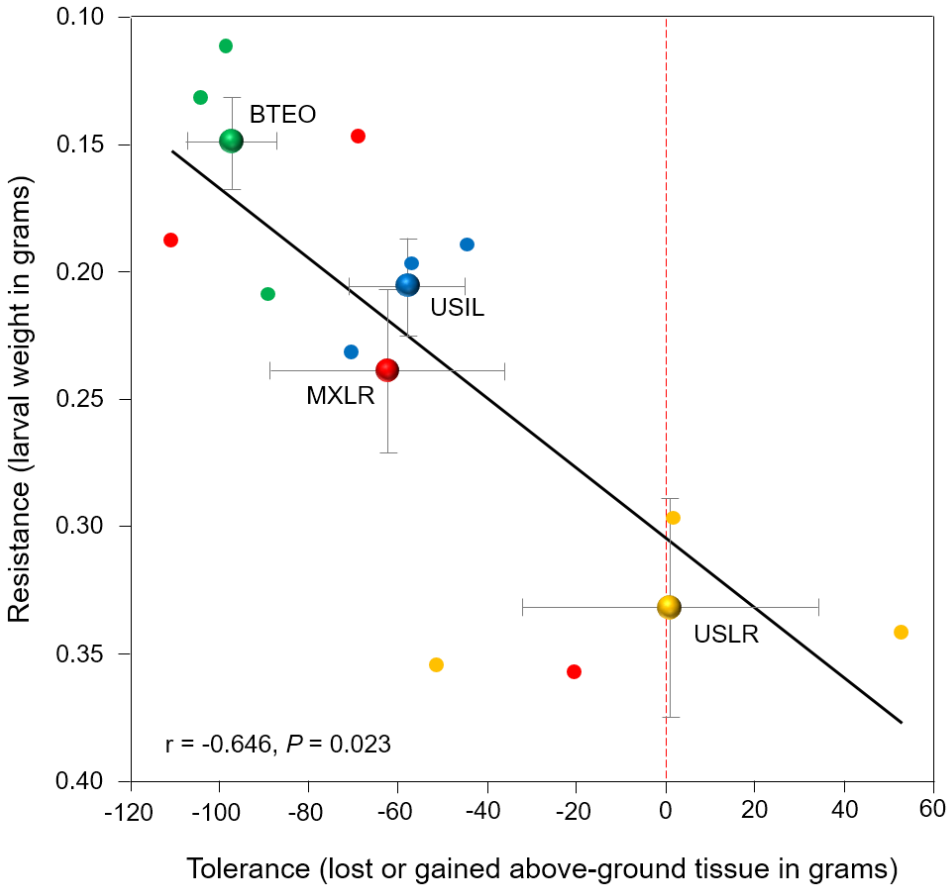

Figure 7. Correlation between resistance and tolerance to root herbivory in *Zea* plant types. Relationship between resistance (expressed as larval weight) and tolerance (expressed as plant tissue loss or gain) to root herbivory by Western corn rootworm larvae in 12 plant accessions (small circles), with three accessions corresponding to each of four plant types (large circles with bi-directional standard error bars). Note that *y*-axis values increase from top to bottom. Plant types are Balsas teosintes (BTEO), Mexican maize landraces (MXLR), US maize landraces (USLR), and US inbred maize lines (USIL). The weight of Western rootworm larvae (g) after 10 days of feeding on each accession was used as a proxy for resistance, while the loss or gain of above-ground tissue (g) of each accession after 10 days of exposure to root herbivory by rootworm larvae was used as a proxy for tolerance. Inset is Pearson's correlation *r* statistic corresponding to the 12 plant accessions. The red, dotted vertical line on *x*-axis indicates tissue compensation (i.e. no tissue lost nor gained); means to the left of the dotted line are suggestive of undercompensation for tissue loss, and means to the right are suggestive of overcompensation for tissue loss.

Discussion

This study addressed whether maize defense, in the forms of resistance and tolerance to root herbivory, was mediated by domestication, spread, and breeding processes that spanned divergent environments and thousands of years and kilometers. To that end, we studied resistance and tolerance to Western corn rootworm feeding in four host plants that encompass those processes: Balsas teosinte, Mexican landrace maize, US landrace maize, and US inbred line maize. Specifically, we assessed the performances of WCR larvae and host plant types as proxies for resistance, and the performances of host plant types as affected by WCR feeding as proxies for tolerance. We expected to find that maize resistance against WCR was weakened with domestication, spread, and breeding, and that tolerance to WCR increased as resistance decreased. Our results were consistent with our expectations, though not entirely. On one hand, maize resistance indeed decreased from Balsas teosintes to US landraces, i.e. with maize domestication and spread, though, surprisingly, the trend seems to have reversed with breeding: US inbred lines showed more resistance to WCR than their US landrace ancestors, so were intermediately resistant rather than least resistant. On the other hand, tolerance indeed increased as resistance decreased, as expected.

Maize Resistance Decreased with Domestication and Spread, but Increased with Breeding

Our results suggested that maize resistance to root herbivory by WCR was weakened with domestication and spread, as we expected, while breeding affected resistance differently than we expected. Specifically, MANOVA revealed a strong multivariate effect of plant type on resistance variables, and *a priori* comparisons showed significant differences between Balsas teosintes and Mexican landraces, as well as between Mexican landraces and US landraces, but not between US landraces and US inbreds. Similarly, ANOVAs of individual dependent variables showed both domestication and spread effects, especially on WCR larval performance (i.e. weight), which was enhanced on Mexican landraces compared to Balsas teosintes, as well as on US landraces compared to Mexican landraces. However, WCR larval performance declined on US inbreds compared to US landraces, in partial contrast to our MANOVA results. Moreover, an *a posteriori* contrast comparison between Balsas teosintes and US inbred lines showed no significant differences in larval weight and lost plant growth ($F_{1, 167} = 4.033$, $P = 0.046$; data not shown; Sidak-corrected critical $P \leq 0.012$). This result may indicate significant allocation of resources to defense against WCR in US inbred lines, as would be expected to support enhanced resistance. Overall, these results suggested that domestication and spread affected resistance, as we anticipated and in agreement with other studies (Bazzaz et al., 1987; Rosenthal and Dirzo, 1997; Rodriguez-Saona et al., 2011), but resistance was partly recovered with breeding, contrary to expected. The optimal plant defense hypothesis predicts that there is a cost of defense,

particularly that metabolic resources cannot be simultaneously used to defend, grow, and reproduce, so that plant fitness increases when herbivory decreases or is absent (Stamp, 2003). This prediction did not seem to apply to US inbred lines, which appeared to compensate root tissue (see below) while decreasing WCR larval weight.

Domestication, spread, and breeding significantly affected WCR performance. These results suggested two, non-exclusive defense strategies related to plant defense biochemistry. First, the nutritional value for WCR in *Zea* host plants may have increased from Balsas teosinte to US landrace maize, but decreased in US inbred maize. Changes in nutrient composition may cause differences in larval weight and development, while maintaining survivorship (Meihls et al., 2018). WCR uses a blend of sugars and fatty acids, but not amino acids, as phagostimulants to accept and feed on maize (Bernklau and Bjostad, 2008). Sucrose, although of non-nutritional value to most insects, is known to be an important phagostimulant, including for larvae of Coleoptera, and may be more relevant for host plant acceptance or rejection than any amino acid considered important for insect development (Chapman, 2003). There are no direct studies, to our knowledge, comparing root nutritional value among *Zea* plants. However, *Zea* has experienced selection in 2-4% of its genome, resulting in numerous biochemical differences among teosintes, landraces, and inbred lines (Dorweiler et al., 1993; Wright et al., 2005; Flint-Garcia et al., 2009; de Lange et al., 2014). Secondly, maize landraces may be down-regulating some secondary metabolites, while maize inbreds may be up-regulating them to levels similar to those in Balsas teosinte. The composition of secondary metabolites has been altered by domestication in various crops, affecting their interactions with specialist

and generalist insects (Da Costa and Jones, 1971; Howe et al., 1976; Gols et al., 2008; Chacon-Fuentes et al., 2015). Typically, generalist herbivores perform better on domesticated plants compared to their wild relatives due to a reduction in secondary metabolites (Rodriguez-Saona et al., 2011; Bellota et al., 2013; Szczepaniec et al., 2013; Turcotte et al., 2014; Bernal et al., 2015; Chen et al., 2015b; Maag et al., 2015b). WCR shifted to maize when the crop reached northern Mexico (Lombaert et al., 2017), and encountering maize landraces with weaker defenses than its original, wild host may have been advantageous for the quasi-specialist WCR (Branson and Ortman, 1967; 1970; Hahn and Maron, 2016). Maize breeding, conversely, may have partly reversed the decreasing trend of secondary metabolite levels, without a concurrent effect on maize productivity. Maize per-plant productivity (but not per-area yields) seems to have reached a maximum several decades ago, so that any productivity costs of chemical defense may be negligible, while concurrent breeding efforts may have inadvertently selected for WCR resistance, as evident for other maize pests (Duvick, 2005). Regardless of the relative importance of either defense strategy, the differences in WCR and seedling responses among plant types in our study was consistent with hypotheses of resistance reductions with domestication and spread (Rosenthal and Dirzo, 1997; Whitehead et al., 2017; Zust and Agrawal, 2017). However, breeding seemingly increased resistance (measured as decreased WCR performance) in US inbred lines, with no apparent cost to productivity. Further below, we discuss conditions under which resistance against WCR may have increased in US inbred maize concurrently with productivity, i.e. yield gains, particularly in the context of

intensive maize agriculture reliant on modern technologies, such as synthetic fertilizers and pesticides, among others.

Maize Tolerance Increased with Domestication and Spread, but Decreased with Breeding

Our results suggested that maize tolerance of root herbivory by WCR was enhanced as resistance decreased with domestication and spread, as expected, while breeding affected tolerance (and resistance) differently than expected. Specifically, MANOVA revealed strong multivariate effects of plant type on tolerance variables, and contrast comparisons revealed increasingly smaller (but significant) differences between WCR-infested and control seedlings (as indicated by *F* and *P* values) in Balsas teosintes, Mexican landraces, and US inbreds, while a significant difference was not found in US landraces. In this regard, US landraces showed the smallest partial η^2 effect size of WCR infestation on seedling growth (partial η^2 = 0.055, 0.000 – 0.101), while effect sizes were 3.6- (partial η^2 = 0.200, 0.099 – 0.273), 2.4- (partial η^2 = 0.134, 0.046 – 0.200), and 1.9-fold greater (partial η^2 = 0.107, 0.027 – 0.168) in Balsas teosintes, Mexican landraces, and US inbreds, respectively (data not shown) (Richardson, 2011). Similarly, our univariate analyses showed that US landraces consistently compensated for tissue losses, while Mexican landraces and US inbreds inconsistently compensated for tissue losses, and Balsas teosintes consistently undercompensated for tissue losses. Finally, measured as total above- or belowground tissue losses, Balsas teosintes lost both above- and belowground tissue with WCR feeding, Mexican landraces and US inbreds lost

aboveground tissue, and US landraces compensated for above- and belowground tissue losses. Taken together, these results suggested that tolerance was strongest in US landraces, weakest in Balsas teosintes, and intermediate in Mexican landraces and US inbreds.

Domestication and subsequent farming could favor tolerance evolution when abiotic factors (e.g., soil nutrients, light availability) mediate the selection of plant defenses against herbivores (Hahn and Maron 2016). Annual crops, grown as they typically are, in resource rich environments are predicted to maximize fitness by allocating resources towards growth and reproduction, and trading-off constitutive resistance to herbivory (Herms and Mattson, 1992; Rosenthal and Dirzo, 1997). Additionally, biotic factors may impose selective pressures on domesticated plants. For example, in Hahn and Maron´s (2016) framework for intraspecific variation of plant defenses, two factors mediate defense evolution to tolerance or resistance: Low physiological stress (selecting for fast growing plants) and herbivory pressure (selecting for induced resistance). Moreover, herbivory pressure may indirectly select for tolerance as some root herbivores are able to manipulate the host to allocate primary metabolites (e.g., carbon, phosphorus, among others) to roots, and increase their host's quality (Robert et al., 2012b). Such allocation may increase the likelihood of root compensation and, therefore, tolerance to root herbivory, and plants able to compensate for root herbivory may be favored by selection (cf. Figure 5 and 6). In parallel, this may explain the increased resistance and weakened tolerance in US inbred lines compared to US landraces. US inbred lines have been bred in contexts of low physiological stress and high WCR herbivory, especially

since the 1940s, compared to the contexts in which their ancestral landraces were grown and selected (see below).

Maize Resistance and Tolerance Trade-Off

Overall, our results showed a negative correlation between resistance and tolerance, consistent with optimal defense hypotheses and our expectation (Herms and Mattson, 1992; Blossey and Notzold, 1995; Zou et al., 2007; Hahn and Maron, 2016). However, we expected that this correlation would be consistent also with the evolutionary transitions between Balsas teosintes and US inbred lines. Usually, trade-offs are observed when fitness is compromised due to competing resource demands, e.g., resistance and fast growth (Agrawal et al., 2010). Natural selection may benefit one or the other depending on their direct or ecological costs (Strauss et al., 2002). Artificial selection, however, may favor a trade-off between a desired trait and a less-desired trait, e.g., selection for productivity weakened resistance, as our results suggested for Mexican landraces. A changing environment and herbivory pressure for US landraces may have led to an adaptive, negative correlation, where maize exposed to WCR under higher resource availability was subjected to strong selection for herbivory tolerance (Agrawal et al., 2010). Furthermore, plant resistance may de-escalate when a plant's herbivore fauna is dominated by mono- or oligophagous insects, such as WCR (Agrawal and Fishbein, 2008; Agrawal et al., 2010). WCR became a pest after maize agriculture spread to North America, and may have been an important selection force shaping the defenses of modern maize in the US. The extended, thousands of years-association between maize and WCR

— punctuated with severe WCR bottlenecks when maize agriculture became dominant in (current) southwestern (ca. 500 YBP) and northern (ca. 180 YBP) USA states — may have led to an evolutionary compromise, with maize gaining tolerance and WCR becoming a specialist (Robert et al., 2012b; Lombaert et al., 2017; Robert et al., 2017).

Disarmed by Agricultural Intensification: Maize Traded Western Corn Rootworm Tolerance for Token Resistance

Our results addressing the effects of maize domestication and spread on defense strategy evolution were consistent with theoretical predictions concerning resistance and tolerance evolution in the contexts of plant productivity-resistance trade-offs and plant resistance-tolerance trade-offs, respectively (Agrawal et al., 2010; Pearse et al., 2017; Zust and Agrawal, 2017). Namely, our results showed that resistance to WCR decreased with both maize domestication and spread, and tolerance increased as resistance decreased, as expected. However, our results addressing the effects of breeding on maize defenses were inconsistent with predictions based on productivity-resistance and resistance-tolerance trade-offs. Specifically, breeding reversed the preceding trend of decreasing resistance and increasing tolerance so that US inbred lines were less tolerant and more resistant to WCR than their ancestral US landraces. We believe that this reversal is a result of agricultural intensification of maize production, particularly the systematic breeding of maize varieties for maximum yield under the umbrella of commercial, synthetic fertilizers, irrigation, and pesticides (Bernal and Medina, 2018). Under such intensification, pesticides provided relief from WCR injury without a metabolic cost to the crop, and fertilizers coupled with

irrigation enhanced plant nutrient levels to support on one hand the productivity increases gained with systematic breeding, and on the other to offset any productivity losses due to WCR and other pests. This intensification period began in the late 1940s with the widespread availability of hybrid maize varieties, chemical fertilizers, and pesticides, and in the context of increasing pressure by WCR, which up to then had not been a significant pest (Perkins, 1982; Palladino and Fitzgerald, 1996; Duvick, 2005; Gray et al., 2009; Smith, 2011; Lombaert et al., 2017). In contrast, the period prior to intensification was characterized by natural and farmer (artificial) selection of maize landraces for broad resistance to environmental stresses, the absence of pesticides and commercial fertilizers, and minimal WCR pressure; this period ended with the deployment of commercial hybrid varieties, and decline of landraces, beginning in the 1930s (Duvick, 2005; Kutka, 2011; Smith, 2011; Bernal and Medina, 2018).

Overall, our results were consistent not only with predictions concerning plant defense evolution in the contexts of plant productivity-resistance trade-offs and plant resistance-tolerance trade-offs, as noted above, but also with predictions concerning defense strategy evolution in the context of variable resource availability and environmental stresses, particularly physiological stress (under low resource availability) and herbivory pressure (Herms and Mattson, 1992; Blossey and Notzold, 1995; Zou et al., 2007; Hahn and Maron, 2016) (Figure 8). We believe that shifts in resource availability, WCR pressure, and farmer selection of maize landraces to systematic breeding of maize inbred lines between the pre-intensification and intensification periods of maize production mediated the evolution of WCR defenses in US inbred maize lines (Duvick,

2005; Gray et al., 2009; Ivezić et al., 2009; Kutka, 2011; Smith, 2011; Lombaert et al., 2017; Mesa et al., 2017). For example, while the slight gain in WCR resistance evident in US inbred lines was not anticipated per expectations under a productivity-resistance trade-off, it was an anticipated result of directed systematic breeding for WCR resistance (and inadvertent selection under WCR pressure), and was associated with a loss of tolerance, as anticipated under a resistance-tolerance trade-off (Duvick, 2005; Agrawal, 2006; Agrawal and Fishbein, 2008; Gray et al., 2009; Ivezić et al., 2009; Agrawal et al., 2010). In Figure 8a, we show how resource availability may have increased (indicated by the arrow's increasingly dark coloration) with maize domestication and spread, as maize — by that time an important food crop — is subjected to site selection and cultural practices aimed at enhancing its productivity. Concomitantly, physiological stress gradually may have lost importance as a driver of herbivore defense evolution as resource availability increased (see horizontal arrow at top of Figure 8, showing how resource availability is relevant to defense evolution at low resource availability, while herbivory pressure is relevant at high resource availability). In Figure 8B, we show how resource availability may have continued to increase and reached its highest level with the breeding transition, particularly with the advent of commercial fertilizers to support cultivation of high-yielding maize cultivars, i.e. intensification. At the same time, WCR emerged as an important pest of maize, and while it may have become a significant driver of herbivore defense evolution, its significance was mediated by the use of insecticides, which became widely available as maize agriculture was increasingly intensified. Altogether, we believe that our results illustrate how the evolution of defense strategies in maize, and perhaps

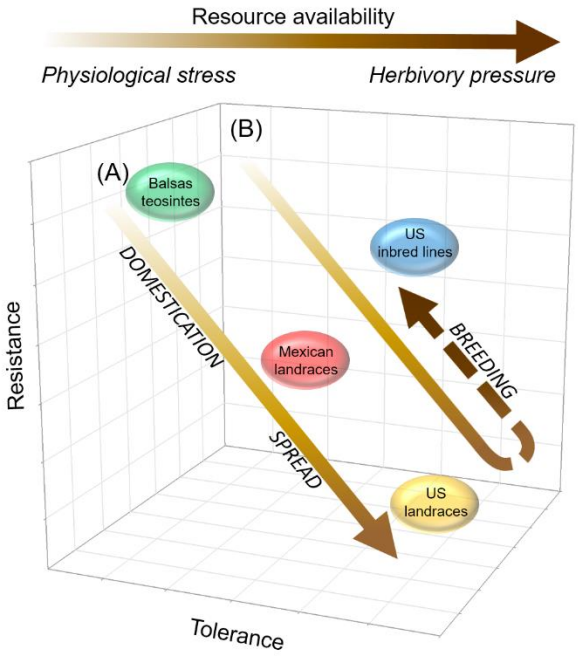

Figure 8. Hypothesized relationship between plant tolerance and resistance in maize, as mediated by agricultural intensification, resource availability, and environmental stress. In this study's context, *Agricultural intensification* refers to widespread cultivation of high-yielding maize cultivars developed through systematic breeding, under the umbrella of chemical inputs, particularly commercial insecticides and synthetic fertilizers, and under increasing WCR pressure (see *Text* for additional details). The high-yielding cultivars are hybrids generated from inbred lines, which require chemical fertilizers (and adequate moisture) and pesticides to reach maximum productivity. The prior, *pre-intensification* period is characterized by widespread cultivation of landrace maize, natural and farmer (artificial) selection of landraces for broad resistance to environmental stresses, absence of fertilizers and pesticides, and minimal WCR pressure. **(A)** Prior to agricultural intensification, resistance to WCR gradually decreases while tolerance increases with maize domestication and spread, as resource availability increases, and as physiological stress gradually loses relevance to defense strategy evolution. **(B)** The trend of WCR resistance loss with WCR tolerance gain is reversed with breeding under agricultural intensification, where resource availability is high, physiological stress is minimized with the advent of chemical fertilizers, and WCR pressure becomes relevant to defense strategy evolution, though its relevance is mediated by insecticide use. In arrows in both **(A)** and **(B),** and in horizontal arrow at top of figure, the lighter to darker gradient in coloration indicates an increasing gradient of resource availability; within this gradient, physiological stress and herbivory pressure are most relevant to defense strategy evolution at the low- and high-resource availability extremes, respectively.

other crops, is predicted by ecological-evolutionary hypotheses predicting defense strategy evolution in the contexts of plant resistance-productivity trade-offs, plant tolerance-resistance trade-offs, and varying resource availability vis-à-vis plant physiological stress and herbivory pressure (Herms and Mattson, 1992; Blossey and Notzold, 1995; Zou et al., 2007; Hahn and Maron, 2016).

CHAPTER III

CONSTITUTIVE AND INDUCED BIOCHEMICAL DEFENSES IN MAIZE WERE MEDIATED BY DOMESTICATION, SPREAD, AND BREEDING

Introduction

Phytohormones and their precursors and derivatives are biochemical compounds that regulate a multitude of plant processes, including defenses against herbivores and pathogens, responses to biotic and abiotic stresses, and regulation of plant growth (Wasternack and Kombrink, 2010; Erb et al., 2011). For example, jasmonic acid (JA) has multiple functions in plants, among which are initiating defense responses against insects and pathogens, and inducing trichome development, among others (Maes and Goossens, 2010; Christensen et al., 2013; Yan et al., 2013). Similarly, salicylic acid (SA) has been associated with seed germination, cell growth, stomatal closure, responses to abiotic stressors, and defense against biotrophic and hemibiotrophic pathogens, among others processes (Vlot et al., 2009). Importantly, however, cross-talk may occur between phytohormones, e.g., antagonism between JA and SA (Aloni et al., 2006; Wasternack and Hause, 2013).

Phytohormones are grouped in classes that include auxins (e.g., indole-3-acetic acid or IAA), jasmonates (e.g., JA), SA, and others, according to their places in different metabolic pathways. Thus, jasmonates are produced by the lipoxygenase pathway, and SA and IAA are produced in the chorismite pathway, though SA can be produced from either isochorismate acid or phenylalanine, whereas IAA is dependent on tryptophan (Davies,

2010; Wasternack and Kombrink, 2010; Dempsey et al., 2011; Zoeller et al., 2012; Zhao, 2014; Christensen et al., 2015; Widemann et al., 2015). Phytohormone production levels vary within and between plant species, and can be shaped by different selective pressures, including natural and artificial selection through herbivory and disease, and crop domestication and breeding, among others (Rosenthal and Dirzo, 1997; Chinchilla-Ramírez et al., 2017; Palmer et al., 2019).

Plant defense responses to aboveground herbivory have been studied extensively (Maschinski and Whitham, 1989; Machado et al., 2016; Rowen and Kaplan, 2016), while responses to belowground herbivory are poorly understood (Kaplan et al., 2008; Luthe et al., 2011; Johnson et al., 2016; Papadopoulou and van Dam, 2016). Nevertheless, it is apparent that belowground herbivory and mechanical wounding trigger plant responses similar to those triggered in aboveground tissues, where both systemic acquired resistance (SAR) and induced systemic resistance (ISR) have been studied extensively (Schmelz et al., 2003; Onkokesung et al., 2010). While SAR and ISR induction leads to heightened defense responses against pathogens and herbivorous insects, different signaling molecules are associated with responses against those stressors (McConn et al., 1997; Schmelz et al., 2003; Erb et al., 2009; Hasegawa et al., 2011). Hormones are responsible for modulating gene transcription, leading to translation into defensive compounds. Furthermore, phyhormones travel through plant vasculatory systems to distant, undamaged tissues, and prepare them for potential attack by pathogens or insects. Accordingly, the concentrations of relevant phytohormones change within plants when SAR or ISR occur (Ballaré, 2011).

Plants under attack by herbivores allocate resources towards defense, which reduces the availability of resources for growth and reproduction (Herms and Mattson, 1992). In this regard, the Optimal Defense Hypothesis postulates that there is a cost for defense, whether constitutive or induced, in plants. However, how plants invest resources in herbivore defense seems to depend on their genetics, the availability of resources, and herbivory pressure (Herms and Mattson, 1992; Hahn and Maron, 2016; Zust and Agrawal, 2017). Thus, crop domestication may affect how plants invest in herbivory defense by re-directing resources to productivity, including yield, rather than defense (Bazzaz et al., 1987; Zust and Agrawal, 2017). Additionally, plant spread to new environments, e.g., by invasive and crop species, and ensuing, novel herbivory pressures may mediate the evolution of resistance (whether constitutive or induced, or whether based on qualitative or quantitative chemical defenses) or tolerance (Herms and Mattson, 1992; Hahn and Maron, 2016). Artificial selection and breeding in crop species have historically benefited productivity (yield) and other human-interest characteristics over resistance to herbivores and pathogens (Rosenthal and Dirzo, 1997; Troyer, 1999; Rodriguez-Saona et al., 2011; Whitehead et al., 2017). Moreover, crops are typically grown in resource-rich environments compared to wild plants, which favors fast growth and tolerance to herbivores and pathogens, rather than resistance (Coley et al., 1985). For example, maize (*Zea mays mays* L.) underwent successive bouts of artificial and natural selection as it was domesticated, grown in increasingly favorable contexts as it became a staple crop, and spread in the Americas and beyond, and most recently, as it underwent systematic breeding directed mostly at enhancing yield (Rosenthal and Dirzo, 1997; Bellota et al.,

2013; Davila-Flores et al., 2013; de Lange et al., 2014; Maag et al., 2015; Chinchilla-Ramírez et al., 2017). Since its initial domestication, these processes have shaped how maize responds to herbivory and disease (Davila-Flores et al., 2013; Fontes-Puebla and Bernal, 2019).

Previous studies suggested that herbivory pressure, resource availability, and agricultural intensification mediated the evolution of maize defenses against root herbivory by Western corn rootworm (WCR) (*Diabrotica virgifera virgifera* LeConte) (Fontes-Puebla and Bernal, 2019). Herbivory pressure by WCR likely contributed to shaping maize defensive strategies, whether based on resistance or tolerance, after the crop spread from central Mexico to North America (Zou et al., 2007; Chen, 2016; Hahn and Maron, 2016; Fontes-Puebla and Bernal, 2019). Maize resistance (e.g., antibiosis) and tolerance (e.g., compensatory growth, enhanced photosynthesis) to WCR depend on the triggering of signaling cues, such as phytohormones. Resistance may imply the synthesis of secondary metabolites triggered by changes in phytohormone levels, whereas tolerance may depend in part on growth-related phytohormones (Vlot et al., 2009; Zhao, 2014; Borrego and Kolomiets, 2016). Thus, constitutive and herbivore-induced phytohormone profiles relevant to maize resistance and tolerance to WCR, and other herbivores may have been mediated by the crop's domestication, spread, and systematic breeding (Wright et al 2005, Fontes-Puebla and Bernal, 2019).

In this study, we tested whether the profiles of constitutive and induced maize phytohormones were mediated by the crop's domestication, spread, and breeding. Specifically, we compared constitutive and induced phytohormone profiles among four

plant types representing the evolutionary and agronomic transitions from maize's wild ancestor to highly-bred, commercial maize cultivars, viz.: Balsas teosinte (*Zea mays* L. spp. *parviglumis* Iltis and Doebley), Mexican maize landraces, US maize landraces, and US maize breeding lines. Each plant type was represented by three plant accessions. The domestication effect was assessed by comparing constitutive and induced phytohormone levels between Balsas teosinte and Mexican maize landraces; the effects of northward spread were assessed by comparing between Mexican landraces and US landraces, and; the effects of breeding were addressed by comparing between US landraces and US inbred lines. Overall, we expected to find a trend in both constitutive and induced phytohormone levels across plant types consistent with effects of domestication, spread, and systematic breeding. Particularly, we expected to find a trend of decreasing levels of phytohormone positively related to resistance, and increasing levels of phytohormones positively related to plant growth from Balsas teosinte to US inbred maize lines. We discussed the results in the context of those expectations, as well as in reference to previously reported results concerning evolution of maize defense against WCR as mediated by artificial and natural selection, geographical spread, and systematic breeding (Fontes-Puebla and Bernal, 2019).

Materials and Methods

Plants and Insects

Four plant types were used, all belonging to the genus *Zea* L., and spanning the evolution of maize from its domestication from its wild ancestor to its subsequent spread

and systematic breeding in North America (Matsuoka et al., 2002; Buckler and Stevens, 2006; Hufford et al., 2012a; van Heerwaarden et al., 2012). The plant types were: Balsas teosinte (immediate ancestor of maize); Mexican landraces (descendants of Balsas teosinte), which served to assess any domestication effects; US landraces (descendants of Mexican landraces), used here to assess any effects of northward spread, and; US inbred lines (derived from US landraces), used for assessing any effects of systematic breeding (Troyer, 1999; Matsuoka et al., 2002; Labate et al., 2003; Buckler and Stevens, 2006; Hufford et al., 2012a; van Heerwaarden et al., 2012). Each plant type included three accessions: "El Cuyotomate," "Talpitita," and "El Rodeo" for Balsas teosinte; Palomero Toluqueño, Chalqueño, and Cacahuacintle for Mexican landraces; Lancaster Sure Crop, Reid Yellow Dent, and Gourdseed for US landraces, and; Mo17, B73, and W438 for US inbred lines (Table 1). The seeds were obtained from a variety of sources, as described in Table 1.

Seeds were germinated in Petri dishes (150 × 15mm) within moistened, absorbent paper towels for 5 d (Balsas teosinte and inbred lines) or 4 d (Mexican and US landraces). Teosinte seeds were removed from their fruit cases using a nail clipper before germinating. Seed surface sterilization was not necessary. After germination, each seedling was transplanted to a cone-tainer (4 × 25 cm, diam × length) (Stuewe & Sons, Tangent, OR, USA), which had been modified with chiffon mesh to prevent WCR larvae from escaping through drainage holes, and allowed to grow for an additional 10 d; water was provided as needed. Growing conditions were 25 ± 2 °C, 50% RH, and 12:12 photoperiod (L:D). The soil used was Baccto® premium potting soil (Michigan Peat Co., Houston, TX, USA),

which was sifted through a 60-mesh sieve prior to transplanting seeds to facilitate subsequent root harvest (see below).

WCR eggs, diapause strain, were provided by USDA-ARS-North Central Agricultural Research Laboratory (Brookings, SD, USA). Eggs were incubated in Petri dishes (150 × 15mm) at 25 ± 2 °C, ~ 80% RH for 12 ± 1 d over moistened absorbing paper. Neonate first-instar larvae (< 24 h after eclosion) were used in all assays.

Constitutive, Western Corn Rootworm-Induced, and Total Biochemical Compounds

Differences in levels of biochemical compounds (i.e. analytes) were assessed in maize seedlings free of WCR herbivory or exposed to WCR herbivory for three exposure times. Constitutive analytes were assessed in seedlings free of WCR herbivory, while total analytes were assessed in seedlings exposed to WCR herbivory for 8, 24, or 48 h; WCR-induced analytes were estimated as the difference between total and constitutive analytes, i.e. induced analytes = total analytes – constitutive analytes. To this aim, 10 neonate WCR larvae were placed in individual cone-tainers holding a ~15 d-old seedling (Robert et al., 2012), and allowed to feed for intervals of 8, 24, or 48 h; in parallel, one set of seedlings (of similar size and number of leaves) was not exposed to WCR larvae. The assay included three biological replicates for each exposure time as well as for non-exposed seedlings per each plant accession, i.e. nine biological replicates per plant type; each biological replicate consisted of four seedlings. After the exposure times concluded for seedlings exposed to WCR, seedlings were rinsed with running water to carefully cleanse their roots of soil, after which the roots were excised, flash-frozen, and kept at -80 °C until ground; seedlings

not exposed to WCR were thus processed at the time WCR larvae were placed in exposed seedlings, i.e. at 0 h. A mortar-pestle was used to grind the root tissues in liquid nitrogen. The ground tissue was kept at -80 °C until analyte extraction (Christensen et al., 2013).

Biochemical Compounds Extraction and Quantification

Twelve analytes considered relevant to the study were measured: 12-oxophytodienoic acid (12-OPDA), Jasmonic acid (JA), Jasmonyl-Isoleucine (JA-Ile), 12-carboxy-jasmonyl-Isoleucine (12COOH-JA-Ile), 10-oxophytoenoic acid (10-OPEA), 10-oxophytodienoic acid (10-OPDA), Death acid with 4 carbons in the carboxylic side chain (DA4), Azelaic acid (AZA), Coumaric acid (COU), Benzoic acid (BNZ), Salicylic acid (SA), and indole-3-acetic acid (IAA).

For biochemical compounds extraction, a 104.3 ± 0.651 mg portion of ground tissue from each plant accession was mixed with 500 µL of alcohol-based extraction buffer containing 10 µM of isotopically labeled internal standards: d-ABA ([2H6](+)-cis, trans-abscisic acid; Olchemlm cat# 0342721), d-ACC (1-Aminocyclopropane-2,2,3,3-d4-carboxylic acid; Sigma cat#736260), d-IAA([2H5] indole-3- acetic acid; Olchemlm cat# 0311531), d-JA (2,4,4-d3; acetyl-2,2-d2 jasmonic acid; CDN Isotopes cat# D-6936), and d-SA (d6- salicylic acid; Sigma cat#616796), with further 30 min agitation at 4 °C in darkness; 500 µL of dichloromethane were added and agitated for another 30 min at 4 °C followed by centrifugation (13,000 g/5 min) at 4 °C in darkness. The supernatant was removed and the remaining organic solvent was evaporated under N_2 (g) flow. The pellet was re-solubilized in 150 µL of MeOH, shaken for 1 min and centrifuged (14,000 g/2

min). The supernatant then was analyzed by LC/MS (HPLC 1200 series rapid resolution coupled to MS G6410A series triple quadrupole, QqQ; Agilent Technologies, Santa Clara, CA) equipped with an ESI source. The column used was a Zorbax ECLIPSE XDB-C18 rapid resolution HT 4.6 × 50 mm 1.8 µm p.s. column following the mobile phases and elution conditions as described by (Chinnapandi et al., 2019).

Statistical Analyses

Preliminary analyses did not reveal a significant effect of exposure lengths (8, 24, 48 h) on biochemical compound responses to plant types, so all subsequent analyses considered herbivory by WCR as an independent variable, with two levels: without herbivory (= not exposed to WCR), and with herbivory (= exposed to WCR, independently of duration of exposure). Specifically, a two-Way ANOVA for the interaction 'time × plant type' was performed where the dependent variable was analyte level (pmol/g FW) and the independent variables were 'time' (8, 24, and 48 h) and 'plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines). All data were transformed to $\ln(x + 1)$ or $\sqrt{x}$ to meet normality. The significance level for interaction was $P \leq 0.05$.

Independent multivariate analyses of variance (MANOVA) were applied to evaluate whether constitutive, induced, and total analyte levels were affected by domestication, spread, and breeding. Constitutive analyte levels were calculated as per-replicate levels measured without the exposure to WCR; induced analyte levels were calculated as the per-replicate level measured after exposure to WCR larvae minus the

corresponding per-replicate constitutive level; total defense levels corresponded to the analyte levels measured after exposure to WCR, as described above. The independent variables were 'plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines), and 'accessions' (as described above in *Plants and Insects*), which were nested within plant type. An additional MANOVA for the 'herbivory × plant type' interaction was performed to assess whether herbivory affected induced (as described above) analyte levels within plant types. Throughout, the dependent variable was analyte concentration (pmol/g of fresh tissue), whether constitutive, induced or total. All data were transformed to ln(x) to meet normality assumptions prior to analyses. *A priori* contrasts were used for paired comparisons between Balsas teosinte and Mexican landraces (i.e. domestication effect), Mexican landraces and US landraces (i.e. spread effect), and US landraces and US inbred lines (i.e. breeding effect). The significance level for contrasts was adjusted, per Sidak´s correction, to $P \leq 0.017$ for each of the three comparisons, to maintain Type 1 error below $\alpha = 0.05$ (Abdi, 2007). Pearson's correlations of canonical scores with dependent variables (i.e. analyte concentration) were used to determine the contributions of each dependent variable to the total variation in the canonical axes of MANOVA's centroid plots; only correlations with r values $\geq |0.50|$, and $P \leq 0.05$ were considered significant. All analyses were conducted using JMP® Pro 14.0.0 software (SAS Institute Inc., 2018).

Results

Constitutive Biochemical Compound Levels

The MANOVA for constitutive analyte level differences between plant types showed a strong significant multivariate effect for plant type (Wilks' $\lambda = 0.015$, $P < 0.001$), but not for accession nested within plant type ($\lambda = 0.007$, $P = 0.325$); variation along the *y* axis (Canonical 1) accounted for 81% of total variation, while variation along the *x* axis (Canonical 2) accounted for 18% of total variation (Figure 9). *A priori* contrasts between plant type pairs showed significant differences between Balsas teosintes and Mexican landraces ($F_{12, 13} = 9.118$, $P < 0.001$), but not between Mexican landraces and US landraces ($F_{12, 13} = 1.904$, $P = 0.104$) nor US landraces and US inbred lines ($F_{12, 13} = 0.357$, $P = 0.944$) (Figure 9). Correlation of canonical scores showed that 10-OPDA ($r = -0.632$, $P < 0.001$), 10-OPEA ($r = -0.563$, $P < 0.001$), DA4 ($r = -0.544$, $P < 0.001$), and AZA ($r = 0.615$, $P < 0.001$) contributed the most to the variation among plant type multi-variate means along the *y*-axis, while SA ($r = 0.579$, $P < 0.001$), AZA ($r = 0.578$, $P < 0.001$), and 12-OPDA ($r = 0.529$, $P < 0.001$) contributed the most to variation among multi-variate means along the *x*-axis (Figure 9).

ANOVA and *a priori* contrasts performed on the analytes with significant correlations to axes *y* or *x*, i.e. 10-OPEA, 10-OPDA, DA4, AZA, 12-OPDA, and SA, showed significant differences among plant types ($F_{3, 24} \geq 3.518$, $P \leq 0.03$), except for SA ($F_{3, 24} = 1.534$, $P = 0.231$) (Table 3). Significant domestication effects ($F_{1, 24} \geq 6.654$, $P \leq$

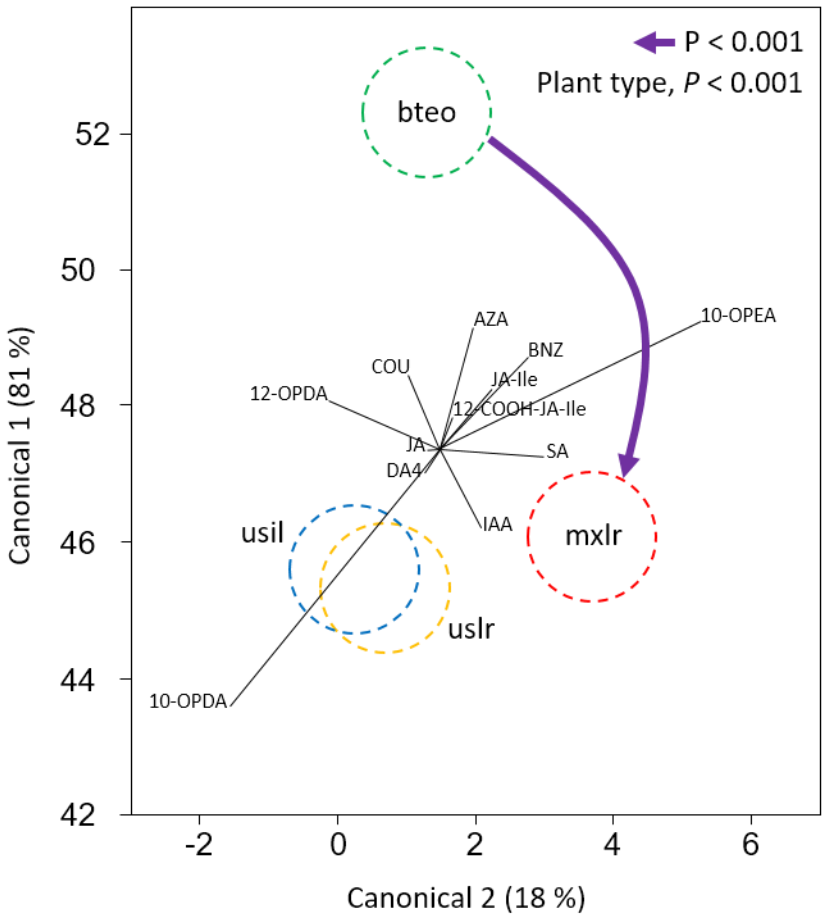

Figure 9. Canonical centroid plot from MANOVA for constitutive biochemical compounds. Wilks $\lambda = 0.015$, $P < 0.001$. Dashed circles represent confident intervals (95%) around the multivariate means of each plant type. The independent variables included in the model were 'plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines), and 'accessions' nested within plant type (not shown here). The dependent variables were: 12-oxophytodienoic acid (12-OPDA), Jasmonic acid (JA), Jasmonyl-Isoleucine (JA-Ile), 12-carboxy-jasmonyl-Isoleucine (12COOH-JA-Ile), 10-oxophytoenoic acid (10-OPEA), 10-oxophytodienoic acid (10-OPDA), Death acid with 4 carbons in the carboxylic side chain (DA4), Azelaic acid (AZA), Coumaric acid (COU), Benzoic acid (BNZ), Salicylic acid (SA), and indole-3-acetic acid (IAA). The solid arrow indicates significant *a priori* contrast ($P < 0.017$, per Sidak corrected) between plant types representing domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLS vs. USIL) transitions. BTEO = Balsas teosintes; MXLR = Mexican landraces; USLR = US landraces; USIL = US inbred lines.

Table 3. Analysis of variance (ANOVA) statistics for constitutive, induced, and total defense significant canonical correlated analytes (r values ≥ |0.50|, and $P \leq 0.05$) from MANOVA and *a priori* contrasts between plant types. Significant *P* values for ANOVA ($P \leq 0.05$) and contrasts ($P \leq 0.017$) are shown in bold.

Analytes	ANOVA			CONSTRASTS					
				Domestication		Spread		Breeding	
	SS	F	*P*	F	*P*	F	*P*	F	*P*
CONSTITUTIVE									
Canonical 1									
10-OPDA	5227.757	5.705	**0.004**	13.779	**0.001**	0.260	0.614	0.015	0.902
10-OPEA	13.744	5.630	**0.004**	12.229	**0.001**	0.037	0.848	0.003	0.954
DA4	14.561	4.736	**0.009**	10.086	**0.004**	0.011	0.916	0.009	0.925
AZA	2.253	8.532	**< 0.001**	2.557	0.122	6.633	**0.016**	0.001	0.970
Canonical 2									
12-OPDA	4.687	3.518	**0.030**	6.654	**0.016**	4.838	0.037	0.575	0.455
AZA	2.253	8.532	**< 0.001**	2.557	0.122	6.633	**0.016**	0.001	0.970
SA	1.296	1.534	0.231	-	-	-	-	-	-
INDUCED									
Canonical 1									
DA4	51.267	23.291	**< 0.001**	46.661	**< 0.001**	0.202	0.653	0.624	0.431
Canonical 2									
SA	15.023	2.892	**0.039**	3.608	0.060	5.399	0.022	0.151	0.698
TOTAL DEFENSE									
Canonical 1									
12-OPDA	10.420	19.276	**< 0.001**	47.843	**< 0.001**	2.133	0.147	9.579	**0.002**
DA4	46.996	14.663	**< 0.001**	21.096	**< 0.001**	0.687	0.409	7.156	0.008

ANOVA d.f. for constitutive = 3, 24; induced and total defense = 3, 96. Contrast d.f. for constitutive = 1, 24; induced and total defense = 1, 96. Data was transformed to $\ln(x + 1)$ or $\sqrt{(x)}$ to meet normality.

Table 4. Constitutive, induced, and total defense significant canonical correlated analyte mean values from ANOVA. Mean values ± SE (r values ≥ |0.50|, and $P \leq 0.05$).

Analytes	Analyte mean values ± SE (pmol/g FW)			
	BTEO	**MXLR**	**USLR**	**USIL**
CONSTITUTIVE				
Canonical 1				
10-OPDA	1164.6 ± 315.55	4329.9 ± 947.58	3590.3 ± 574.81	3576.4 ± 762.87
10-OPEA	1957.2 ± 610.69	7442.9 ± 1720.7	6510.6 ± 1432.9	6710.8 ± 1776.2
DA4	3.39 ± 0.86	24.39 ± 7.20	15.75 ± 1.72	28.75 ± 8.30
AZA	227.34 ± 22.26	187.11 ± 24.61	123.58 ± 8.66	126.75 ± 14.44
Canonical 2				
12-OPDA	5923.8 ± 1471.2	2565.1 ± 679.36	4879.2 ± 958.04	5723.5 ± 809.80
AZA	227.34 ± 22.26	187.11 ± 24.61	123.58 ± 8.66	126.75 ± 14.44
SA	135.76 ± 23.27	296.49 ± 132.31	115.43 ± 10.69	114.33 ± 12.10
INDUCED				
Canonical 1				
DA4	2.322 ± 0.510	17.867 ± 2.830	16.506 ± 5.237	24.51 ± 5.22
Canonical 2				
SA	121.63 ± 41.28	185.12 ± 32.661	62.124 ± 11.319	57.94 ± 14.29
TOTAL DEFENSE				
Canonical 1				
12-OPDA	9004.3 ± 818.55	4023.0 ± 364.14	5132.8 ± 392.23	7213.8 ± 561.25
DA4	2.592 ± 0.546	17.034 ± 3.411	15.133 ± 4.583	27.05 ± 5.13

SE = standard error; FW = fresh weight.

0.016) were detected for 10-OPEA, 10-OPDA, and DA4, the levels of which increased, and for 12-OPDA, the level of which decreased with domestication (Tables 3, 4). A significant spread effect was detected only for AZA ($F_{1, 24} = 6.633$, $P = 0.016$), the level of which decreased (Tables 3, 4). Significant breeding effects were not detected for any of the analytes subjected to ANOVA (Table 3).

Induced Biochemical Compound Levels

A MANOVA on induced analyte levels showed a strong, significant multivariate effect for plant type (Wilks' $\lambda = 0.218$, $P < 0.001$), and for accession nested within plant type ($\lambda = 0.176$, $P < 0.001$); variation along the *y* axis (Canonical 1) accounted for 80% of total variation, while variation along the *x* axis (Canonical 2) accounted for 13% of total variation (Figure 10A). *A priori* contrasts between plant type means showed significant differences between Balsas teosintes and Mexican landraces ($F_{12, 85} = 9.642$, $P < 0.001$), but not between Mexican landraces and US landraces ($F_{12, 85} = 0.229$, $P = 0.099$), nor US landraces and US inbred lines ($F_{12, 85} = 0.190$, $P = 0.205$) (Figure 10A). Correlation analyses showed that DA4 ($r = -0.756$, $P < 0.001$) contributed significantly to the separation among plant type multi-variate means along the *y* axis, while SA ($r = 0.619$, $P < 0.001$) contributed significantly to separating among plant type means along the *x* axis (Figure 10A).

The ANOVA performed on DA4 and SA showed significant differences among plant types for both analytes ($F_{3, 96} \geq 2.892$, $P \leq 0.039$) (Table 3). *A priori* contrasts showed a significant domestication effect for DA4 ($F_{1, 96} = 46.661$, $P < 0.001$), which increased

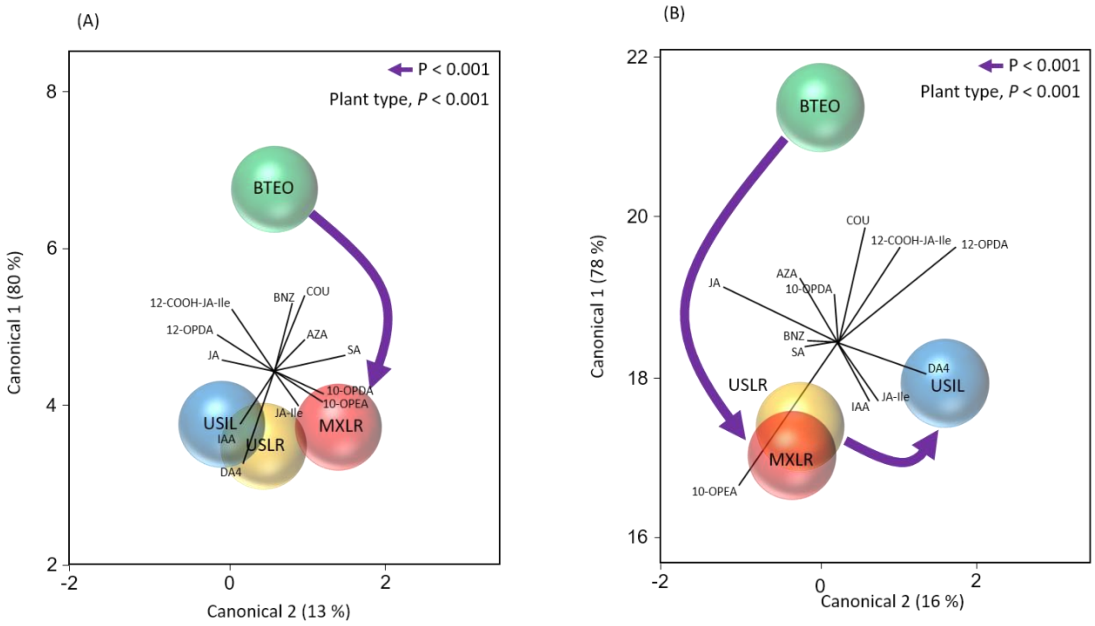

Figure 10. Canonical centroid plots from MANOVA for induced and total defense biochemical compounds. (A) induced and **(B)** total defense analyte variables (Wilks $\lambda = 0.218$, $P < 0.001$; $\lambda = 0.112$, $P < 0.001$, respectively). Spheres represent the 95% confident intervals around the multivariate means of each plant type. The independent variables were 'plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines), and 'accessions' nested within plant type (not shown here). The dependent variables were: 12-oxophytodienoic acid (12-OPDA), Jasmonic acid (JA), Jasmonyl-Isoleucine (JA-Ile), 12-carboxy-jasmonyl-Isoleucine (12COOH-JA-Ile), 10-oxophytoenoic acid (10-OPEA), 10-oxophytodienoic acid (10-OPDA), Death acid with 4 carbons in the carboxylic side chain (DA4), Azelaic acid (AZA), Coumaric acid (COU), Benzoic acid (BNZ), Salicylic acid (SA), and indole-3-acetic acid (IAA). The solid arrows indicate significant *a priori* contrast ($P < 0.017$, per Sidak corrected) between plant types representing domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLS vs. USIL) transitions. BTEO = Balsas teosintes; MXLR = Mexican landraces; USLR = US landraces; USIL = US inbred lines.

between Balsas teosinte and Mexican landraces, but not for SA, and no significant spread and breeding effects for DA4 and SA (Tables 3, 4).

Total Biochemical Compound Levels

A MANOVA for total analyte levels showed significant effects of plant type (λ = 0.112, $P < 0.001$) and accession nested within plant type ($\lambda = 0.153$, $P < 0.001$); variation along the *y* axis (Canonical 1) accounted for 78% of total variation, while variation along the *x* axis (Canonical 2) accounted for 16% of total variation (Figure 10B). *A priori* contrasts between plant types indicated differences between Balsas teosintes and Mexican landraces ($F_{12, 85} = 19.106$, $P < 0.001$), and between US landraces and US inbred lines ($F_{12, 85} = 4.100$, $P < 0.001$), but not between Mexican and US landraces ($F_{12, 85} = 1.701$, $P = 0.081$) (Figure 10B). Correlation of canonical scores showed that 12-OPDA ($r^2 = 0.590$, $P < 0.001$) and DA4 ($r^2 = -0.530$, $P < 0.001$) contributed significantly to variation along the *y* axis; no analyte contributed significantly (i.e., $r > 0.500$, $P < 0.05$) to variation along the *x* axis (Figure 10B).

ANOVAs performed on levels of 12-OPDA and DA4 showed significant differences among plant types ($F_{3, 96} \geq 14.663$, $P \leq 0.001$) (Table 3). *A priori* contrasts between pairs of plant types showed that both 12-OPDA and DA4 differed significantly between Balsas teosinte and Mexican landraces ($F_{1, 96} \geq 21.096$, $P \leq 0.001$), and between US landraces and US inbred lines ($F_{1, 96} \geq 7.156$, $P \leq 0.008$) (Table 3). Specifically, the levels of 12-OPDA decreased after domestication and increased with breeding, while DA4 increased after both domestication and breeding (Table 4).

Interaction Between WCR Herbivory and Plant Types

The MANOVA for the interaction between herbivory and plant type showed a significant multivariate effect ($\lambda = 0.610$, $P = 0.001$); variation along the *y* axis (Canonical 1) accounted for 68% of total variation, while variation along the *x* axis (Canonical 2) accounted for 24% of total variation (Figure 11). *A priori* contrasts within plant types, i.e. infested vs. noninfested with WCR larvae, showed a significant difference for Balsas teosinte ($F_{12, 129} = 4.071$, $P < 0.001$), but not for Mexican landraces ($F_{12, 129} = 1.207$, $P = 0.284$), US landraces ($F_{12, 129} = 1.056$, $P = 0.402$), and US inbred lines ($F_{12, 129} = 1.711$, $P = 0.071$) (Figure 11). Canonical correlation analysis did not find any analytes contributing significantly (i.e. $r > 0.500$, $P < 0.05$) to variation along the *x* or *y* axes.

Two-Way ANOVA (plant type, herbivory) performed on each of the 12 analytes showed significant interaction effects on levels of 12-OPDA, JA, 12-COOH-JA-Ile, DA4, COU, and IAA ($F_{3, 140} \geq 2.682$, $P \leq 0.049$) (Table 5, Figure 12). *A priori* contrasts between WCR-infested and noninfested plant types showed significant increases within Balsas teosinte for 12-OPDA, JA, DA4, and COU, and a significant decrease for IAA ($F_{1, 140} \geq 6.916$, $P \leq 0.009$) (Figure 13A, B, D-F); DA4 and IAA increased significantly in US inbred lines, (Figure 13D, F) ($F_{1, 140} \geq 8.273$, $P \leq 0.004$), but no significant changes were evident in Mexican or US landraces ($P \geq 0.028$) (Table 5).

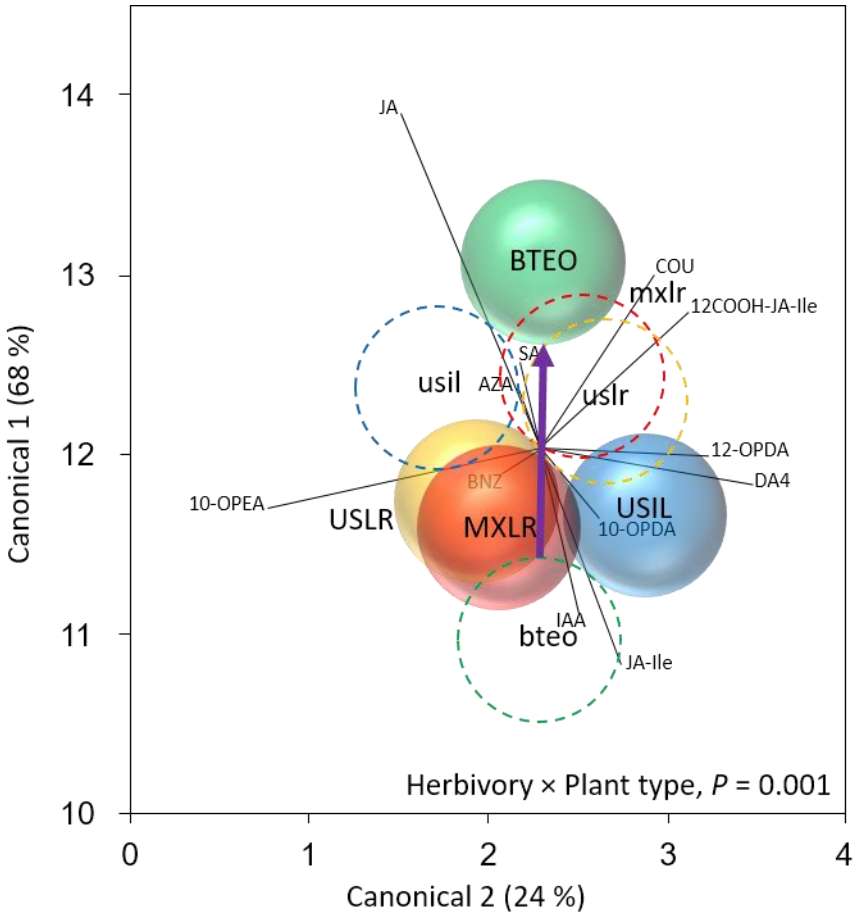

Figure 11. Canonical centroid plot from MANOVA for Western corn rootworm infested (WCR) and noninfested analyte variables. Wilks $\lambda = 0.610$, $P < 0.001$. Dashed circles and solid spheres represent confident intervals (95%) around the multivariate means of WCR-noninfested and -infested, respectively. The model included the interaction term 'Herbivory × plant type', where herbivory denotes WCR-infested and noninfested independent variables whereas plant type included Balsas teosintes, Mexican landraces, US landraces, and US inbred lines. The dependent variables were: 12-oxophytodienoic acid (12-OPDA), Jasmonic acid (JA), Jasmonyl-Isoleucine (JA-Ile), 12-carboxy-jasmonyl-Isoleucine (12COOH-JA-Ile), 10-oxophytoenoic acid (10-OPEA), 10-oxophytodienoic acid (10-OPDA), Death acid with 4 carbons in the carboxylic side chain (DA4), Azelaic acid (AZA), Coumaric acid (COU), Benzoic acid (BNZ), Salicylic acid (SA), and indole-3-acetic acid (IAA). The solid arrow indicates significant *a priori* contrast ($P < 0.012$, per Sidak corrected) between bteo and BTEO plant type. bteo, BTEO = Balsas teosintes noninfested or infested, respectively, by WCR; mxlr, MXLR = Mexican landraces; uslr, USLR = US landraces; usil, USIL = US inbred lines.

Table 5. Two-Way ANOVA statistics for WCR noninfested and infested analyte variables and *a posteriori* contrasts within plant types. Significant values for ANOVA ($P \leq 0.05$) and contrasts ($P \leq 0.017$) are shown in bold.

Analytes	Two-Way ANOVA			CONTRASTS							
				bteo vs BTEO		mxlr vs MXLR		uslr vs USLR		usil vs USIL	
	SS	$F_{12, 29}$*	*P*	$F_{1, 140}$	*P*	$F_{1, 140}$	*P*	$F_{1, 140}$	*P*	$F_{1, 140}$	*P*
Lipoxygenase pathway											
13-LOX branch											
12-OPDA	4.325	3.610	**0.015**	7.095	**0.008**	1.976	0.162	3.658	0.057	0.140	0.708
JA	310.434	3.225	**0.024**	8.635	**0.003**	3.429	0.066	0.036	0.848	0.801	0.372
JA-Ile	2.166	0.913	0.436	-	-	-	-	-	-	-	-
12-COOH-JA-Ile	5470.888	4.082	**0.008**	5.077	0.025	4.899	0.028	3.247	0.073	3.105	0.080
9-LOX branch											
10-OPDA	493.983	0.413	0.743	-	-	-	-	-	-	-	-
10-OPEA	0.093	0.038	0.989	-	-	-	-	-	-	-	-
DA4	18.568	4.301	**0.006**	8.494	**0.004**	0.237	0.626	0.201	0.654	8.273	**0.004**
AZA	1.450	1.843	0.142	-	-	-	-	-	-	-	-
Chorismate pathway											
COU	2.594	2.682	**0.049**	7.866	**0.005**	1.303	0.255	1.273	0.261	0.286	0.593
BNZ	18.389	0.236	0.871	-	-	-	-	-	-	-	-
SA	1.568	1.165	0.325	-	-	-	-	-	-	-	-
Tryptophan pathway											
IAA	568.695	4.468	**0.005**	6.916	**0.009**	1.428	0.234	0.739	0.391	8.790	**0.003**

* d.f.. Data was transformed to $\ln(x + 1)$ or $\sqrt{(x)}$ to meet normality.

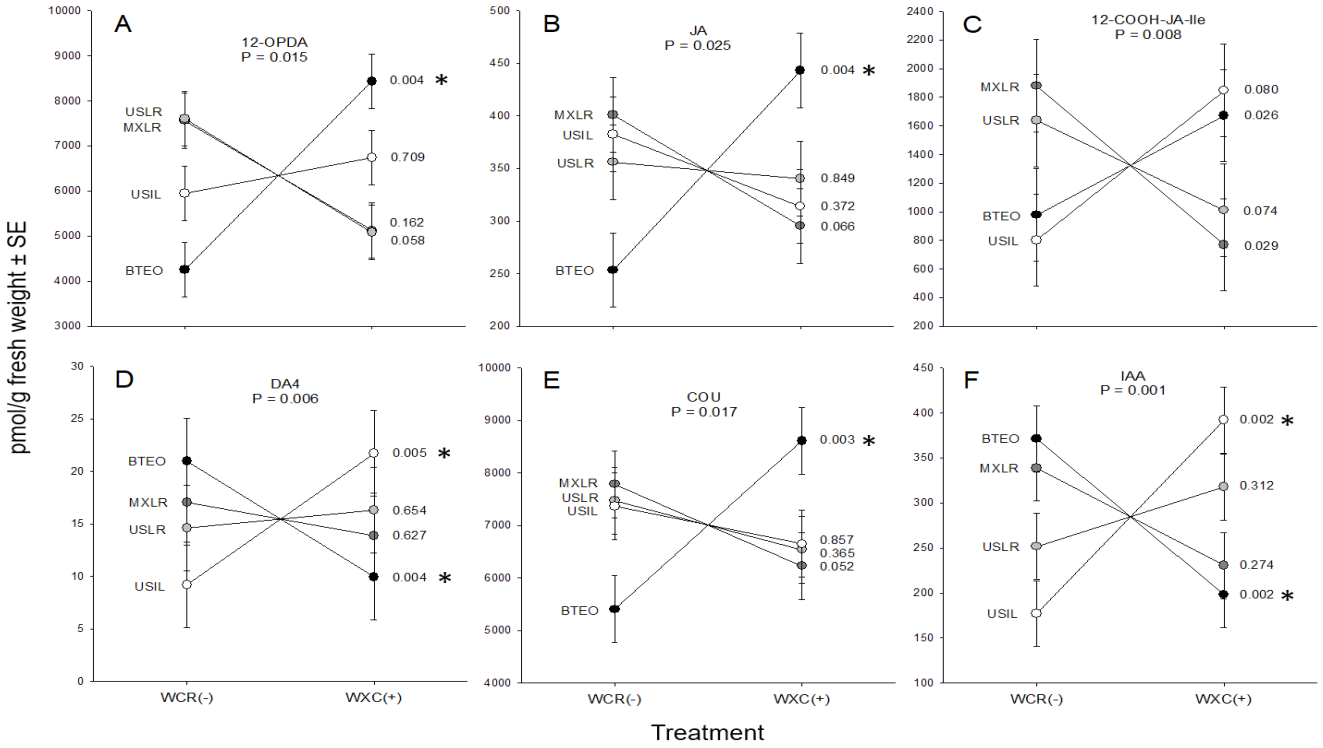

Figure 12. Interaction plots for Two-Way ANOVA depicting significant noninfested and infested by Western corn rootworm biochemical compound variables within each plant type. $F_{3,\ 140} \geq 2.682$, $P \leq 0.05$. Inset in each plot are the bivariate ANOVA statistics for the herbivory × planta type effect on (A) 12-OPDA, (B) JA, (C) 12-COOH-JA-Ile, (D) DA4, (E) COU, and (F) IAA. The y-axis represents mean analyte levels in pmol/g of fresh weight ± SE, whereas the x-axis represents the treatment. WCR (-) = noninfested by Western corn rootworm; WCR (+) = infested by western corn rootworm. A priori contrasts between noninfested and infested plant types' P values are shown on the right side of the plot and significant contrasts ($P \leq 0.012$, per Sidak corrected) are indicated by an asterisk (*). Plant types are indicated on the left side of the plot. BTEO = Balsas teosintes; MXLR = Mexican landraces; USLR = US landraces; USIL = US inbred line.

Discussion

This study addressed whether constitutive and induced levels of biochemical compounds related to maize resistance and tolerance to Western corn rootworm were mediated by domestication, spread, and breeding, processes spanning thousands of maize and WCR generations, and divergent environments across ~20 ° latitude and ~3000 m elevation. To this aim, seedlings of four *Zea* plant types representing those processes: Balsas Teosintes, Mexican landraces, US landraces, and US inbred lines were exposed or not. Constitutive, induced, and total analyte levels were quantified and contrasted between plant types. I expected to find analyte levels differing among plant types representing the domestication, spread, and breeding processes. Specifically, I expected to find a decrease in putative resistance-enhancing analytes and an increase in IAA, a growth-promoting hormone, from Balsas teosinte to modern maize inbred lines. The results were partially consistent with the predictions. On one hand, resistance-enhancing analytes decreased after domestication and spread, as expected, and the growth-related hormone IAA remained unchanged after domestication and spread, contrary to expected. On the other hand, breeding increased the levels of both pathogen resistance-enhancing analytes and the growth-related hormone IAA, as expected.

The 12 analytes measured in this study, 12-OPDA, JA, JA-Ile, 12COOH-JA-Ile, 10-OPEA, 10-OPDA, DA4, AZA, COU, BNZ, SA, and IAA occur in three biochemical pathways: lipoxygenase pathway, with two branches, 13-LOX and 9-LOX (deoxygenation at carbon position 13 or 9, respectively); chorismic acid pathway, and; tryptophan-

dependent pathway (Figure 13) (Wasternack and Kombrink, 2010; Dempsey et al., 2011; Zoeller et al., 2012; Zhao, 2014; Widemann et al., 2015). The jasmonates 12-OPDA, JA, JA-Ile, and 12COOH-JA-Ile belong to the 13-LOX branch and 10-OPEA, 10-OPDA, DA4 belong to the 9-LOX branch of the lipoxygenase pathway; AZA, also forms part of the lipoxygenase pathway, but it remains unknown how it is biosynthesized. The salicylates COU, BNZ, and SA belong to the chorismite pathway. Jasmonates and salicylates are associated with plant defenses, and some analytes of this group may regulate plant development and physiological stress response as well (Vlot et al., 2009; Yan et al., 2013); the lipoxygenase pathway is particularly relevant to WCR resistance (Christensen et al., 2013; Alouw, 2015; Borrego and Kolomiets, 2016). The hormone IAA belongs to the tryptophan derived pathway, which is directly related to plant growth (Zhao, 2010) (Figure 13).

Domestication, Spread, and Breeding Favored the Synthesis of 9-lipoxygenases Over 13-lipoxygenases

The results suggested that constitutive, induced (= total - constitutive) and total (measured after exposure to WCR larvae) analyte levels associated with enhanced herbivory defense decreased with domestication and spread, but increased with breeding. The MANOVA showed a significant multivariate effect of plant type on lipoxygenase pathway-derived analyte levels. *A priori* contrasts between plant types representing domestication, spread, and breeding showed differences between Balsas teosintes and Mexican landraces, and between Mexican landraces and US landraces, but not between

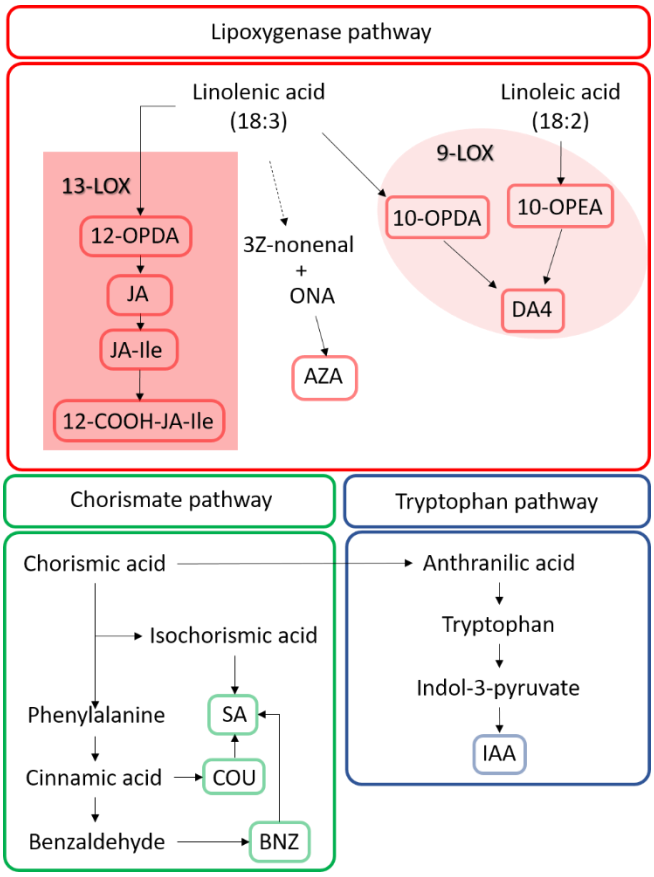

Figure 13. Place of the 12 analytes measured in this study in three metabolic pathways involved in plant defense and growth. The pathways are presented in abbreviated form. The 12 analytes are indicated by their placement within a rectangle. The Lipoxygenase pathway includes 13-LOX and 9-LOX branches, with derivatives oxidized in the 13 and 9 carbon, respectively; the analytes within the lipoxygenase pathway are 12-oxophytodienoic acid (12-OPDA), Jasmonic acid (JA), Jasmonyl-Isoleucine (JA-Ile), 12-carboxy-jasmonyl-Isoleucine (12COOH-JA-Ile) from the 13-LOX branch, 10-oxophytodienoic acid (10-OPDA), 10-oxophytoenoic acid (10-OPEA), and death acid with 4 carbons in the carboxylic side chain (DA4) from the 9-LOX branch, and Azelaic acid (AZA). The analytes within the chorismate pathway are Salicylic acid (SA), Benzoic acid (BNZ), and Coumaric acid (COU). Indole-3-acetic acid (IAA) is the sole hormone within the tryptophan pathway.

US landraces and US inbred lines in constitutive levels (Figure 10). For induced levels, contrasts showed differences only between Balsas teosintes and Mexican landraces, whereas for total analyte levels contrasts showed significant differences between Balsas teosintes and Mexican landraces, as well as between US landraces and US inbred lines (Figures 11A and 11B). ANOVA and *a priori* contrast comparisons showed that constitutive and total 12-OPDA (13-LOX branch) levels decreased with domestication, but total 12-OPDA levels increased with breeding. Constitutive AZA levels decreased with spread, and the constitutive 10-OPEA and 10-OPDA (9-LOX branch) increased with domestication, whereas constitutive, induced and total DA4 (9-LOX branch) levels increased with domestication (Table 5). These results suggest that Balsas teosinte may be allocating more resources towards 13-LOX pathways for constitutive and induced defense against herbivory and disease, rather than to 9-LOX and chorismite pathway derivatives. However, they also suggest that synthesis of 9-LOX-derived analytes was enhanced after domestication, over synthesis of 13-LOX-derived analytes. This increase in 13-LOX oxylipins and decrease of 9-LOX oxylipins in the results may be related to the fact that 9-LOX oxylipins cross-talk with 13-LOX oxylipins, particularly JA hormone biosynthesis in maize roots (Gao et al., 2008).

The results suggest that Balsas teosinte's total analyte response relies on 13-LOX branch of the lipoxygenase pathway. 12-OPDA is a JA precursor that has its own signaling role in defense responses, unlike other jasmonates (Stintzi et al., 2001). For example, 12-OPDA enhances callose accumulation and the resistance1-Cysteine Protease in maize upon attack by the corn leaf aphid (*Rhopalosiphum maidis* Fitch) (Varsani et al., 2019).

During physiological stress, 12-OPDA maintains homeostasis in the plant, hence it is considered a cell protector (Park et al., 2013). Perhaps, high 12-OPDA constitutive levels in Balsas teosintes may be used to maintain cell homeostasis upon physiological stress. Balsas teosintes, which grow wild, and frequently relegated to marginal environments, are adapted to grow under comparatively higher physiological stress than maize landraces and inbred lines, which are grown in conditions suitable for agriculture (Sánchez González and Ruiz Corral, 1995; Wilkes, 1997; Hufford et al., 2012a; Ureta et al., 2012; de Lange et al., 2014; Bellon et al., 2018; Fontes-Puebla and Bernal, 2019). The reduction of 12-OPDA levels in Mexican landraces relative to Balsas teosintes may be a consequence of artificial selection favoring growth in a context of lower physiological stress under agriculture. With domestication, the transcription factor *teosinte branched1* (*tb1*) was selected as part of the architectural improvements for increasing productivity. *tbi* regulates apical dominance, bud dormancy, and is related to JA biosynthesis regulation, which is particularly relevant to maize because high accumulation of JA reduces cell growth, whereas little to no accumulation of JA promotes branching (Borrego and Kolomiets, 2016; Dong et al., 2019). However, it seems that breeding under herbivory pressure selected for this hormone in US inbred lines, even when this maize is grown under much less physiological stress than maize landraces (Duvick, 2005). In a recent study, US inbred lines showed stronger resistance and lower tolerance relative to US landraces, plausibly resulting from systematic breeding and agricultural intensification (Fontes-Puebla and Bernal, 2019). Thus, modest resistance to WCR in US inbred lines may partly be due to increased levels of 12-OPDA.

Lower levels of constitutive AZA after domestication suggest that maize may be allocating resources to synthesize other 9-LOX derivatives, instead of AZA. Derivatives of 9-LOX have been documented to influence root growth, among other developmental characteristics, as well as mediating defensive responses by acting directly as phytoalexins against pathogens (Hwang and Hwang, 2010; Cecchini et al., 2019). AZA is a signal associated with SAR, particularly as an inducer of SA accumulation upon pathogen attack (Jung et al., 2009; Gao et al., 2015). For example, Wu et al. (2013) mention that AZA plays an important role in maize response to the sugarcane mosaic virus. However, Cecchini et al. (2019) proposed that AZA functions as a signal in multiple systemic immunity programs, not exclusively inducing SAR, and may cause morphological changes in roots by inhibiting primary root growth and increasing lateral root density. Interestingly, Balsas teosinte grows denser roots than modern maize (Gaudin et al., 2011; Gaudin et al., 2014). Given the ambiguity of AZA functionality, high constitutive levels of this compound may be related to either priming against pathogens or functions related to root growth.

Domestication enhanced the synthesis of the 9-LOXs 10-OPEA, 10-OPDA, and DA4, over 13-LOX jasmonates. Christensen et al. (2015) named the 9-LOX derivatives death acids (DAs) because they were found in high concentrations in necrotic tissue from pathogen-infected maize. Nevertheless, 9-LOX derivatives can regulate JA biosynthesis as well as cell death upon pathogen attack (Gao et al., 2008; Hwang and Hwang, 2010). High levels of 10-OPEA can impair growth not only in pathogens, but in insects and plant cells too, e.g., suppress growth of *Fusarium verticilloides* and *Aspergillus flavus*, and

inhibit growth of *Helicoverpa zea* (Christensen et al., 2015). In contrast, Gao et al. (2007) found that some 9-LOX derivatives may promote fungal growth, e.g., synthesis of the mycotoxin fumonisin B1 by *F. verticilloides* was inhibited when the *ZmLOX3*, a 9-LOX gene, was inactivated in maize. Whether DAs act as defense signals or growth regulators, increased constitutive levels of 10-OPDA, 10-OPEA and DA4 suggest that *Zea* plants may allocate resources to follow the 9-LOX branch instead of the 13-LOX branch. Apparently, selection for changes in plant architecture (i.e. branching in teosintes) and enhanced growth (i.e. higher productivity in maize) in *Zea* plants switched the metabolic pathway taken by plants in their constitutive and induced, and, therefore, total responses against herbivory and disease. The results showed that after domestication, the constitutive and induced levels of DAs increased relative to pre-domestication. However, previous studies showed greater larval weights of WCR and compensation of root tissue on Mexican landraces compared to Balsas teosinte, suggesting that DAs may not be as effectual on WCR as they are on the foliage feeder *H. zea* (Christensen et al., 2015; Fontes-Puebla and Bernal, 2019).

Root Herbivory Triggers Expression of Jasmonates in Balsas Teosinte, and a Death Acid and Auxin in US Inbred Lines

The results showed that Balsas teosinte produced higher levels of herbivory resistance-enhancing phytohormones after root herbivory than maize, and that with systematic breeding, inbred maize lines expressed higher levels of pathogen resistance- and growth-related analyses (Constantino et al., 2013; Christensen et al., 2015; Varsani et

al., 2016). MANOVA showed a significant interaction between herbivory and plant type. Contrast comparisons between WCR-infested and non-infested plant types showed a significant difference only for Balsas teosinte. ANOVA and *a posteriori* contrast comparison within plant types for each analyte showed that in Balsas teosinte, 12-OPDA, JA (13-LOX branch), and COU (from chorismate pathway), increased with WCR feeding, whereas DA4 (9-LOX branch) and IAA (from tryptophan pathway) decreased. Additionally, US inbred lines responded to WCR herbivory by significantly increasing DA4 and IAA levels.

Jasmonates play important roles in plant defense against chewing insects, such as WCR, whereas COU is either a SA or lignin and flavonoids precursor involved in defense against pathogens and piercing-sucking insects (Yalpani et al., 1993; Lee et al., 1995; Wasternack and Kombrink, 2010; Wasternack and Hause, 2013). Wild plants tend to be more resistant to herbivory than domesticated plants, as domestication favored fast-growth and high productivity over defense (Rosenthal and Dirzo, 1997; Rodriguez-Saona et al., 2011; Davila-Flores et al., 2013; de Lange et al., 2014; Fontes-Puebla and Bernal, 2019). Additionally, geographical spread, along with exposure to new biotic and abiotic stressors can reshape plant defense responses (Blossey and Notzold, 1995; Zou et al., 2007; Erb et al., 2011a; Chen, 2016; Fontes-Puebla and Bernal, 2019). Thus, increased resource availability and novel herbivory pressure following domestication and crop spread may select for fast-growing plants leading to tolerance as a defense strategy (Zou et al., 2007; Hahn and Maron, 2016). However, systematic breeding, agricultural intensification (e.g. use of fertilizers and insecticides to support high crop productivity and offset insect

injury), and continuous herbivory pressure (e.g., WCR in USA Corn Belt since ~1950s) may have unexpectedly selected for resistance in maize, without measurably affecting growth (Baker, 1972; Duvick, 2005; Fontes-Puebla and Bernal, 2019). In a previous study, US inbred lines regained modest resistance to WCR, while losing tolerance, compared to US landraces (Fontes-Puebla and Bernal, 2019). The results showed that WCR-infested US inbred lines responded by increasing DA4 and IAA levels, which suggests that DA4 may be providing modest resistance (Christensen et al., 2015), while increased IAA levels may be providing modest tolerance through root compensation, as shown in that previous study. Interestingly, US landraces did not show significant differences in analyte levels between WCR-infested and non-infested plants, even though previous studies showed that they are highly tolerant, but not resistant to WCR (Fontes-Puebla and Bernal, 2019). This suggests that tolerance, through compensation of tissues lost to herbivory, is not mediated by a particular analyte and its concentration, e.g., IAA, but by a combination of analytes. Likewise, the results suggested that high DA4 and IAA levels in US inbred lines may be related to the increasing resistance and decreasing tolerance to WCR, relative to US landraces.

CHAPTER IV

RECRUITMENT OF WESTERN CORN ROOTWORM BY CONSTITUTIVE ROOT VOLATILES FROM *ZEA* SPP. WAS MEDIATED BY DOMESTICATION, SPREAD, AND BREEDING

Introduction

The interactions between herbivorous insects and plants are innumerable given their abundances and diversities. The antagonism implicit in interactions between herbivore insects and plants is at the base of an arms race in which plants continuously adapt to fend off herbivorous insects, and insects continuously counter-adapt to overcome plant defenses (Ehrlich and Raven, 1964; Mello and Silva-Filho, 2002; Schoonhoven et al., 2005). This arms race is driven by adaptation through natural selection, and is widely evident in plants and associated insects in natural settings. However, it may be evident too in crop plants and their insect pests in agricultural settings, especially when crop plants are subject to artificial selection in which farmers select seed in one cropping season to plant in the subsequent season (Chen and Schoville, 2018).

Multiple factors are involved in how plants respond to attacks by insects and how insects counter-respond (Schoonhoven et al., 2005; Stout, 2013; Chen and Schoville, 2018). In natural habitats, the selective environment for plants is frequently shaped by chance (e.g., seedlings evading or being found by herbivores), rather than by design, e.g., specialist pests searching for hosts in extensive monocrops devoid of non-crop, alternative hosts. Nevertheless, selective pressure occurs in both scenarios, though with different

intensities and durations. In agricultural settings, environmental stresses differ from those prevalent in natural habitats, e.g., herbivory pressure may be intense, and resource availability high, which may mediate crop plant evolution relative to evolution of wild plants. Whether in natural habitats or agricultural settings, plant evolution is explained, in part, through optimal defense hypotheses, including hypotheses centered on resource availability and herbivory pressure (Agrawal et al., 2010; Erb et al., 2011; Machado et al., 2016; Robert et al., 2017; Zust and Agrawal, 2017; Chen and Schoville, 2018).

Domestication, geographical spread, and systematic breeding have modified the interactions between crop plants and insects relative to crop wild ancestors and their herbivores (Macfadyen and Bohan 2010, Chen et al. 2015a, Wang et al. 2018). Among the changes between plant and insect interactions are those driven by changes in the diversity of signaling chemicals (i.e., semiochemicals) emitted by plants and the responses that they may trigger in herbivores (Rodriguez-Saona et al., 2011; Bellota et al., 2013; Szczepaniec et al., 2013; Turcotte et al., 2014; Bernal et al., 2015; Chen et al., 2015b; Maag et al., 2015a). Plants use semiochemicals for various ends as a result of their coevolution with other organisms, including conspecifics, herbivore insects, and the natural enemies of these insects. Herbivorous insects, for example, may be variably affected by plant semiochemicals depending on the strength of their relationship with a plant, e.g. monophagous vs. polyphagous insects using plant kairomones while searching for a host plant (Zhu-Salzman et al., 2005; Speed et al., 2015). At the same time, predatory insects searching for prey may benefit from herbivore-induced plant volatiles (HIPV) acting as synomones (Züst et al., 2012).

Herbivorous insects generally rely on chemical cues to find and select hosts (Bruce et al., 2005). If a semiochemical emitted by a plant species facilitates host location by its herbivores, the plant is challenged and consequently adapts (Zangerl and Berenbaum, 2005). Therefore, it is expected that plants producing allomones derived from kairomones are better defended than those whose kairomones remain unchanged, especially after herbivores evolve to sequester kairomones for their own defense. For example, maize (*Zea mays mays* L.) produces the insecticide 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) which provides defense against *Diabrotica balteata* (LeConte) and *Spodoptera littoralis* (Boisduval), but not against Western corn rootworm (*Diabrotica virgifera virgifera* LeConte) (WCR). WCR not only tolerates DIMBOA, but use it to locate host tissues, and accumulates the related benzoxazinoid glucosides HDMBOA-Gl and MBOA-Glc for defense against entomopathogenic nematodes and their lethal symbionts (Robert et al., 2017). Additionally, WCR uses the induced volatiles (E)-β-caryophyllene (EβC) and ethylene for host location and selection (Robert et al., 2012b; Robert et al., 2017). Importantly, EβC is used also by entomopathogenic nematodes parasitic on WCR for locating WCR hosts (Rasmann et al., 2005).

To date, no constitutive blend or particular semiochemical emitted by maize roots has been identified as attractive to WCR larvae, though the primary metabolite CO_2 has been suggested as a strong mediator of WCR foraging behavior (Johnson and Gregory, 2006). However, the output of an individual-based model of WCR larval behavior as affected by root volatiles suggested additional, attractive cues other than CO_2 (Schumann et al., 2018). Furthermore, variation in expression among *Zea* spp. may not be limited to

one particular terpene, such as EβC. Semiochemicals other than EβC, and plausibly relevant to host finding, may also be produced at variable levels among *Zea* spp. and maize cultivars, affecting WCR recruitment.

In previous studies, *Zea* resistance and tolerance to WCR, and synthesis of defensive biochemical compounds were shown to be affected by domestication, spread, and breeding, consistent with predictions of optimal defense, varying resource availability, and herbivory pressure hypotheses (Fontes-Puebla and Bernal, 2019). In this study, I hypothesized that domestication, spread, and breeding mediated the recruitment of WCR by *Zea* plant spanning those evolutionary processes. To test this hypothesis, I performed two bioassays to compare between recruitment (i) by each of four *Zea* plant types (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines) relative to a-non-host (sorghum, *Sorghum bicolor* (L.) Moench), and, (ii) by each maize plant type relative to its ancestral plant type (viz. Mexican landraces vs. Balsas teosinte, US landraces vs. Mexican landraces, and US inbred lines vs. US landraces). Parallel to these assays, I tentatively identified the constitutive volatile organic compounds produced by seedling roots of the different *Zea* plant types, and tested for domestication, spread, and breeding effects, and compared and contrasted with the results of the recruitment bioassays. In an additional bioassay, I addressed whether recruitment of WCR was mediated by root volume, a question that was prompted by partial results of bioassays (i) and (ii). Finally, question-driven, *post hoc* statistical comparisons of selected plant types or accessions of plant types were conducted in order to identify particular volatiles that may warrant future study because they may prove to be especially refractory or attractive to WCR. Overall, I

expected to find that recruitment and volatile profile variation among *Zea* plant types were mediated by domestication, spread, and breeding. Specifically, I expected to see a recruitment trend in which WCR larvae, given a choice, would be more strongly recruited by the least-resistant *Zea* plant types identified in previous studies. I also expected to identify similarities and differences between volatile profiles among *Zea* plant types that would be consistent with any trend evident in recruitment strength. I discussed my results in the context of plant defense evolution mediated by domestication and artificial selection, geographical spread, and systematic breeding.

Materials and Methods

Plants and Insects

In this study, four different *Zea* plant types were used as hosts, and sorghum (*S. bicolor*) as a non-host control in one assay (see below) (Branson et al., 1969; Strnad and Dunn, 1990). While sorghum is considered a non-host, a prior study showed that it recruits WCR larvae when offered opposite maize in a two-choice setting (Branson et al., 1969; Strnad and Dunn, 1990). The four *Zea* plant types were selected to represent the evolution of maize from its wild ancestor to modern, Mid-western USA maize. These plant types were: Balsas teosinte (hereafter BTEO), the immediate ancestor of maize; Mexican landraces (MXLR), the immediate descendents of BTEO; US landraces (USLR), immediate descendents of MXLR, and; US inbred lines (USIL), descendents of USLR (Troyer, 1999; Matsuoka et al., 2002; Labate et al., 2003; Merrill et al., 2009; Sánchez,

2011; van Heerwaarden et al., 2012) (Table 1). Importantly, each plant type was represented by three accessions to better-represent genetic variation within each plant type (Table 1).(USDA-ARS, 2015)

Seeds of each accession were germinated in Petri dishes (150 × 15mm) within moistened paper towels for 5 or 6 d at 25 ± 2°C, ~80% RH, indirect 12:12 photoperiod (L:D); a preliminary germination test showed no need for seed surface sterilization. BTEO, USIL, and sorghum required one additional day to germinate, so were initiated 1 day prior to seeds of the other plant types. The fruit case covering BTEO seeds was removed with a toenail clipper before setting for germination. The 5 or 6 d-old seedlings were used in both recruitment and volatiles assays (see below).

Western corn rootworms eggs, diapause strain, were provided by the USDA-ARS-North Central Agricultural Research Laboratory (Brookings, SD, USA). The eggs were removed from their soil medium by rinsing in water, placed directly within a folded, moistened paper towel, and incubated for 5 d at 25 ± 2°C, ~80% RH, under darkness for 12 ± 1 d. Thirty neonate (< 24h after eclosion) larvae were used per replicate (seedling) in all three recruitment assays described below.

Recruitment Assays

Three two-choice assays were conducted consisting of trials in which groups of 30 neonate WCR larvae were placed at the center of an arena and allowed to forage freely for seedling roots during 2 h. In each assay, the arena was divided into three zones: two recruitment zones, each holding at least one seedling, which were separated by one, central

buffer zone (~ 1 cm-wide transverse band), at the center of which larvae were released. Seedlings were set on each recruitment zones and the whole arena was covered with 1 cm-deep layer of sifted soil (60-mesh strainer), emulating soil conditions. The arena was covered with cling film (Press'n Seal®, Glad Products, Oakland, CA) to conserve soil moisture and allowed to stabilize for 4 h before WCR were released. Once the larvae were released, the arena was sealed again to prevent larvae from escaping. Each trial ended after 2 h, at which time thin cardboard dividers were placed between the buffer and recruitment zones to keep larvae from moving between zones; each arena was then frozen until WCR larvae were recovered and counted. Arenas were thawed to recover larvae: Larvae recovered from a recruitment zone, including from corresponding seedling roots, were scored as having been recruited by the seedling in that particular zone, while larvae recovered from the buffer zone were excluded from statistical analyses.

Recruitment by Zea Plant Types Relative to Sorghum

This assay's goal was to compare the recruitment of neonate WCR larvae between successive pairs of plant types (BTEO, MXLR, USLR, USIL) representing each transition: domestication, spread, and breeding. Recruitment by each plant type was assessed in comparison to sorghum. While sorghum is considered a non-host plant for WCR, prior studies showed that it recruited WCR larvae when offered in opposition to maize in 2-host choice experiments, as noted above (Branson et al., 1969; Strnad and Dunn, 1990). I expected that recruitment would be stronger in the least resistant of each consecutive pair

of plant types: i.e. stronger in MXLR compared to BTEO, in USLR compared to MXLR, and in USIL compared to USLR.

For this assay, round arenas (~13.5 cm diam, 147.71 cm^2) were prepared as described above. One *Zea* seedling was placed in one recruitment zone and one sorghum seedling was placed in the opposite zone, so that they were ~5 cm away from the buffer zone (Hiltpold and Turlings, 2008). Thirty-four total replicate trials, each involving 30 WCR larvae, were conducted: 9 for BTEO, 8 for MXLR, 9 for USLR, and 8 for USIL. The proportion of larvae observed to have been recruited by a plant type in each trial was converted to its arc-sine $\sqrt{x}$ value, and converted values were subjected to (i) analysis of variance (ANOVA) to compare WCR recruitment between plant types, and (ii) one-sample *t*-tests to compare recruitment by each plant type relative to sorghum. The ANOVA model included as independent variables plant type (BTEO, MXLR, USLR, USIL), and the difference in weights between *Zea* and sorghum seedlings within each trial (= weight in grams of *Zea* seedling – weight in grams of sorghum seedling). The difference in weights between *Zea* and sorghum seedlings was included in ANOVA to account for any correlation between the volumes of seedlings and their emissions of any constitutive volatiles. The one-sample *t*-tests compared against a hypothetical proportion of 0.5, indicative of equal recruitment by the *Zea* plant type and sorghum seedlings within individual trials.

Recruitment by a Derived Plant Relative to an Ancestral Plant

This assay's goal was to directly compare WCR recruitment between plant types within each transition, i.e., BTEO vs. MXLR for the domestication transition, MXLR vs. USLR for spread, and USLR vs. USIL for breeding. I expected that recruitment would be greater by the derived versus ancestral plant type within each transition, i.e., MXLR > BTEO, USLR > MXLR, and USIL > USLR.

For this assay, rectangle arenas (22 × 6.5 cm, 145.2 cm^2) were prepared as described above, and held three seedlings per plant type (= one seedling of each plant type's three accessions) in each recruitment zone. In these arenas, as in the circular arenas (see above), the distance between buffer zone and seedlings was ~5 cm. Recruitment was defined as described above. Sixteen total replicate trials, each involving 30 WCR larvae, were conducted, with four trials per each transition: domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLR vs. USIL). The proportions of larvae observed to have been recruited by each plant type within each trial were converted to arc-sine $\sqrt{x}$ values, and subjected to ANOVA to compare WCR recruitment between plant types within each transition. The ANOVA model included the independent variable 'plant type' nested within transition: BTEO and MXLR nested within domestication; MXLR and USLR nested within spread, and; USLR and USIL nested within breeding. Additionally, the ANOVA model included the difference in weights between plant types offered in each trial as an independent (co)variable, as explained above; within each trial, the difference was calculated as weight of ancestral minus weight of derived plant type (i.e. BTEO – MXLR for domestication transition, MXLR – USLR for spread, and USLR – USIL for

breeding). If ANOVA indicated a significant nested effect, *a priori* contrast comparisons were used to compare between ancestral and derived plant types within each transition, with a significance level adjusted for three total comparisons, per Sidak's correction, to $P \leq 0.017$ (Abdi, 2007).

Mediation of Recruitment by Root Volume

This assay was prompted by the results of the two prior recruitment assays, which showed that the observed recruitment of WCR was mediated in part by the differences in weights of seedlings in each trial (see *Results*). The assay's goal was to assess whether seedling root weights — assumed to mediate in part the volume of volatile emissions — determine the recruitment strength of WCR larvae. To this end, seedlings of one BTEO accession and one USIL accession were offered to WCR larvae: *Talpitita* BTEO and *Mo17* USIL. were simultaneously offered to WCR larvae at three pre-determined ratios of root weights: Talpitita 1:1 Mo17 (= 583.97 mg: 584.03 mg), Talpitita 2:1 Mo17, and Talpitita 1:2 Mo17. The results of a prior study showed that WCR performance (= larval weight after 10 d) did not differ on these accessions (Fontes-Puebla and Bernal, 2019), and preliminary analysis of results from the assay *Recruitment by a* Zea *Plant Type Relative to Sorghum* suggested that WCR recruitment was similar for these accessions (data not shown). Therefore, I expected that WCR would (i) not discriminate between the two accessions when they were offered at equal root weights, and (ii) would be recruited more strongly by the accession offered at the 2-fold root weight in a given trial.

Rectangular arenas, as described above (*Recruitment by a Derived Plant Relative to an Ancestral Plant*), were used for this assay. Three replicates per weight ratio, each involving 30 WCR larvae, were conducted. A one sample *z*-test was applied to the frequencies of larvae recovered from the recruitment zone corresponding to the *Talpitita* BTEO accession in each of the three weight ratios, with the null hypothesis that half of all recovered larvae (i.e. proportion = 0.5) would be recruited by this accession (Abdi, 2007).

Volatiles Assay and Analyses

Constitutive, volatile organic compounds were collected from excised roots of BTEO, MXLR, USLR, and USIL. To that aim, 5 d-old roots were excised, flash-frozen with liquid nitrogen, and ground in liquid nitrogen with a mortar and pestle (Köllner et al., 2008; Erb et al., 2011). The ground tissue was transferred to 5 mL vials with screw caps and stored at -80 °C until weighed and analyzed. The assay included three biological replicates for each plant accession, i.e., nine biological replicates per plant type; each biological replicate consisted of 100 ± 5 mg of frozen tissue. The sample was transferred to a 1.8 mL glass vial with septum screw cap under liquid nitrogen bath and stored at -80°C until analyzed (< 72 h).

The headspace root volatiles were collected through solid-phase microextraction (HS-SPME) by a 65µm polydimethylsiloxane/divinylbenzene (PDMS-DVB) coated fiber (Manual Stableflex, Supelco, Bellefonte, PA, USA) and preconditioned at 250°C/30min under He stream (3mL/min). A warm sand bed was used to raise the headspace's temperature to 38 ± 2°C. The fiber holder was inserted through the septum and the fiber

exposed for 10 min. Collected volatiles were desorbed directly to an Agilent 7890 B gas chromatographer coupled to a 5977 B mass spectrometer with a splitless injector held at 250°C. The column used was a 30m×250µm×0.25µm, Agilent 19091S-433UI, HP-5ms, USA, with an initial temperature of 40°C held for 5 min, then ramped to 250°C by increments of 20°C/min. Chromatograms were analyzed through MSD ChemStation (version F.01.03.2357, 2005-Agilent Technologies). Tentative identification of detected compounds was made by comparing mass spectra and retention times with those published in NIST17 and Gothenburg Department of Chemical Ecology mass spectral library (NIST, 2017). The identification and concentration estimation of β-caryophyllene (EβC) was obtained by the use of a standard (Sigma-Aldrich, W225207) and a calibration curve.

Multivariate Analysis of Variance (MANOVA) followed by Pearson's correlation of canonical scores with volatiles were conducted to evaluate whether the peak areas of the constitutive root volatiles differed among the four plant types, indicating domestication, spread, and breeding effects. The independent variables were 'plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines) and 'accessions' nested within plant type (three accessions per plant type, see Table 1). The dependent variables were the peak areas from detected constitutive root volatiles. Peak areas were transformed to $\ln(x+1)$ before analyses. *A priori* contrasts were used to compare between Balsas teosinte vs. Mexican landraces, Mexican landraces vs. US landraces, and US landraces vs. US inbred lines with a critical P of 0.017, per Sidak's correction (Abdi, 2007). Pearson's correlations of canonical scores with volatiles peaks were used to determine the contribution of each volatile to the total variation in the canonical axes that

constitute the MANOVA's centroid plot. Pearsons r values $\geq |0.50|$, and P values ≤ 0.05 were considered significant.

An Analysis of variance (ANOVA) was performed on each volatile ($P \leq 0.05$) detected in all four plant types. The independent variables included 'plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines) and 'accessions' nested within plant type (three per plant type, see Table 1), followed by *a priori* contrasts to compare between successive plant types, as explained above.

Simpson's diversity index and corresponding species evenness index used in ecology to measure biodiversity of a habitat were used to assess volatile diversity and evenness between plant types (Morris et al., 2014). The diversity index accounts the number of volatiles and their abundance, whereas the evenness index refers to how close in number each volatile is between plant types (Simpson, 1949; Pielou, 1966).

Similarities Between Constitutive Volatile Emissions of Balsas Teosinte and US Inbred Maize Lines

This analysis was prompted by the results of the assay *Recruitment by a Derived Plant Relative to an Ancestral Plant.* The goal was to assess similarities between the volatile profiles of Balsas teosinte and US inbred lines (see results for *Volatiles Analyses*) in light of the similarly low recruitment levels evident for either plant type in that assay. The volatiles for BTEO and USIL were analyzed with MANOVA, as described above, though the model, in this case, included plant type as the only independent variable; the dependent variables were all the volatiles produced by both plant types, i.e., including

volatiles produced by one but not the other plant type. Following MANOVA, correlation analyses were used to identify the volatiles that most contributed to any separation between multivariate means, as described above. Finally, two-sample *t*-tests were used to compare mean levels of each volatile between BTEO and USIL.

Volatiles that Characterize both Talpitita Balsas Teosinte and Mo17 Maize Inbred Line

This analysis was prompted by the results of the assay *Mediation of recruitment by root volume*. The goal was to infer on individual volatiles plausibly underlying the differences in recruitment between the *Talpitita* BTEO and *Mo17* USIL accessions. Two-sample *t*-tests were used to compare mean levels of each volatile produced by Talpitita Balsas teosinte and Mo17 inbred line. All statistical analyses were performed using JMP software (SAS Institute Inc., 2018).

Results

Recruitment Bioassays

Recruitment by Zea Plant Types Relative to Sorghum

In two-choice assays against sorghum, recovery of WCR larvae from recruitment zones of the *Zea* plant types did not differ between any of the plant type pairs representing the domestication, spread or breeding transitions, contrary to our expectation (Plant type $F_{3, 29} = 1.070$, $P = 0.378$; Weight difference $F_{1, 29} = 11.570$, $P = 0.002$) (Figure 14). In contrast, WCR larvae were consistently recovered more frequently from each of the *Zea*

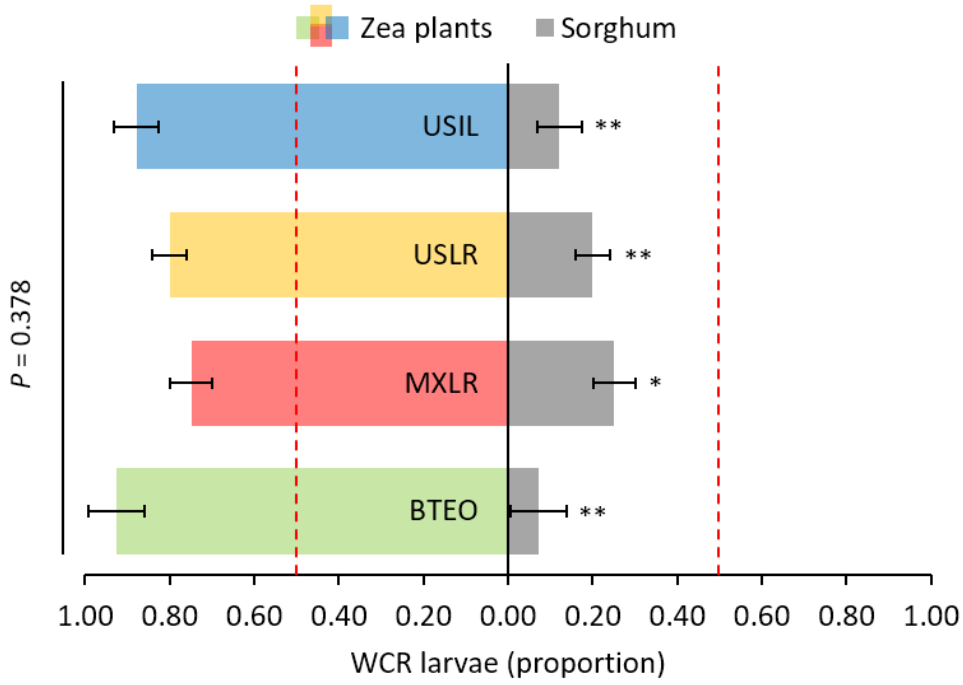

Figure 14. Recruitment of WCR by *Zea* plants relative to sorghum. Proportions (± SE) of neonate Western corn rootworm larvae recovered from roots of one of four plant types (Balsas teosinte, Mexican landraces, US landraces, and US inbred lines) offered in opposition to sorghum in two-way choice assays; Larvae proportions above 0.50, indicated by red dashed lines, were considered recruited by the plant type. The plant types are ordered from most ancestral (bottom) to most derived (top). A proportion increased from bottom to top was expected. ANOVA (with root mass as co-variable) did not revealed significant differences across proportions corresponding to plant types ($F_{3, 29}$ = 1.070, P = 0.378); asterisks besides each bar indicate significance of difference to expected 0.50 proportion or no preference, * < 0.01, ** < 0.001. BTEO = Balsas teosinte, MXLR = Mexican landraces, USLR = US landraces, USLR = US inbred lines.

plant type recruitment zones compared to the sorghum zone, as expected (Balsas teosinte, one-sample $t = 5.51$, $P = 0.001$; Mexican landraces, one-sample $t = 3.79$, $P = 0.007$; US landraces, one-sample $t = 5.62$, $P = 0.001$; US inbred lines, one-sample $t = 5.98$, $P = 0.001$). Overall, these results suggested that neonate WCR larvae do not forage randomly for hosts. Additionally, they indicated that while the assay may have been insufficiently sensitive to detect a trend of recruitment strength (relative to sorghum) across plant types, if it exists, the assay was sufficiently sensitive for comparing WCR larval recruitment strengths between plant pairs, and it uncovered the relevance of root volume for WCR recruitment.

Recruitment by a Derived Plant Relative to an Ancestral Plant

Recruitment of WCR larvae was greater by derived plant types compared to ancestral plants ($P \leq 0.019$) in the domestication (Balsas teosintes vs. Mexican landraces) and spread (Mexican landraces vs. US landraces) transitions, as expected; however, in the breeding transition ($P < 0.001$) fewer larvae were recruited by the derived (US inbred lines) compared to the ancestral (US landraces) plant type (Plant type [Transition] $F_{3,19} = 13.062$, $P < 0.001$; Weight difference $F_{1, 19} = 0.159$, $P = 0.694$) (Figure 15). Overall, these results suggested that recruitment of WCR is stronger by i) derived over ancestral plants, except under the breeding transition, and, at the same time; ii) less-resistant over more-resistant plants, per resistance levels assessed in a prior study (Fontes-Puebla and Bernal, 2019). Additionally, these results suggested that the Balsas teosintes and the US inbred lines may be refractory to WCR larvae, compared to the Mexican and US landraces.

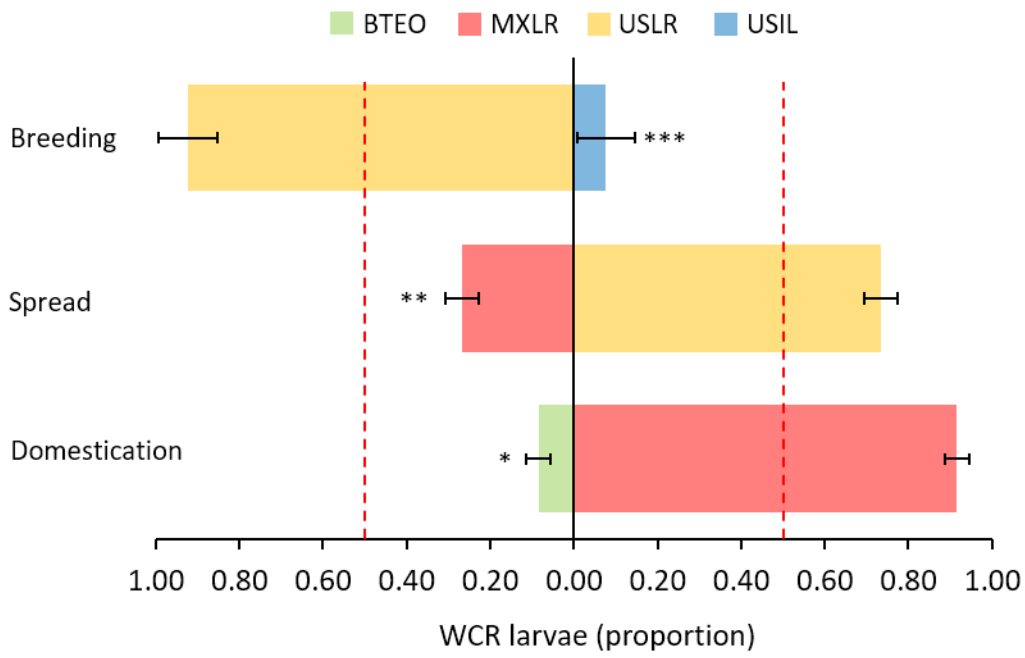

Figure 15. Recruitment of WCR by a derived plant relative to an ancestral plant. Neonate Western corn rootworm larvae (± SE) recovered from roots of four plant types, Balsas teosinte (BTEO), Mexican landraces (MXLR), US landraces (USLR), and US inbred lines (USIL), when offered in 2-way choice assays representing three agricultural transitions: (i) domestication, (ii) spread, and (iii) breeding. The domestication transition is represented by Balsas teosinte vs. Mexican landraces, the spread transition by Mexican landraces vs. US landraces, and the breeding transition by US landraces vs. US inbred lines. The transitions are ordered chronologically from bottom to top, and within each transition the plant types (bars) are ordered left and right as ancestral and derived, respectively. Larvae proportions above 0.50 (red dashed lines) were considered recruited by the plant type. Within each transition we expected greater recovery of larvae in the derived plant type (right side of the plot). ANOVA indicated significant differences between plant types within transitions (Plant type [Transition] $F_{3,19} = 13.062$, $P < 0.0001$); asterisks beside each bar indicate significance of difference to expected 0.50 proportion (red dashed line). Asterisks beside bars indicate *P* value corresponding to an *a priori* contrast comparison within each pair of plant types, * = 0.002, ** = 0.019, *** < 0.001.

Mediation of Recruitment by Root Volume

Unlike our expectations, recruitment of WCR larvae was significantly higher in every case by *Talpitita* BTEO compared to *Mo17* USIL: when *Talpitita* BTEO root weight was less than that of *Mo17* USIL ($z = 2.51$, $P = 0.012$); when *Talpitita* BTEO root weight exceeded that of *Mo17* USIL ($z = 3.34$, $P = 0.001$), and; when *Talpitita* BTEO and *Mo17* USIL root masses were equal ($z = 5.00$, $P < 0.0001$) (Figure 16). Across all trials, 77% of WCR larvae were recovered from the *Talpitita* BTEO recruitment region ($z = 6.01$, $P < 0.0001$). These results showed that differences in recruitment of WCR larvae by *Talpitita* BTEO or *Mo17* USIL were largely independent of these accession's root volumes. Moreover, these results suggested that *Mo17* USIL was refractory to WCR larvae compared to *Talpitita* BTEO, and that its refractoriness was likely independent of the volumes of constitutive volatiles produced by those accessions.

Volatiles Analyses

At least 22 constitutive volatiles were recorded across *Zea* plant types (Table 6, Figure 17a). Mexican and US maize landraces produced more volatiles, 15 each, than Balsas teosinte, with 14, and US inbred lines with 12. Balsas teosintes, Mexican and US maize landraces produced the same number of exclusive volatiles with 3, whereas US inbred lines had no exclusive volatiles relative to their ancestors (Figure 17a, Table 6). Both volatile diversity and evenness increased significantly with domestication, i.e., from

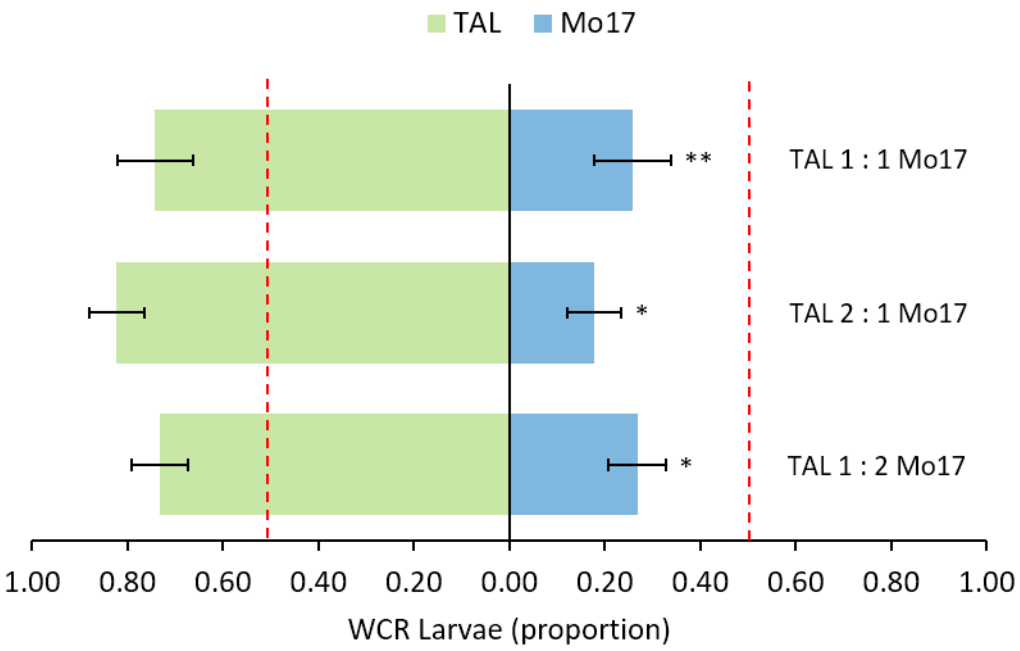

Figure 16. **Mediation of recruitment by root volume.** Recovery of Western corn rootworm larvae from roots of the Balsas teosinte accession Talpitita (*Talpitita* BTEO) compared to the maize inbred line accession MO17 (*Mo17* USIL) when root weights were manipulated so that one or the other plant's root weight is 2-fold greater or smaller, or their root weights are equal. Recovery of larvae was expected to be positively correlated with root weight, so that: (i) more larvae would be recovered from *Talpitita* BTEO when its root weight was greater than that of *Mo17* USIL (TAL 2 : 1 Mo17); (ii) fewer larvae would be recovered from *Talpitita* BTEO when its root weight was smaller than that of *Mo17* USIL (TAL 1 : 2 Mo17), and; (iii) similar numbers of larvae would be recovered from *Talpitita* BTEO and *Mo17* USIL when their root weights were equal (TAL 1 : 1 Mo17). In each case, more larvae were recovered from *Talpitita* BTEO compared to *Mo17* USIL: TAL 1 : 2 Mo17, $z = 2.51$, $P = 0.012$; TAL 2 : 1 Mo17, $z = 3.34$, $P = 0.001$, and TAL 1 : 1 MO17, $z = 5.00$, $P < 0.0001$.

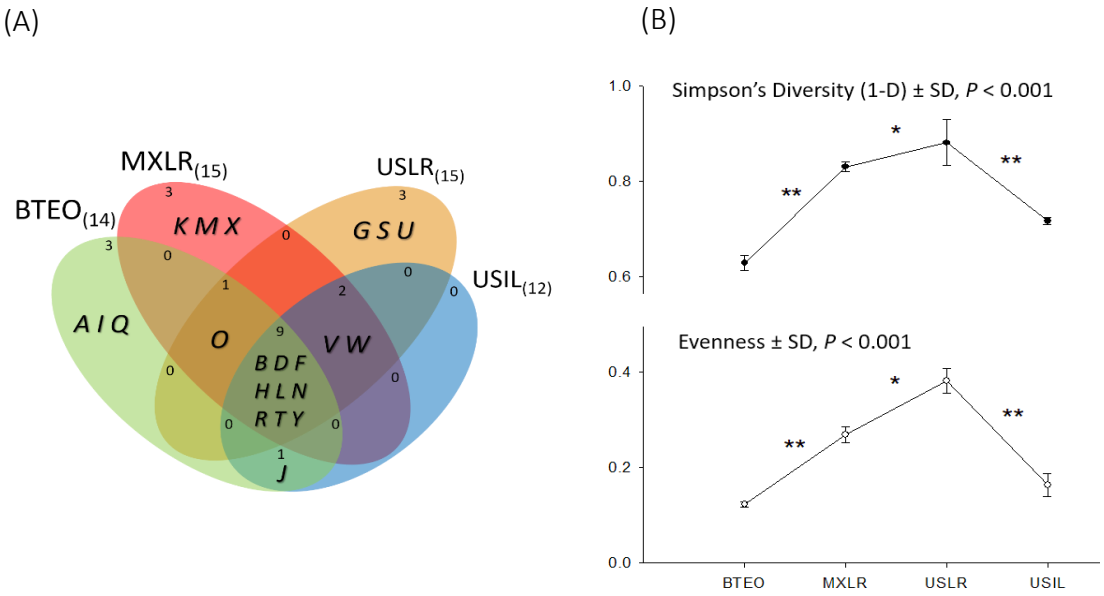

Figure 17. Constitutive root volatiles among four *Zea* plant types. (A) Venn diagram showing constitutive root volatiles among four *Zea* plant types. Letters represent each volatile (see table 1 for identification). The numbers inside the ovals indicate the quantity of shared volatiles among the spliced shapes. The number within parenthesis indicate the total number of volatiles per corresponding plant type. The size of each oval is proportional to the total volatiles of each plant type. **(B)** Constitutive volatiles diversity (Simpson's, 1-D) and evenness across plant types; *y*-axis indicate indices and *x*-axis indicate plant types. Overall significant *P* values are inset in each plot. Asterisks indicate significant differences in each transition between the ancestor and derived plant type representing domestication (BTEO vs MXLR), spread (MXLR vs USLR), and breeding (USLR vs USIL), * 0.001, ** < 0.0001. BTEO = Balsas teosintes, MXLR = Mexican landraces, USLR = US landraces, USIL = US inbred lines.

Table 6. Average peak areas for constitutive volatiles from *Zea* plant types.

	VOLATILE	Average peak area			
		BTEO	**MXLR**	**USLR**	**USIL**
A	(S)-β-macrocarpene	4.19E+05	n.d.	n.d.	n.d.
B	1-octen-3-ol	2.00E+07	3.75E+06	2.26E+06	2.20E+06
D	(E)-2-hexenal	5.11E+04	2.15E+04	1.08E+06	2.54E+05
E	(E)-2-nonenal	n.d.	n.d.	n.d.	n.d.
F	(E)-3-hexen-1-ol	3.43E+05	2.19E+06	3.29E+06	4.99E+06
G	α-muurolene	n.d.	n.d.	4.41E+05	n.d.
H	Acoradiene	4.00E+05	1.16E+05	1.34E+05	4.04E+04
I	α-cubebene	1.94E+06	n.d.	n.d.	n.d.
J	Azulene	1.20E+06	n.d.	n.d.	3.88E+04
K	β-selinene	n.d.	2.02E+05	n.d.	n.d.
L	**β-caryophyllene**	**7.68E+05**	**1.06E+06**	**1.17E+06**	**1.22E+05**
M	Chloromethyl-octyl-ether	n.d.	1.28E+06	n.d.	n.d.
N	Copaene	1.03E+06	3.84E+05	1.67E+06	2.83E+05
O	δ-cadinene	2.12E+05	1.39E+04	4.67E+05	n.d.
Q	Decanol	7.12E+05	n.d.	n.d.	n.d.
R	Eucalyptol	2.64E+06	2.02E+06	2.43E+06	3.61E+05
S	γ-muurolene	n.d.	n.d.	2.89E+05	n.d.
T	Geosmin	1.75E+06	1.06E+06	1.91E+06	1.38E+06
U	Germacrene D	n.d.	n.d.	3.75E+05	n.d.
V	Heptanal	n.d.	6.45E+04	6.69E+04	1.37E+04
W	Hexanal	n.d.	5.70E+06	4.73E+06	8.40E+06
X	Nonanal	n.d.	1.74E+05	n.d.	n.d.
Y	p-cymene-2,5-diol	2.56E+06	9.17E+05	2.22E+06	1.22E+06

Balsas teosinte to Mexican landraces, and again with spread, i.e., from Mexican landraces to US landraces (Figure 17b). However, both volatile diversity and evenness decreased significantly with breeding, i.e., from US landraces to US inbred lines. These results suggested that both artificial and natural selection, by farmers and environmental stresses, respectively, increased the diversity and evenness of volatiles produced by maize, but both volatile diversity and evenness were decreased by systematic breeding. Multivariate analyses showed significant effects (Wilk's λ = 2.25E-06, $P < 0.001$) of domestication, spread, and breeding, as evident from the separation of multivariate means for constitutive volatiles corresponding to the four plant types, Balsas teosinte, Mexican landrace maize, US landrace maize, and US inbred maize lines (Figure 18). *A priori* contrast comparisons showed that the domestication (Balsas teosintes vs. Mexican landraces, $F_{8, 17} = 231.32$, $P < 0.001$), spread (Mexican landraces vs US landraces, $F_{8, 17} = 81.28$, $P < 0.001$), and breeding (US landraces vs US inbred lines, $F_{8, 17} = 129.05$, $P < 0.001$) transitions significantly affected the volatile profiles of the four plant types.

Univariate analyses showed 20 volatiles with significant variation between plant types ($F_{3, 24} \geq 3.135$, $P \leq 0.044$) (Table 7). The transition effects on levels of these individual volatiles were grouped into six patterns, patterns I-VI (Figure 19, Table 7). A strong domestication effect is evident in patterns I and II, correspondingly indicated by a significant decline or increase between plant types BTEO and MXLR for volatiles (S)-β-macrocarpene, 1-octen-3-ol, acoradiene, α-cubebene, azulene, decanol, (E)-3-hexen-1-ol, heptanal, and hexanal, followed by no subsequent changes. In pattern III, a significant domestication effect is indicated by decreases in the emission of volatiles copaene,

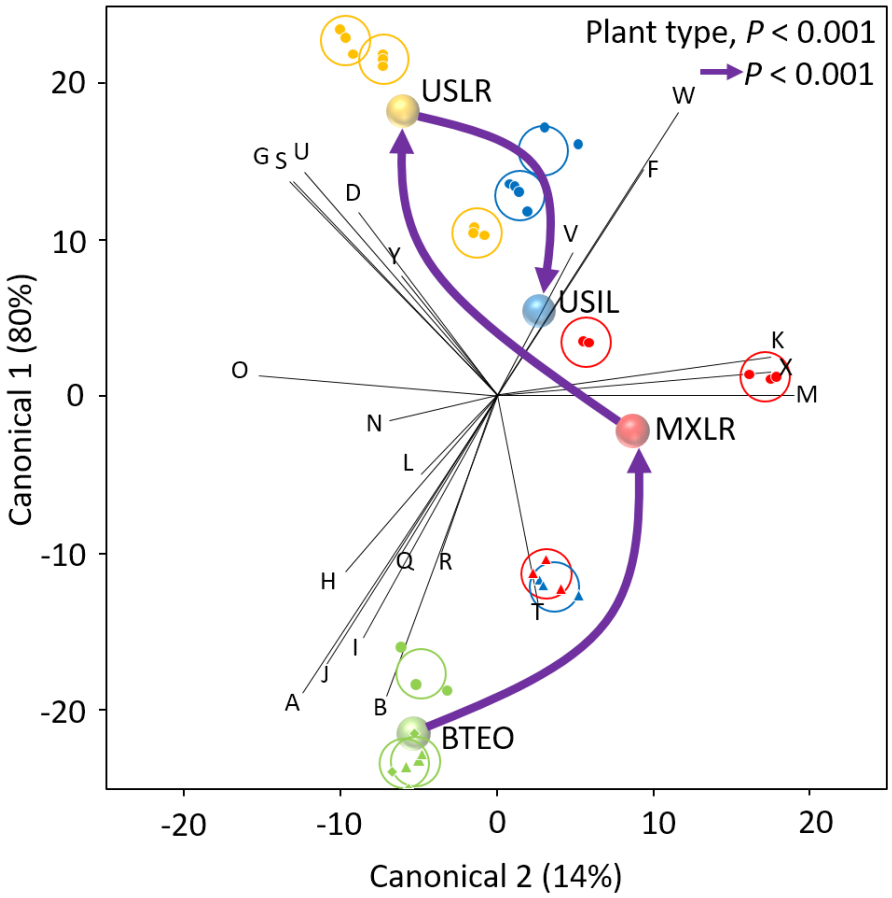

Figure 18. Canonical centroid plot from the MANOVA for constitutive root volatiles from *Zea* spp. Wilks' λ = 2.245E-06, *P* < 0.001. Solid filled circles represent 95% confident intervals around multivariate means for each plant type. The model included the independent variables 'Plant type' (Balsas teosintes, Mexican landraces, US landraces, and US inbred lines), and 'accessions' nested within plant type (three accessions per plant type denoted by empty solid circles) —see table 1. The dependent variables were the peak areas of constitutive root volatiles from *Zea* spp. (Table 2). Arrows indicate significant differences between plant types representing domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLR vs. USIL) (*a priori* contrasts with critical *P* of 0.017, per Sidak correction). BTEO = Balsas teosintes, MXLR = Mexican landraces, USLR = US landraces, USIL = US inbred lines.

Table 7. Analysis of Variance among *Zea* plant types and accessions nested within plant types for constitutive root volatiles. The transition effect shows *a priori* contrasts between plant types representing domestication, spread, and breeding.

Pattern	Volatiles	ANOVA statistics			Transition effect		
		Adj r^2	Plant type *P* value	Accession *P* values	DOM	SPR	BRE
1	A	0.866	< 0.001	0.142	↓	↔	↔
	B	0.915	< 0.001	0.843	↓	↓	↔
	H	0.457	0.005	0.018	↓ 0.002	↔	↔
	I	1.000	< 0.001	< 0.001	↓	↔	↔
	J	0.886	< 0.001	0.457	↓	↔	↔
	Q	0.312	0.010	0.169	↓ 0.006	↔	↔
2	F	0.540	< 0.001	0.258	↑	↔	↔
	V	0.496	0.044	0.002	↑ 0.020	↔	↔
	W	0.998	< 0.0001	< 0.001	↑	↔	↑ 0.005
3	N	0.804	< 0.001	< 0.001	↓ 0.013	↑ 0.003	↓
	O	0.783	< 0.001	< 0.001	↓	↑	↓
	T	0.305	0.178	0.032	↔	↔	↔
	Y	0.998	< 0.001	< 0.001	↓	↑	↓
4	K	0.996	< 0.001	< 0.001	↑	↓	↔
	M	1.000	< 0.001	< 0.001	↑	↓	↔
	X	1.000	< 0.001	< 0.001	↑	↓	↔
5	D	0.250	0.017	0.303	↔	↑ 0.005	↔
	G	1.000	< 0.001	< 0.001	↔	↑	↓
	S	1.000	< 0.001	< 0.001	↔	↑	↓
	U	0.854	< 0.001	0.028	↔	↑	↓
6	L	0.995	< 0.001	< 0.001	↔	↔	↓
	R	0.863	< 0.001	< 0.001	↔	↔	↓

Red downward arrows and blue upward arrows indicate a significant decrease and increase of volatile peak areas, respectively by $P < 0.001$ unless specified within the cell. Gray horizontal left-right arrows indicate no difference ($P > 0.05$). Table 1 lists the volatiles' names for A-Z. DOM = domestication, SPR = spread, BRE = breeding.

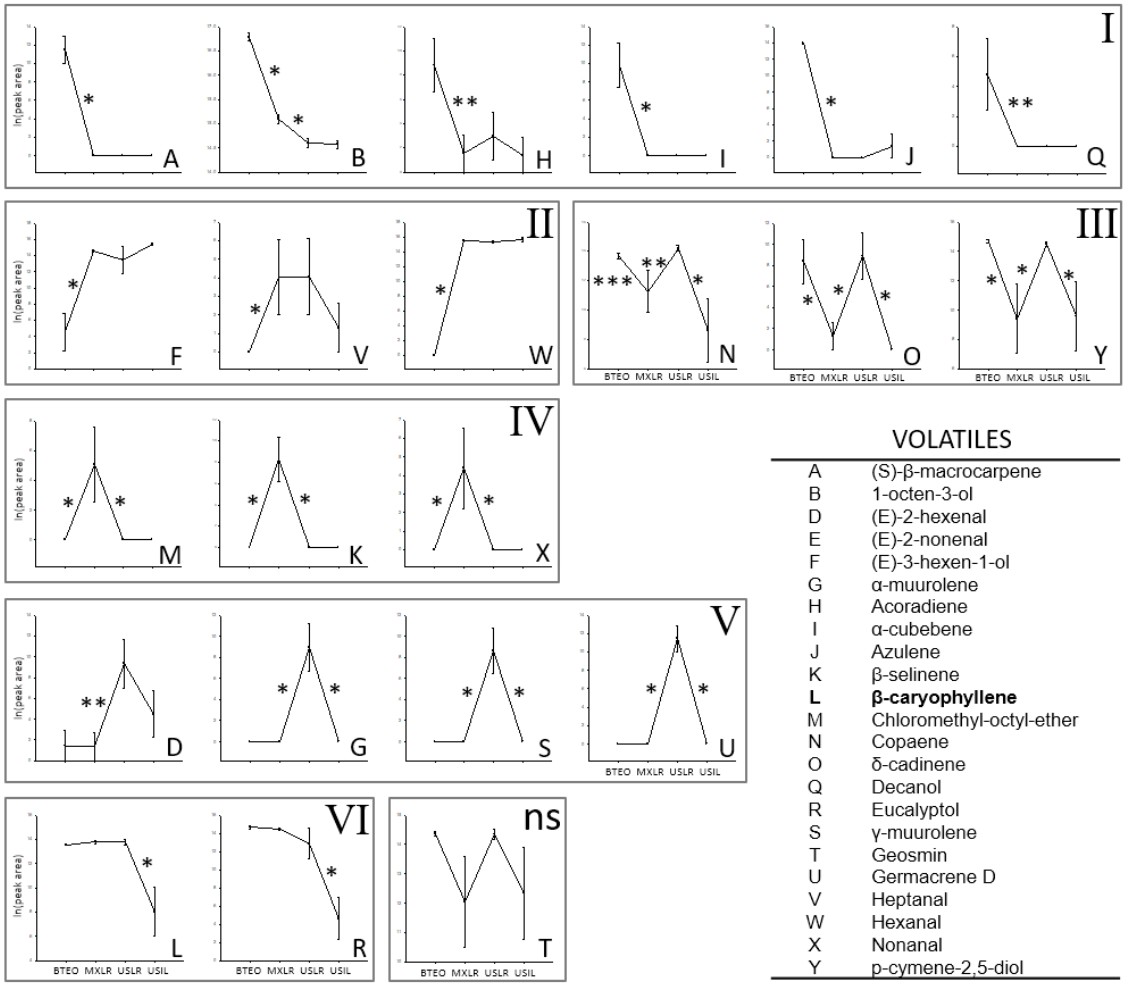

Figure 19. Constitutive root volatile univariate trends among the four *Zea* plant types. All $P \leq 0.010$, except T at the middle bottom, which is not significant at $P = 0.178$; see Table 3 for ANOVA statistics for volatiles across plant types showing the effects of domestication, spread, and breeding on levels of individual volatiles (upper-case letters inset in each plot are designations for each volatile and the names are listed in the bottom right). The *y*-axis represents the volatile's peak areas in ln(*x+1*); the *x*-axis represents the four plant types. Asterisks indicate significant differences between plant types representing domestication (BTEO vs. MXLR), spread (MXLR vs. USLR), and breeding (USLR vs. USIL) (*a priori* contrasts with critical *P* of 0.017, per Sidak correction), * < 0.001, ** < 0.005, *** = 0.013. BTEO = Balsas teosintes, MXLR = Mexican landraces, USLR = US landraces, USIL = US inbred lines.

δ-cadinene, and p-cymene-2,5-diol in plant type MXLR relative to plant type BTEO, followed by a recovery with spread (i.e. from MXLR to USLR) and decline to domestication levels with breeding (i.e. from USLR to USIL). In pattern IV, levels of volatiles chloromethyl-octyl-ether, β-selinene, and Nonanal increased with domestication, and decreased with spread to remain unchanged with breeding, so that the levels of these volatiles are similar to pre-domestication levels. In pattern V, there was a spread effect which increased the levels of (E)-2-hexenal, α-muurolene, γ-muurolene, and germacrene D, and a breeding effect which decreased levels to those of domestication. Finally, in pattern VI, emission of volatiles EβC, and Eucalyptol were high and conserved from plant types BTEO through USLR, but a significant breeding effect decreased their production significantly (Figure 19). Additionally, the ANOVA showed significant nested effects ($F_{8, 24} \geq 2.971$, $P \leq 0.018$, where acoradiene, α-cubebene, and δ-cadinene peak areas varied within BTEO; β-selinene, EβC, copaene, heptanal, hexanal, and nonanal varied within MXLR; EβC, γ-muurolene, heptanal, and hexanal varied within USLR; and EβC, copaene, eucalyptol, and hexanal varied within USIL ($F_{2, 24} \geq 3.424$, $P \leq 0.042$) (Table 7).

Altogether, these results showed that aggregate volatiles profiles and production of individual volatiles both changed with maize domestication, spread, and breeding, so that significant differences at the aggregate and individual volatile levels are evident between relevant plant types. Moreover, these results highlighted that domestication and breeding were the most effectual of the transitions, as evident in our multivariate and univariate analyses (Figures 18, 19). Thus, volatile levels in modern maize, i.e. USIL, were determined by domestication (patterns I and II) or breeding (pattern VI).

Additionally, the production of volatile EβC, was only affected (negatively) by breeding (Figure 19).

Similarities Between Volatile Emissions of Balsas Teosinte and US Inbred Maize Lines

Balsas teosintes and US maize inbred lines produced a total of 16 constitutive volatiles combined; Balsas teosintes exclusively produced four of these, whereas US inbred lines produced two (Figure 20a). MANOVA discriminated between volatile multivariate means corresponding to BTEO and USIL ($F_{1, 16}$ = 6887.1, P = 0.010). Correlation analyses showed that volatiles (S)-β-macrocarpene, 1-octen-3-ol, acoradiene, α-cubebene, azulene, EβC, copaene, δ-cadinene, and eucalyptol, were significantly negatively correlated with canonical 1 scores (Pearson's r -0.507 to -0.980, $P \geq 0.032$), while volatiles (E)-3-hexen-1-ol and hexanal were significantly positively correlated (respectively, Pearson's r = 0.998, $P < 0.0001$, and Pearson's r = 0.765, $P < 0.001$); the remaining volatiles, (E)-2-hexenal, decanol, geosmin, heptanal, and p-cymene-2,5-diol, were not significantly correlated with canonical 1 nor 2 scores (Pearson's $r \leq |0.470|$, $P \geq 0.049$). Coincidently with the MANOVA and correlation results, the levels of volatiles (S)-β-macrocarpene, 1-octen-3-ol, acoradiene, α-cubebene, azulene, EβC, copaene, δ-cadinene, and eucalyptol, were significantly lower ($t \geq 2.351$, $P \leq 0.046$, 8 df), and those of (E)-3-hexen-1-ol and hexanal were significantly higher (respectively, t = 57.228, $P < 0.0001$, and t = 4.749, P = 0.001, 8 df) in USIL compared to BTEO, while those of (E)-2-hexenal, decanol, geosmin, heptanal, and p-cymene-2,5-diol, did not differ between the two plant types ($t \leq 1.000$ to 1.998, $P \geq 0.081$, 8 df) (Figure 20b). Of the latter volatiles,

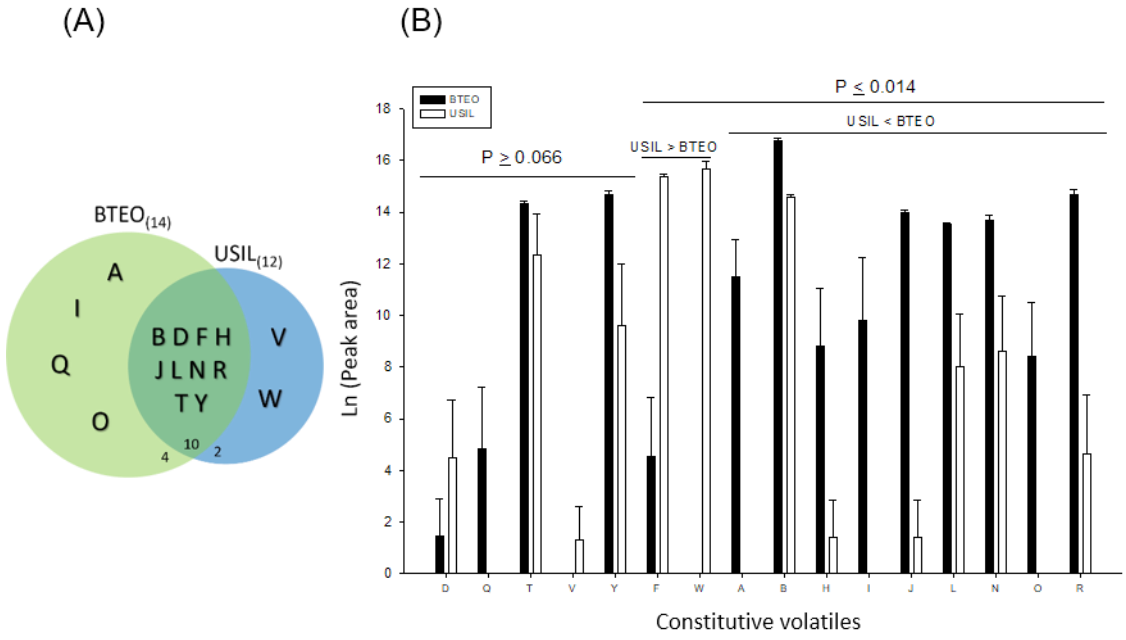

Figure 20. Constitutive root volatiles among Balsas teosintes and US inbred lines. (A) Venn diagram for volatiles shared and not shared by plant types Balsas teosintes (green) and US maize inbred lines (blue). Letters inside the circles represent each volatile (see table 2 for identification). The numbers inside the circles indicate the quantity of shared volatiles among the spliced shapes. The number within parenthesis indicate the total number of volatiles per corresponding plant type. The size of each circle is proportional to the total volatiles of each plant type. **(B)** Peak areas of volatiles in ln(*x+1*) ± SE shared or not shared by plant types Balsas teosintes (black columns) and US maize lines (white). Volatiles D, Q, T, V, Y (columns at left) do not differ between Balsas teosintes and US maize lines (P = 0.066 to 0.347); note that D, Q, and V occur at low levels or are absent, while T and V occur at comparatively high levels. All remaining volatiles (A through W, from left-of-center to far right) differ between Balsas teosintes and US maize lines (P = 0.014 to < 0.0001). See Table 2 for corresponding volatile names.

however, three were produced at relatively low levels or not produced by one or the other plant type: (E)-2-hexenal, 4.5±2.2 vs. 0.0±0.0; decanol, 4.8±2.4 vs. 0.0±0.0, and; heptanal, 1.3±1.3 vs. 0.0±0.0) (Figure 20b).

Overall, these results suggested that the volatile profiles of Balsas teosintes and US inbred lines are similar in the presences of volatiles geosmin and p-cymene-2,5-diol, and/or absences of volatiles (E)-2-hexenal, decanol and Heptanal. Consequently, these results suggest that volatiles (E)-2-hexenal, decanol, geosmin, Heptanal, and p-cymene-2,5-diol may underlie the apparent refractoriness of both Balsas teosinte and US inbred lines to WCR larvae (see Figure 15).

Volatiles that Characterize both Talpitita Balsas Teosinte and Mo17 Maize Inbred Line

The accessions *Talpitita* BTEO and *Mo17* USIL produced a combined total of 12 constitutive volatiles; *Talpitita* BTEO produced four exclusive volatiles, whereas *Mo17* USIL produced two (Figure 21a). Both accessions produced similar levels ($t \leq 2.00$, $P \geq 0.144$) of five of the 12 volatiles, i.e. (S)-β-macrocarpene, (E)-2-hexenal, (E)-3-hexen-1-ol, δ-cadinene, and geosmin, while the remaining seven volatiles differed between those accessions ($t \geq 4.80$, $P \leq 0.04$) (Figure 21b). In volatiles that differed between the accessions, particularly, volatiles azulene and eucalyptol were produced by *Talpitita* BTEO but not *Mo17* USIL; production of volatiles 1-octen-3-ol, EβC, copaene, and p-cymene-2,5-diol was higher in *Talpitita* BTEO compared to *Mo17* USIL, and *Mo17* USIL, but not *Talpitita* BTEO, produced volatile W. Average relative amounts (± SE) of the volatile EβC were calculated through the use of a calibration curve, and *Talpitita* BTEO

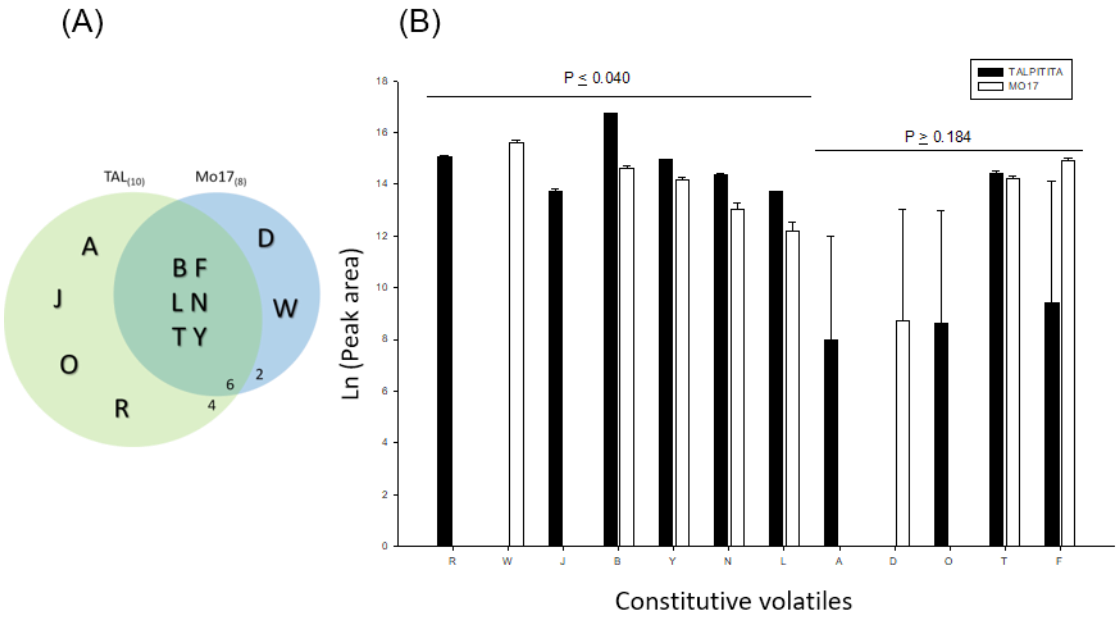

Figure 21. Constitutive root volatiles among *Talpitita* BTEO and *Mo17* USIL. (A) Venn diagram for volatiles shared and not shared by the accessions *Talpitita* BTEO (light green) and *Mo17* USIL (light blue). Letters inside the circles represent each volatile (see table 2 for identification). The numbers inside the circles indicate the quantity of shared volatiles among the spliced shapes. The number within parenthesis indicate the total number of volatiles per corresponding plant type. The size of each circle is proportional to the total volatiles of each plant type. **(B)** The volatiles that differed significantly (shared or exclusive) between the two accessions are grouped on the left. On the right are volatiles that occur at similar levels. Peak areas of volatiles in $\ln(x+1)$ ± SE shared or not shared by the accessions *Talpitita* Balsas teosintes (black columns) and *Mo17* USIL (white). Volatiles J and R (columns at left) are exclusive of *Talpitita* BTEO, whereas W is exclusive to *Mo17* USIL. See Table 2 for corresponding volatile names.

(2.050 ± 0.003 ng/g FW) was found to produce 1.3-fold more EβC than *Mo17* USIL (1.544 ± 0.042 ng/g FW) (t = 12.14, P = 0.007, 2 d.f.).

Overall, these results suggested that any effects of root volume on recruitment of WCR larvae by roots is overridden by effects of constitutive volatiles emitted by roots. Additionally, these results suggested that volatiles 1-octen-3-ol, Azulene, EβC, Copaene, Eucalyptol, and p-cymene-2,5-diol, individually or in combination, may render *Talpitita* BTEO more attractive to WCR larvae than *Mo17* USIL, while production of volatile Hexanal may render *Mo17* USIL refractory to WCR larvae.

Discussion

I hypothesized that domestication, spread, and breeding mediated recruitment of WCR larvae by constitutive volatiles emitted by roots of four *Zea* plant types: Balsas teosinte, Mexican landrace maize, US landrace maize, and US inbred line maize. This hypothesis was tested with bioassays that: (i) indirectly compared recruitment (i.e., relative to the non-host plant sorghum) of WCR between Balsas teosinte and Mexican landrace maize, Mexican landrace maize and US landrace maize, and US landrace maize and US inbred maize, and; (ii) directly compared recruitment between those pairs of plant types. In parallel, the constitutive volatiles emitted by roots of the different plant types were tentatively identified and compared, and similarities and dissimilarities were sought among their corresponding volatiles profiles that could explain the results of the recruitment bioassays, in part. Finally, focused comparisons were made between the volatile profiles of selected plant types (BTEO, USIL) and accessions (*Talpitita* Balsas

teosinte, *Mo17* US inbred maize) to infer whether particular volatiles may prove refractory or attractive to WCR larvae were made. Overall, I expected to find a trend in which recruitment of WCR larvae would be stronger by less resistant plants, as characterized in previous studies. I also expected to identify similarities and differences among the profiles of constitutive volatiles of the *Zea* plant types consistent with differences in the recruitment levels of those plant types. The results largely confirmed the expectations as domestication, spread, and breeding mediated recruitment of WCR neonate larvae by the *Zea* plant types. Specifically, less resistant plant types were more strongly recruited WCR larvae compared with more resistant plant types. Interestingly, volatile diversity and evenness increased significantly with domestication and spread, but decreased significantly with breeding. Finally, the recruitment of WCR larvae by *Zea* plant types seemed to be independent of their root volumes, and possibly of volumes of volatile emissions, but dependent on volatile profiles specific to each *Zea* plant type.

Recruitment Bioassays

Domestication, Spread, and Breeding Mediated WCR Larvae Recruitment by Zea *Plant Types*

The first assay's results suggested that the foraging behavior of neonate WCR larvae is not random, as anticipated (Robert et al., 2012a; Robert et al., 2012c; Schumann et al., 2018). Specifically, while we did not find significant differences in recruitment within the pairs of plant types that we compared, WCR larvae were consistently recruited

more strongly by the *Zea* plants than by the sorghum plants (Figure 14). Sorghum appeared refractory to WCR, pushing recruitment towards *Zea* plants, contrary to the similar levels of recruitment reported previously for maize and sorghum (Branson et al., 1969). In contrast, the second assay's results, which compared directly between ancestral and derived host plants, showed that recruitment of WCR larvae by maize plants was significantly enhanced by domestication (Balsas teosinte vs. Mexican landrace) and spread (Mexican landrace vs. US landrace) (Figure 15). Surprisingly, however, breeding appeared to have significantly weakened recruitment of WCR larvae, as indicated by the stronger recruitment by US inbred maize compared to US landrace maize (Figure 15). These results are consistent with expectations based on previous studies (Fontes-Puebla & Bernal 2019). In a prior study, WCR performance decreased after feeding on resistant *Zea* plant types compared to less-resistant plant types, including on US inbred lines vs. US landraces, which suggests that neonate WCR larvae are more attracted to lesser-resistant plants (Fontes-Puebla & Bernal 2019).

An earlier study suggested that CO_2 was the only cue that WCR used to find its host, though this volatile is not a species-specific cue (Bernklau and Bjostad, 1998). However, other studies highlighted challenges inherent to experimentally discriminating among potential cues relevant to WCR larvae, e.g., discriminating between primary and secondary metabolites that may act as attractants (Hibbard and Bjostad, 1988; Hibbard et al., 1994; Bernklau and Bjostad, 1998). More recently, it was found that WCR uses EβC induced after initial infestation to locate host roots and aggregate in a density-dependent manner (Robert et al., 2012a; Robert et al., 2012c). EβC can be produced and emitted

constitutively in maize, which may mediate an initial attraction, likely complementing the effects of CO_2 and other volatiles (Bernklau and Bjostad, 1998; Erb et al., 2011). Perhaps, once host roots are encountered and injured, they may release induced EβC, so amplifying recruitment of WCR larvae (Robert et al., 2012a). These findings increase the likelihood that a blend of chemicals, rather than individual chemicals, released from a host plant may mediate WCR host location.

Root semiochemicals vary inter- and intraspecifically, likely due to intra-specific genetic diversity mediated by natural and artificial selection (Wright et al., 2005; Block et al., 2019). In *Zea*, for example, resistance to root herbivory was reduced with domestication and spread, but systematic breeding in the context of agricultural intensification and increased WCR herbivory pressure seemed to have inadvertently selected for resistance (Fontes-Puebla and Bernal, 2019). Furthermore, one of the most characteristic changes resulting from domestication in maize is architectural (Dorweiler et al., 1993). These phenotypic changes affected root density and size as they vary between the ancestor and derived *Zea* spp. (Gaudin et al., 2011; Gaudin et al., 2014). These changes in root size and density across transitions were evident between the seedlings used for this study, and mediated in part the recruitment of WCR. Roots from Balsas teosintes, for example, were thin with more lateral and seminal roots in comparison with maize landraces, which had fewer lateral roots and a visibly thicker main root, or with inbred maize lines which had few to none lateral roots but shared thickness with landraces (pers. observ.). Regardless, WCR larvae responded differently to the available hosts, from the most ancestral to the most derived *Zea* plant types.

Root Volume Alone Does Not Mediate the Recruitment of WCR Larvae to its Host.

I assessed whether seedling root weights determined the recruitment of WCR larvae, prompted by the results of two preceding assays. The recruitment of WCR larvae by the accessions *Talpitita* Balsas teosinte and *Mo17* US inbred line was independent of root volume, contrary to expected. The analysis showed that in every weight ratio, *Mo17* was refractory to WCR larvae compared to *Talpitita*, suggesting that such refractoriness may be due to their volatile profile independently of their root volume (Figure 16). In a recent study, Robert et al. (2012a) found that WCR larvae preferred infested over healthy plants when both plants emitted the same volume of CO_2, but when the healthy plant emitted more CO_2 than the infested plant, WCR larvae preferred the healthy plant. They also found that WCR larvae were attracted to the phytohormone ethylene. Root exudates in the rhizosphere, including CO_2 and ethylene, provide cues to soil-dwelling insects and other organisms, partly dependent on root volume (Baetz and Martinoia, 2014; Haichar et al., 2014; Rasmann and Turlings, 2016). Therefore, it is reasonable to think that different root weights will result in different concentrations of exudates and CO_2. However, in light of our results, it seems that WCR larvae are opting for quality (i.e. volatile blend) rather than volume, at least under our experimental conditions.

Volatile Assays

The results suggested that domestication and spread increased the breadth, diversity, and evenness of volatiles profiles of Mexican and US landrace maize, while

breeding decreased them in US inbred lines (Figure 17a, b). The MANOVA showed significant multivariate effects of domestication, spread, and breeding, and *a priori* contrasts showed that the three transitions significantly affected the constitutive root volatile profiles of the four plant types (Figure 18). Furthermore, ANOVA on each constitutive root volatile showed significant differences in 20 out of 24 volatiles detected in *Zea* plant types, and revealed six patterns of effects (Figure 19, Table 7). Interestingly, constitutive EβC in roots was detected in two of the three accessions within US inbred lines tested contrary to what Köllner et al. (2013) reported. This volatile is especially important because it has been identified as a cue used by WCR to locate its host (Robert et al., 2012a), and because my results from the recruitment assays suggested that additional volatiles may be involved in WCR host foraging.

Volatile organic compounds have multiple functions in plant metabolism, and some may form part of the synthesis of other compounds that perform a variety of functions, such as sterols and plant hormones associated with developmental processes (Block et al., 2019). This variability in function may allow some volatiles to be conserved across populations and species. The volatiles detected in this study were grouped into 6 patterns, based on whether they were affected by one or more transitions, i.e. domestication, spread, and breeding. Volatiles in pattern I showed a strong effect of domestication, specifically occurring at high levels in Balsas teosinte and being absent or at low levels in maize. Of the volatiles under this pattern, volatiles 1-octen-3-ol and azulene may be fundamentally important to Balsas teosintes as they occur at consistently higher levels and show little to no variation compared to maize. This suggests that volatiles

1-octen-3-ol and azulene are important to Balsas teosinte regardless of environmental conditions. However, whether volatiles 1-octen-3-ol and azulene are related to defense or growth in Balsas teosinte is unclear. Similarly, the variation evident in volatiles (S)-β-macrocarpene, acoradiene, α-cubebene, and decanol may indicate that while they may be important to Balsas teosinte, their importance depends on local environmental conditions, given the considerable variation surrounding their means. In contrast, the apparent irrelevancy of volatiles 1-octen-3-ol and azulene, and (S)-β-macrocarpene, acoradiene, α-cubebene, and decanol to maize seems a result of artificial selection during domestication, and may reflect alleles that are absent or not expressed in maize, perhaps due to a trade-off with other traits.

The levels of volatiles (E)-3-hexen-1-ol, heptanal, and hexanal, under pattern II, increased with domestication, from nil or low levels in Balsas teosinte to high levels in maize. Thus, these volatiles may be of little relevance to Balsas teosinte, but highly relevant to maize, though with some variability. For example, volatile heptanal shows high variability in all three maize types (Mexican and US landraces, and US inbred lines), while volatiles (E)-3-hexen-1-ol and hexanal show less variability. Thus, volatiles under pattern II seem to be relevant to maize, though their relevance seems to be dependent on local environmental conditions, and perhaps the goals of systematic breeding.

Pattern III, IV, and V seem to have variable relevance for landraces only, as copaene, δ-cadinene, and p-cymene-2,5-diol apparently are significant for Balsas teosinte and not so much for landraces and chloromethyl-octyl-ether, β-selinene, nonanal, acoradiene, α-muurolene, γ-muurolene, and germacrene D seems only relevant to

landraces. For US inbred lines, however, little to none of these volatiles appear to be relevant. Finally, under pattern VI, systematic breeding reduced the levels of EβC and eucalyptol, as both are consistently high from Balsas teosintes to US landraces. In particular, EβC was reduced from high levels with little to no variation in Balsas teosintes and maize landraces to low levels with considerable variation in US inbred maize. This loss of EβC with breeding has significant implications for maize-WCR-natural enemy interactions, and hence for WCR management in US maize, as noted in prior studies (Rasmann et al., 2005; Robert et al., 2017).

Overall, patterns I, II, and VI in constitutive volatiles emissions may suggest that the emission of volatiles by maize in significantly higher or lower concentrations relative to Balsas teosinte may have significant implications for agricultural production and crop spread. Artificial selection by farmers initially targeted traits associated with productivity or yield (Wright et al., 2005), and with crop spread selection would focus on adaptation to novel environments while maintaining yields (Villa et al., 2005; van Heerwaarden et al., 2012; Swarts et al., 2017). However, as agricultural production increasingly intensified with the advent of synthetic pesticides and fertilizers within the last 100 years, systematic breeding seems to affected the syntheses of constitutive root volatiles of patterns I, II, and VI, so that US inbred lines produce high yields but are comparatively poorly defended against herbivores (Fontes-Puebla and Bernal, 2019).

Breeding Made Maize Less Attractive to WCR

Domestication significantly modified the constitutive volatile blend of Mexican landrace maize relative to Balsas teosinte, which may have rendered the former more attractive to WCR larvae than the latter. It is plausible that a subset of volatiles refractory to WCR larvae in Balsas teosinte were conserved through domestication, spread, and breeding, or recovered with breeding. Thus, I found that volatiles (E)-2-hexenal, decanol, geosmin, heptanal, and p-cymene-2,5-diol were emitted at similar levels in Balsas teosintes and US inbred maize lines, and may be responsible for the apparent refractoriness of these plant types. Additionally, I found that volatiles (S)-β-macrocarpene, (E)-2-hexenal, geosmin, δ-cadinene, and (E)-3-hexen-1-ol were emitted at similar levels in *Talpitita* Balsas teosinte and *Mo17* US inbred maize, and may further narrow the number of similarities between Balsas teosinte and US inbred maize lines and point to volatiles that may underlie refractoriness in these plant types. Volatiles (E)-2-hexenal and geosmin are shared in both the Balsas teosinte – US inbred maize and *Talpitita* – *Mo17* comparisons, and the low level of the first and high level of the second may merit further research into any effects they may have on WCR foraging.

Interestingly, the volatile EβC, a known host searching and aggregation cue for WCR, was produced constitutively by US inbred maize B73 and Mo17, though it was reported as not being produced constitutively nor induced by WCR in roots of B73 maize, nor in insect-injured leaves of Mo17 (Robert et al., 2012a; Robert et al., 2012c; Köllner et al., 2013). Also, my results showed that inbred maize line W438, which is the third USIL accession included in this study, did not constitutively produce EβC. The inconsistency in

emission of EβC among USIL accessions suggests that breeding efforts have variably affected this trait. Regardless, constitutive EβC levels are consistently high, and were unchanged with domestication and spread, but decreased significantly with breeding (Figure 19L, 20b, 21b). Moreover, there is little variation in EβC levels from Balsas teosinte through US landraces, which suggests that this trait may be of high and conserved relevance previous to breeding, i.e. prior to agricultural intensification. Additionally, the comparison between *Talpitita* Balsas teosinte and *Mo17* inbred maize showed that *Talpitita* produced more EβC than *MO17*, which may explain the strong recruitment of WCR by *Talpitita* relative to *Mo17*. Overall, *Talpitita*'s distinctive profile may render this accession more attractive to WCR larvae than *Mo17*, while hexanal, along with the absence of eucalyptol and azulene, may render *Mo17* refractory.

CHAPTER V

CONCLUSION

Resistance to WCR decreased with both maize domestication and spread, and tolerance increased as resistance decreased, as expected. However, the effects of breeding on maize defenses were inconsistent with predictions based on productivity-resistance and resistance-tolerance trade-offs. Specifically, breeding reversed the preceding trend of decreasing resistance and increasing tolerance so that US inbred lines were less tolerant and more resistant to WCR than their ancestral US landraces. These results were consistent with differences in biochemical compound levels between plant types representing domestication, spread, and breeding. Domestication had consequences on how resources are allocated within the lipoxygenase pathway, where levels of 13-LOX branch derivatives were reduced and levels of 9-LOX branch were increased. Spread did not seem to have effected major changes in biochemical compound levels. However, systematic breeding, along with agricultural intensification and continuous WCR herbivory pressure, seems to have contributed to increase a 9-LOX derivative and IAA levels which, in the context of WCR and maize inbred line performances, suggested that DA4 may be contributing to partial resistance to WCR, and IAA to WCR tolerance through root compensation. While domestication and spread decreased maize's resistance in favor of productivity, such improvement came along with a more diverse volatile profile recruiting novel enemies, such as WCR. Breeding, however, seems to have changed the volatile profile of maize by reducing the diversity of compounds it emits constitutively, and rendering it less attractive

to WCR. I believe that subsets of volatiles were conserved or enhanced with domestication and spread, and may be of agronomic importance, e.g., they may be related to productivity or defense against WCR.

Undoubtedly, domestication was a consequential process that significantly affected maize growth, reproduction, and herbivore defense. Similarly, its spread from present-day Mexico exposed maize to new environments and confronted it to novel suites of herbivores, which adopted the novel crop as a host because of its abundance and advantages, e.g., weakened defenses, superior nutritional value, refuge from natural enemies. With domestication and spread, the distribution and abundance of maize increased beyond those of Balsas teosinte, its wild ancestor, and with those increases maize broadened its genetic diversity as it was challenged by novel abiotic and biotic stresses (Hufford et al., 2012a; Hufford et al., 2012b; Bellon et al., 2018). Breeding in the last ~100 years narrowed maize's genetic diversity to increase its productivity in the context of resource-rich environments (including fertilizers, irrigation, and pesticides) in which tolerance- and resistance-based defenses were favored or neglected through systematic breeding, mainly for yield. In parallel, and partly as a consequence of the increasingly resource-rich environment in which maize was cultivated, WCR became a significant pest of maize in the US. As maize agriculture intensified beginning in the mid-1900s, it seems that tolerance as a basis for WCR management was neglected in deference to chemical control, though a small degree of WCR resistance was gained through breeding. Thus, it seems that US maize inbred lines, the parents of commercial hybrid varieties, are neither tolerant nor resistant to WCR, so are reliant on external means of

defense against this pest, such as insecticides. Overall, my results suggested that the evolution of defense strategies in maize is predicted by ecological-evolutionary hypotheses seeking to explain defense strategy evolution in plants generally, within the contexts of plant resistance-productivity trade-offs, plant tolerance-resistance trade-offs, and varying resource availability vis-à-vis plant physiological stress and herbivory pressure. Future studies should be conducted regarding OPR/LOX expression in both root and shoot constitutive and induced biochemical compounds for the four Zea plant types. An assessment of constitutive and induced root volatiles in vivo as well as olfactory bioassays testing the volatile blends/fractions identified herein or after in vivo bioassays on WCR attraction may provide more clarity to the recruitment results. Evaluation of the host nutritional quality to WCR among the four Zea plant types is suggested to complement recruitment results.

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