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Early developmental shifts in root exudation profiles of five *Zea mays* L. genotypes

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ABSTRACT

Root exudates impact soil-plant-microbe interactions and play important roles in ecosystem functioning and plant growth. During early plant development the root rhizosphere may change drastically. For maize (*Zea mays* L.), one of the world's most important crop species, little is known about root exudation patterns during early plant development. We determined abundance and composition of root exudation among maize genotypes from five inbred lines across three early plant development stages (Emergence, V1–2, and V3–4). We characterized the exudates for non-purgeable organic carbon and performed non-targeted metabolomics with high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Across all genotypes, plant development stage had a significant influence on both abundance and composition of exudates. Exudation rates (mg C per cm^2 root area d^{-1}) were highest in the emergence stage and logarithmically decreased with plant development. In the emergence stage, the roots released predominantly sugars (most indicative: glucose and fructose) and the metabolite richness was generally higher than in later stages. Secondary compounds (e.g. phenolics, benzoxazinoids, or mucilage) increased significantly in later development stages. Differences in the composition of exudates between genotypes may be related to their respective development strategies, with genotypes accumulating more biomass releasing relatively more compounds related to root establishment (growth and rhizosphere development, e.g. mucilage, fatty and organic acids) and slower developing genotypes relatively more metabolites related to maintenance and defense (e.g. phenolics). Our results shed light onto the early dynamics of maize root exudation and rhizosphere establishment, over a phenotypical spectrum of genotypes.

1. Introduction

Root exudates encompass all organic and inorganic carbon-based compounds that fine roots release into the soil (Hawes et al., 2000; Pinton et al., 2009). These compounds provide energy to soil microbes through readily available carbon and influence microbial assembly (Munoz-Ucros et al., 2021) and activity via chemical signaling (York et al., 2016). Root exudates directly impact the soil physical environment, regulating water resources, acting as signaling molecules, mediating microbial decomposition, stabilizing soil aggregates, and promoting carbon loss from protective associations with minerals (Jones et al., 2009; Haichar et al., 2014; Shabtai et al., 2024).

Plant root exudate composition exhibits a chemically diverse range of compounds: sugars, organic acids, amino acids, fatty acids, enzymes, vitamins, growth regulators, and secondary metabolites such as benzoxazinoids, phenolics, and terpenes (Azaizeh et al., 1995; Rasmann et al., 2005; Hu et al., 2018; Zhalnina et al., 2018). The composition of exudates may shift throughout plant development (Gransee and Wittenmayer, 2000). In slender white oat (*Avena barbata* Pott ex Link) and arabidopsis (*Arabidopsis thaliana* (L.) Heynh.), root released compounds

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changed from mostly sugars to mostly carboxylic acids, amino acids, and phenolics over the plants' life cycle (Gransee and Wittenmayer, 2000; Vargas et al., 2009; Chaparro et al., 2013; Zhalnina et al., 2018). These shifts can contribute to succession in the rhizosphere microbiome (Zhalnina et al., 2018). Sugars may play an important role in developing symbiotic plant-microbe interactions during early plant life stages (Vargas et al., 2009; Lemoine et al., 2013; Iannucci et al., 2017) and aromatic compounds such as phenolics can improve plant defense and function as signaling molecules against heterotrophs during vegetative plant stages (Lattanzio et al., 2006; Iannucci et al., 2017). Organic acids potentially further improve plant establishment and survival by enhancing nutrient solubilization and promoting changes in soil pH (Shi et al., 2011; Iannucci et al., 2017). Studies have further observed that root exudation tends to decrease from vegetative to reproductive plant stage, which is likely due to a greater allocation of resources towards reproductive organs (Gransee and Wittenmayer, 2000; Santangeli et al., 2024). However, dynamics in root exudation in the early development stages, from first emergence to vegetative growth, a crucial time of rhizosphere development, remain underexplored.

Maize is an important global crop for human consumption, animal feed, and the biofuels industry (Shiferaw et al., 2011). Over the course of its development, maize grows a complex root system with multiple root types (Hochholdinger, 2009). The root system is composed of embryogenetic primary and seminal roots, followed by lateral and shoot-borne roots ('crown- and brace roots') formed after germination (Feldman, 1994). Developmentally, maize aboveground structures can be coarsely categorized into vegetative ("V") and reproductive stages ("R") (Nielsen, 2019). Despite the major importance of carbon allocation dynamics in crops, where the primary focus is to maximize carbon assimilation and yield, little is known about the differences in the specific composition and quantity of maize root exudation (Gransee and Wittenmayer, 2000; Santangeli et al., 2024), especially during very early maize development, i.e. emergence from the soil, when plants start to interact with their soil environment. Among these developmental categories, root exudation patterns are particularly interesting in the early stages of plant and rhizosphere establishment in the soil (Cotton et al., 2019; Williams and de Vries, 2020; Yu et al., 2021).

In this study, we determined differences in the abundance and composition of root exudates across five common maize inbred lines ('B73', 'HP301', 'Mo17', 'NC350', and 'P39') over three distinct stages of early plant development (VE, V1–2, and V3–4). These genotypes were selected to represent a broad range of the maize phenotypic spectrum (Flint-Garcia et al., 2005; Yu et al., 2008; Buckler et al., 2009). We hypothesized that exudation rate per unit of root (e.g. root surface area) would be higher in the VE than in later development stages across all genotypes to accommodate the fast growth rate and establishment of the root system. We also hypothesized that genotypes that developed more biomass per development stage would have greater exudation rates compared to less developed genotypes (Infante et al., 2018). We further hypothesized that during the earliest stage of maize development (VE), primary compounds such as sugars, amino acids, and organic acids would be in higher abundance since these compounds can contribute to soil priming. Conversely, root exudates from later developmental stages (V1–2 and V3–4) would be composed of primarily secondary compounds, e.g. phenolics. Finally, we investigated differences in maize root exudate metabolomic profiles between genotypes and development stages to determine the role of plant development in shaping root exudate profiles. We hypothesized that the exudate metabolome of genotypes that developed more (root) biomass would be dominated by compounds related to root growth, while the metabolome of genotypes with less biomass would contain mostly compounds needed for maintenance and defense.

2. Material & methods

2.1. Plant material and experimental setup

The five maize genotypes (inbred lines 'B73', 'HP301', 'Mo17', 'NC350', and 'P39') used in the study are included among the 26 founders of the U.S. maize nested association mapping panel (Gage et al., 2020). B73 (Reid yellow dent; stiff stalk synthetic) and Mo17 (Lancaster; non-stiff stalk) are temperate-adapted lines that represent two important U.S. heterotic groups and are widely used in genomic studies (Schnable et al., 2009; Eichten et al., 2011; Sun et al., 2018). HP301 ('popcorn'), NC350 ('tropical'), and P39 ('sweet corn') are representatives of three other subpopulations and have available de novo genome assemblies (Hufford et al., 2021). Plants were cultivated in 0.5 L pots in sand (Commercial Grade Quikrete, Quikrete, Atlanta, GA, USA) in the Guterman greenhouse complex at Cornell University (42°26'52''N, 76°27'39''W) in August 2021 under ambient light conditions. Six biological replicates per maize line were planted to sample at each of three plant development stages: "VE" (emergence), "V1–2" (1–2 leaves fully developed), and "V3–4" (3–4 leaves fully developed), totaling 18 plants per line. Sampling times were determined based on observed plant developmental changes since the genotypes had different rates of development.

2.2. Exudate sampling

Exudates were sampled using an adapted cuvette collection method (Phillips et al., 2008). First, the maize root system was carefully removed from the sand and gently rinsed with Milli-Q (DD) water to remove attached sand particles. Either the whole root system (VE and V1–2 stages) or a root segment containing only primary, seminal, and lateral roots (i.e. no crown root, V3–4 stage) was selected and placed into an autoclaved 30 ml plastic syringe (AIR-TITE, Virginia Beach, VA, USA) containing glass beads (1 mm diameter; Propper, Long Island City, NY, USA). The crown- and remaining lateral roots not inserted into the syringes of the plants in the V3–4 stage were potted in 1.5 L pots in sand (Supplementary Figure 1). The top was sealed with a rubber stopper, the roots were flushed with DD water three times and syringes were left for 24 h wrapped in aluminum foil. Six syringes filled with glass beads but without roots were added as blank controls to normalize for potential contamination during syringe setup. After 24 h, roots were flushed with DD water twice and after another 24 h, root exudates were collected. We added 30 ml of DD water to the syringes and extracted the solution into sterile glass vials (VOA Sampling Vial, RESTEK, Bellefonte, PA, USA) using a membrane pump. Exudates were filtered through sterile nylon filters (0.22 μm, Celltreat, Pepperell, MA, USA) and immediately frozen at −20°C until analysis. All consumables were acid-washed in 1 M HCl before use.

2.3. Sample processing and root scanning

Following exudate collection, the sampled roots were cut, removed from the syringe, rinsed with tap water, placed in a plastic bag, and kept refrigerated. Three roots per genotype and development stage were scanned with a flatbed scanner (Epson Perfection V850 Pro, SEIKO Epson Corporation, Suwa, Nagano, Japan) at 1200 dpi and analyzed for root length, surface area, and number of tips with RhizoVision Explorer (Version 2.0.2, Table 1) (Seethepalli and York, 2020). All roots and green leaves were dried at 65°C for 24–36 h, and their dry masses recorded (Table 1). Specific root area ($cm^2\ g^{-1}$) was calculated for scanned roots and used to estimate root area of the roots that were not scanned. We corrected the root scans by counting tips of all scanned VE stage roots manually, calculating a genotype specific correction factor between software and manual counted tips that was applied to the software counted tips of V1–2 and V3–4 development stages.

Table 1
Biomass and morphological characteristics of leaves and roots of 5 genotypes of maize plants harvested at 3 development stages (VE, V1-2, V3-4).

Genotype	DevelopmentStage	Rootmass (mg)	Leafmass (mg)	Root length (cm)	Root surface area (cm^2)	Root tips (n)	Root diameter (mm)
B73	VE	17.7 (7.4)^{A}	32.9 (4.2)^{A}	31.1 (18)^{A}	6.7 (2.7)^{A}	28 (20)^{A}	0.92 (0.21)^{A}
	V1–2	95.9 (24.6)^{B}	123.5 (13.2)^{B}	259.6 (16.6)^{B}	45.7 (5.1)^{B}	268 (16)^{B}	0.55 (0.03)^{A}
	V3–4	1341.4 (239.3)^{C}	1749.7 (116.7)^{C}	-	-	-	-
	V3-4 (cuvette)	88.0 (43.1)^{B}	-	401.7 (199.6)^{B}	36.4 (12.3)^{B}	431 (206)^{B}	0.32 (0.06)^{B}
HP301	VE	9.2 (3.4)^{A}	10.4 (1.7)^{A}	10.1 (5.4)^{A}	2.6 (1.1)^{A}	13 (10)^{A}	0.96 (0.13)^{A}
	V1–2	39.8 (16.8)^{B}	46.2 (5.9)^{B}	76.8 (22.5)^{B}	13.1 (4.2)^{B}	83 (22)^{B}	0.53 (0.02)^{B}
	V3–4	486.2 (145.0)^{C}	684 (189.3)^{C}	-	-	-	-
	V3–4 (cuvette)	68.2 (14.4)^{B}	-	303.1 (56.9)^{B}	46.8 (3.4)^{C}	299 (55)^{B}	0.50 (0.06)^{B}
Mo17	VE	19.5 (2.7)^{A}	27.1 (5.3)^{A}	26.5 (8.5)^{A}	9.2 (2.7)^{A}	21 (7)^{A}	1.13 (0.05)^{A}
	V1–2	72.2 (11.7)^{B}	86.7 (11.6)^{B}	134.8 (23.2)^{B}	25.5 (2.7)^{B}	148 (35)^{AB}	0.61 (0.04)^{B}
	V3–4	540.7 (90.5)^{C}	1041.1 (101.1)^{C}	-	-	-	-
	V3–4 (cuvette)	114.4 (44.7)^{B}	-	347.9 (48.6)^{B}	45.7 (2.8)^{B}	328 (32)^{B}	0.43 (0.05)^{B}
NC350	VE	11.0 (1.8)^{A}	22.6 (3.1)^{A}	44 (10.2)^{A}	8.0 (0.9)^{A}	53 (17)^{A}	0.63 (0.10)^{A}
	V1–2	44.9 (22.0)^{AB}	59.4 (14.5)^{B}	81.4 (14.9)^{A}	11.5 (2)^{A}	75 (19)^{A}	0.44 (0.00)^{A}
	V3–4	2268.4 (388.4)^{C}	3980.3 (729.1)^{C}	-	-	-	-
	V3–4 (cuvette)	84.4 (41.1)^{B}	-	156.6 (22)^{A}	18.1 (1.6)^{A}	163 (13)^{A}	0.37 (0.02)^{A}
P39	VE	18.8 (4.0)^{A}	25.1 (3.4)^{A}	35.2 (10.6)^{A}	6.3 (1.6)^{A}	41 (11)^{A}	0.59 (0.06)^{AB}
	V1–2	53.5 (15.3)^{A}	58.3 (5.6)^{B}	60.5 (14)^{A}	12.7 (0.9)^{A}	56 (19)^{A}	0.72 (0.11)^{A}
	V3–4	656.2 (140.5)^{C}	1080.2 (270.6)^{C}	-	-	-	-
	V3–4 (cuvette)	171.8 (44.3)^{B}	-	552.9 (274.1)^{B}	67.9 (30.4)^{B}	686 (352)^{B}	0.40 (0.03)^{B}

*For the V3–4 stage, root characteristics are given for the whole plant and for the part that was investigated for root exudation in a cuvette. Values are displayed as means (± 1SE). Capital letters indicate significant ($p < 0.05$; determined using linear mixing models, see Methods) differences between development stages for each genotype.

2.4. TOC and metabolome analysis

A 9 ml aliquot of the exudate samples was analyzed for non-purgeable organic carbon (NPOC) with a TOC-analyzer (TOC-LCSH/CSN, Shimadzu Scientific Instruments, Columbia, MD, USA) coupled to an autosampler unit (ASI-L, Shimadzu Scientific Instruments) after adding 100 µl of 1 M HCl and purging with CO_2 free gas for 2 min prior to analysis. A five-point calibration curve (2, 5, 10, 20, 100 mg NPOC l^{-1}, Supplementary Figure 2) was performed before analysis and a measurement precision of ± 1.6 mg l^{-1} (1 SE) was determined using a lab standard (KHP-potassium hydrogen phthalate; 100 mg NPOC l^{-1}). Measured NPOC quantities were normalized to root surface area (Brunn et al., 2022), root biomass, root length and number of root tips. The remaining samples were lyophilized, each redissolved in 10 ml of 4°C methanol (Millipore-Sigma, Burlington, MA, USA) and subsequently vortexed for 2 min. Samples were spiked with 0.100 mM of 2-amino-3--bromo-5-methylbenzoic acid (internal standard) and vortexed again for 3 min. The exudates were analyzed with LC-MS/MS using a modified protocol from Zhou et al. (2019). Chromatography was performed on a Dionex UltiMate 3000 UPLC stack (Thermo-Fisher, Waltham, MA, USA) equipped with an Ultimate 3000 RS pump, autosampler, Column Compartment, and diode array detector (DAD). The LC-Stack was coupled to a Thermo Q-Exactive Hybrid Quadrupole Orbitrap mass spectrometer. Chromatographic separation was achieved using a Titan™ C18 UHPLC reverse phase column (2.1 mm x 100 mm (1.9 µm), Agilent Technologies, Santa Clara, CA, USA) with a flow rate of 0.5 ml min^{-1} and a column temperature of 40°C. The mobile phases consisted of a 0.1 % formic acid solution mixed with Optima (Thermo-Fisher) LC-MS grade water (eluent A) and a 0.1 % formic acid solution mixed with Optima LC-MS grade acetonitrile (eluent B). Details for machine setup and feature extraction can be found in supplementary methods and examples for typical total ion chromatograms in Supplementary Figure 3.

2.5. Statistical analysis

All statistical analyses were performed with R version 4.2.3 (R Development Core Team, 2023) in RStudio version 2023.06.0 + 421 (RStudio Team, 2023). Significant differences ($p < 0.05$) in leaf and root biomass, root morphology and exudation rates between development stages and genotypes were tested using a linear mixed effect model (R package nlme, version 3.1–162, Pinheiro et al., 2018) with biomass, root surface area, number of tips, root diameter, root length and NPOC/root surface area as dependent and development stage and genotype as fixed factors with replicate number as a random factor. Residuals of the model data were checked for normality with a Shapiro-Wilk test and the data were log-transformed to meet residual normal distribution. Model data were checked for variance homogeneity (Levene test; R package car, version 3.1–2, Fox and Weisberg, 2019). Significant differences between the genotypes within each development stage and between the development stages within each genotype were revealed with the emmeans post-hoc test (R package emmeans, version 1.8.5, Searle et al., 1980). Exudation rates were correlated with root surface area, root dry biomass, root length, and number of root tips by fitting a power function (nls from stats package, version 4.2.3, Bates and Watts, 1988). The R^2 and *p*-values of the correlation were determined after power transformation and linear regression of the data. Metabolites were normalized to the internal standard and obvious contaminants (i.e. non-plant products) were removed. Used data annotations included all compounds with an 80 % mz confidence score, including unannotated compounds assigned as unknown. Compound richness was calculated using the alpha function (Microbiome package, version 1.23.1) and calcDiv functions (Chemodiv package, version 0.3.0, type = "HillDiv" and q = 0, Petrén et al., 2023) and statistically compared using an ANOVA and followed by a posthoc Tukey HSD test (stats package, version 4.2.3). For beta diversity comparisons, the data were transformed with autoscaling (mdatools package, version 0.14.1, Kucheryavskiy, 2020) and compared with a PERMANOVA (Adonis2 test, vegan package, version 2.6–4, Anderson, 2001) and with a non-metric multidimensional scaling analysis (NMDS, vegan package, version 2.6–4) both with a Euclidean distance. To identify shifts in compound functional groups between development stages, compounds were grouped according to their functional class and compared with a Kruskal-Wallis test (stats package, version 4.2.3) and *p*-values were adjusted according to the Bonferroni method to account for multiple-testing. For visualization of functional groups, data were transformed into relative abundance. We performed an indicator species analysis (indicspecies package, version 1.7.14, De Cáceres and Legendre, 2009) to determine whether any functional groups were associated with particular genotypes. Values are displayed as means ± 1 standard error (1SE) from 6 biological replicates per genotype and development stage.

3. Results

3.1. Root development

Root and leaf masses, root lengths, surface areas, and number of root tips increased as the plants developed, while the average root diameter decreased with development stage. Root biomass in the V1–2 stage was four-fold greater than in the VE stage across genotypes, while root biomass in V3–4 was five-fold greater than in V1–2 in Mo17, 10–12-fold greater than in V1–2 in B73, HP301 and P39, and 49-fold greater than in V1–2 in NC350 (Table 1). Root development from V1–2 to V3–4 was therefore significantly ($p < 0.001$) higher in NC350 than in all other genotypes. In the V3–4 stage, root biomass was significantly higher in the NC350 genotype than in the Mo17 ($p < 0.05$) and the HP301 genotypes ($p < 0.01$). Total root mass was lower than total leaf mass over all genotypes across all development stages ($p < 0.001$ for each development stage over all genotypes; Table 1). In the roots sampled for exudates in the cuvette, root length, surface area and number of tips were significantly lower in the VE stage than in the two later stages in B73, HP301 and Mo17, while no difference was found between development stages in NC350. In P39, the V3–4 stage had a significantly higher root length, surface area, and number of tips than the first two stages that were not different from each other (Table 1). Root diameter was significantly higher in the VE than in the V3–4 stage in all genotypes but NC350 and P39, which showed the same trend (Table 1). Across all development stages, root diameter in NC350 was significantly lower than in Mo17 ($p < 0.05$) with the other genotypes in between.

3.2. Root carbon exudation rates

Exudation rates were highest for maize roots sampled at the VE development stage and decreased by approximately one order of magnitude per development stage in the V1–2 and the V3–4 stages for each of the five studied genotypes (Fig. 1). During the VE stage, the mean root exudation rate across the five genotypes ranged from 0.162 ± 0.059 mg cm^{-2} d^{-1} (B73) to 0.678 ± 0.309 mg cm^{-2} d^{-1} (HP301). The variation in mean exudation rates among genotypes decreased in the V1–2 stage, (0.018 ± 0.008 mg cm^{-2} d^{-1} (NC350) to 0.099 ± 0.053 mg cm^{-2} d^{-1} (HP301)), and further decreased in the V3–4 stage, when mean exudation rate across all genotypes was 0.004 ± 0.001 mg cm^{-2} d^{-1} (Fig. 1). There were no differences in exudation rates among genotypes within any of the development stages.

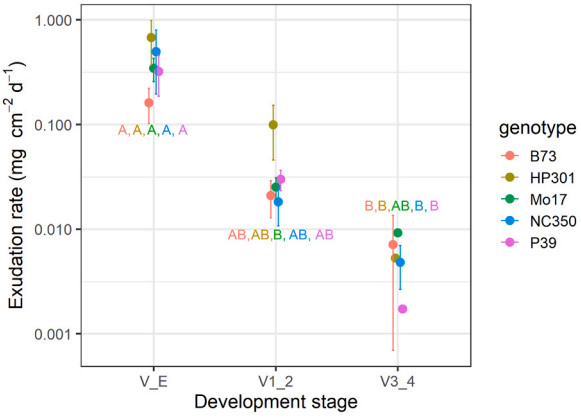

Fig. 1. Exudation rates (mg C per cm^2 root surface area and day; mean ± 1SE) for the different genotypes across three studied growth stages. Different genotypes are shown by different colors and letters indicate significant differences between development stages for each genotype ($p < 0.05$). Exudation rates decrease by approx. one order of magnitude per development stage. Within development stages there were not differences between genotypes. Note the y-axis is on log-scale.

Variation in root exudation rates was the highest in the VE development stage (Fig. 1). We found that exuded carbon during the VE stage correlated negatively with measured root characteristics (Fig. 2), indicating that across all maize genotypes, plants with smaller root area, lower root biomass, shorter root length, and fewer root tips had higher exudation rates (Fig. 2).

3.3. Compound richness across development stages

For both the MS/MS positive and negative ionization modes, we saw a trend of decreasing compound richness with increasing development stages (Fig. 3). We observed a significant interaction between genotype and development stage for the positive mode ($F = 3$, $p = 0.02$) and a significant main effect of development stage for the negative mode ($F = 11$, $p \leq 0.001$). Differences between development stages for the negative mode were primarily driven by lower compound richness in the V3–4 stage (VE vs V1–2: $q > 0.05$; VE vs V3–4: $q \leq 0.001$; V1–2 vs V3–4: $q \leq 0.001$; for genotype specific differences in compound richness with development stage, see Supplementary Figure 5).

3.4. Root exudation metabolome profiles

For both the positive and negative modes, we observed a significant interaction between development stage and genotype for the root exudation profiles (Positive: $F_{8,46} = 2$, $R^2 = 0.22$, $p \leq 0.001$; Negative: $F_{8,47} = 1$, $R^2 = 0.15$, $p = 0.02$; Fig. 4; Supplementary Figure 6).

3.5. Functional groups of exuded metabolites across development stages

We combined compounds into functional groups to identify metabolic shifts according to the plant development stages (Fig. 5A). We identified that 13 and eight functional groups significantly differed between development stages for the positive and negative modes, respectively (Supplementary Figure 7). Of these functional groups, six and five functional groups showed increases, or decreases, corresponding with plant development over time in the positive and negative modes, respectively (Fig. 5A). Sugar abundance significantly decreased with increasing development stage (both modes $q \leq 0.001$). We saw the same pattern for fatty amides ($q = 0.002$), while phenolics ($q \leq 0.001$; positive mode, unadjusted $p = 0.01$, $q = 0.2$; negative mode), benzoxazinoids ($q = 0.01$), polysaccharides mucilage ($q = 0.003$), quinolines ($q \leq 0.001$), fatty acids ($q = 0.009$), indoles ($q = 0.02$), and organic acids ($q = 0.007$) all increased with plant development and had the greatest abundance in the V3–4 stage (Fig. 5A). Within each of the functional groups we determined the compound that most significantly affected the decreasing or increasing trend with development stage (Fig. 5B). For the sugars, glucose (positive mode) and fructose (negative mode) decreased the most with increasing development stage, while docosanamide prominently decreased with development stage in the fatty amide group. Compounds that drove the increase in relative abundance with plant development included 8-hydroxyquinoline (quinolines), 4-coumaric acid (phenolics; positive mode), hydroxybenzaldehyde (phenolics; negative mode), HBOA (benzoxazinoid), and citric acid (organic acid) (Fig. 5B).

3.6. Functional groups of exuded metabolites across genotypes

We then compared the relative abundance of the functional groups with a clear up- or downregulation with development stage (Fig. 5A) between genotypes and development stage (Fig. 6). The functional groups accounted for 15–45 % (positive mode) or 60–80 % (negative mode) of all functional groups in the samples within the MS/MS compound dataset. Sugars were dominant in all genotypes in the VE stage, especially in B73 and NC350 (positive mode), while their relative abundance decreased sharply in the later development stages (notably in B73, NC350 and P39 in the negative mode). In the positive mode,

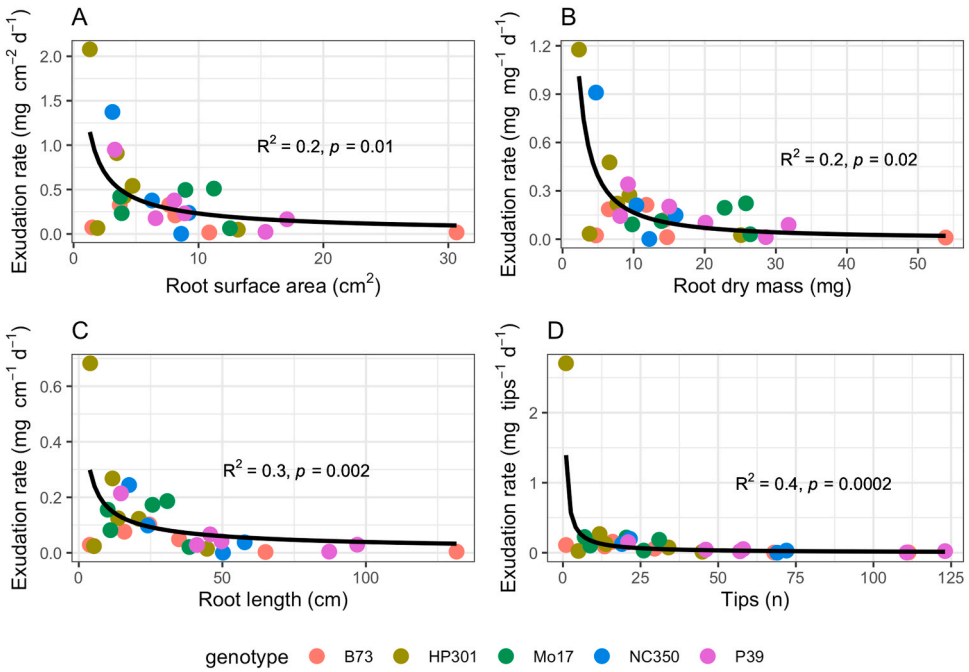

Fig. 2. Correlation between exudation rates and root surface area (A), root dry biomass (B), root length (C) and number of root tips (D) in the VE plant development stage, respectively. The coefficient of determination and *p*-value for the regressions were estimated from power transformations and linear regressions of the data. Different genotypes are shown by different colors. Negative correlations between exudation rates and root surface area, mass, length and number of tips can still be detected if statistical outliers are removed from the regression (Supplementary Figure 4). There was, however, no biological reason to exclude the high values from our analysis.

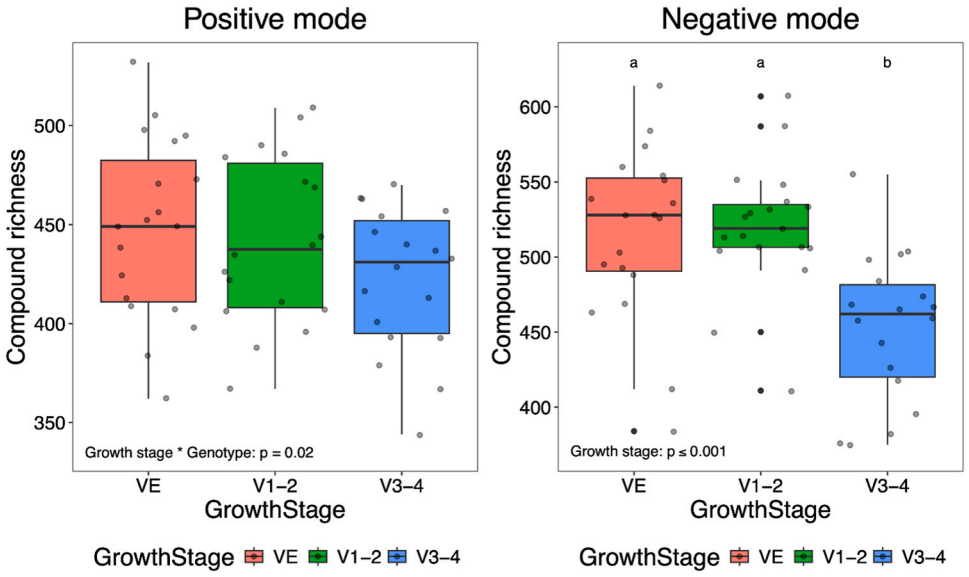

Fig. 3. Compound richness for MS/MS positive and negative ionization modes across all 5 studied genotypes. Colors indicate the three observed development stages.

polysaccharide mucilage increased strongly in the V1–2 stage in B73 and NC350 and remained high in the V3–4 stage, where it was also relatively increased in P39. In the negative mode, the abundance of fatty acids increased in later development stages, notably in NC350 and P39, as well as the abundance of organic acids, prominently in NC350, P39 and HP301 in the V3–4 stage. The relative abundance of organic acids was higher in Mo17 than in the other genotypes in the VE stage and did not increase further with plant development (Fig. 6), while the abundance of phenolics (positive mode) and sugars (negative mode) was higher in Mo17 in the V3–4 stage than in the other genotypes (Fig. 6).

Finally, we performed an indicator species analysis to determine whether any functional groups were associated with particular genotypes. We observed that sesquiterpenes, modified amino acids, and sugars were indicative of B73 ($p = 0.04$), HP301 ($p = 0.001$), and NC350 ($p = 0.03$), respectively. Polysaccharide mucilage compounds were associated with B73 and NC350 ($p = 0.04$).

4. Discussion

We identified differences in both the abundance and composition of maize root exudation across five genotypes through three early development stages. We found that the exudation rate per unit of root surface

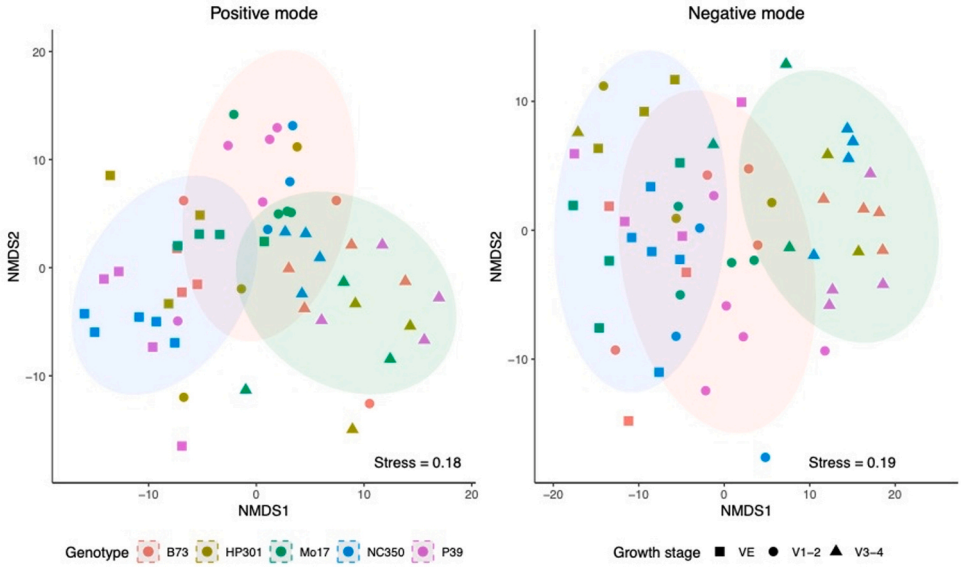

Fig. 4. NMDS ordination of maize exudates for both MS/MS ionization modes. Axes 1 and 2 are shown (positive mode is a 3D ordination (for axis 3 see Supplementary Figure 6) and negative mode is 2D ordination). Different genotypes are shown by different colored points and different shapes represent different maize growth stages. 90 % ellipses are shown for the different maize growth stages, with blue, red, and green being VE, V1–2, and V3–4, respectively.

area was greatest in the VE development stage regardless of genotype and decreased logarithmically as the plants developed (Fig. 1), confirming our hypothesis that maize had higher root exudation rates during early development stages. Even within the VE development stage, plants with a smaller root system exuded more carbon (per root area, length, weight, or number of tips) than plants with larger root systems (Fig. 2). We expected that the maize genotypes that developed more biomass per development stage (especially NC350 in the V3–4 stage; Table 1) would exude more carbon than genotypes with smaller root systems or leaf area. However, we did not find significant differences between genotypes in exudation rates in any of the development stages (Fig. 1). Our results confirmed the hypothesis that the composition of the exudates in the VE development stage would be dominated by sugars (primary metabolites) while in later stages, other and especially secondary metabolites became more abundant. While there were no differences between genotypes in the total amount of exuded carbon, we detected that NC350, the genotype with the largest root system in the V3–4 stage, had a higher relative contribution of mucilage, fatty and organic acids in the V3–4 stage, whereas in Mo17, the genotype with the smallest increase in root area, phenolic compounds played a more dominant role in the V3–4 development stage.

4.1. Root carbon exudation rates

Roots that are actively growing also exude more carbon (Jones et al., 2009; Gao et al., 2024) and plants have higher exudation rates per root mass during the vegetative state than at maturity (Gransee and Wittenmayer, 2000; Santangeli et al., 2024). Our results add to these findings, with the highest exudation rates found in the emergence stage (Fig. 1). In fact, especially during emergence, plants with smaller root systems allocated more carbon per root area to their exudates than plants with more developed root systems (Fig. 2). We found the highest amounts of exudation in the VE and V1–2 stages in the HP301 genotype (albeit not significant, Fig. 1), which also had the lowest above- and belowground biomass at each development stage (Table 1), supporting the idea that the size of the root system has a significant influence on the individual root exudation rate. The negative relationship between root exudation and root system size in the VE stage (Fig. 2) indicates that carbon exudation activity per single 'unit of root' (e.g. a root tip) decreases rapidly with root system development in emerging plants. We did not find a relationship between greater exudation and smaller root systems in the later development stages, supporting the finding that roots are most active in releasing exudates during very early development. With later development stages, exudation rates per root area would likely even further decrease (Santangeli et al., 2024). We normalized our exudation rates per root surface area. When scaled to the whole root system, it has been suggested that with larger root systems in the later development stages, overall root exudation will also be higher than in younger plants, even if exudation per root surface area is greater in earlier development stages. Therefore, the overall influence of the plant on its surrounding soil volume should increase with plant development (Prikryl and Vancura, 1980; Aulakh et al., 2001; Santangeli et al., 2024). However, in our experiment focusing on very early development stages, we found that carbon released per plant per day in the VE stage (1.9 ± 0.3 mg per plant d^{-1}; not shown) was roughly double than in the later V1–2 stage (0.9 ± 0.1 mg per plant d^{-1}; not shown), despite a larger root system in the V1–2 stage (Table 1). This highlights the importance of root exudation as a crucial establishment mechanism (York et al., 2016). Only with the significant increase in root mass in the V3–4 stage (Table 1), total exudation per plant was higher than in the VE stage (8.9 ± 5.9 mg per plant d^{-1}; not shown). In the V3–4 stage, some roots were touching the borders of the containers, potentially limiting root growth. Therefore, total exudation may be even higher in maize plants in the V3–4 stage not restricted by pot size. In fact, exudation rates in field grown maize were about three times (28.8 mg per plant d^{-1}) higher at a similar development stage (Santangeli et al., 2024). However, when compared to maize plants grown hydroponically (Groleau-Renaud et al., 1998), our estimated values exceed previously reported exudation rates of similar aged maize plants (0.2 – 1.2 mg per plant d^{-1} at ages from 4 – 16 days), demonstrating the difficulty of comparing absolute values of exudation rates between studies (Brunn et al., 2022), despite similar root and shoot biomass. We conclude that across genotypes a) the stage of plant development was the greatest driver of exudation and b) during early development, the size of the root system significantly impacted root exudation where plants with similar sized root systems also exuded the same amount of carbon into the soil (Fig. 1, Fig. 2).

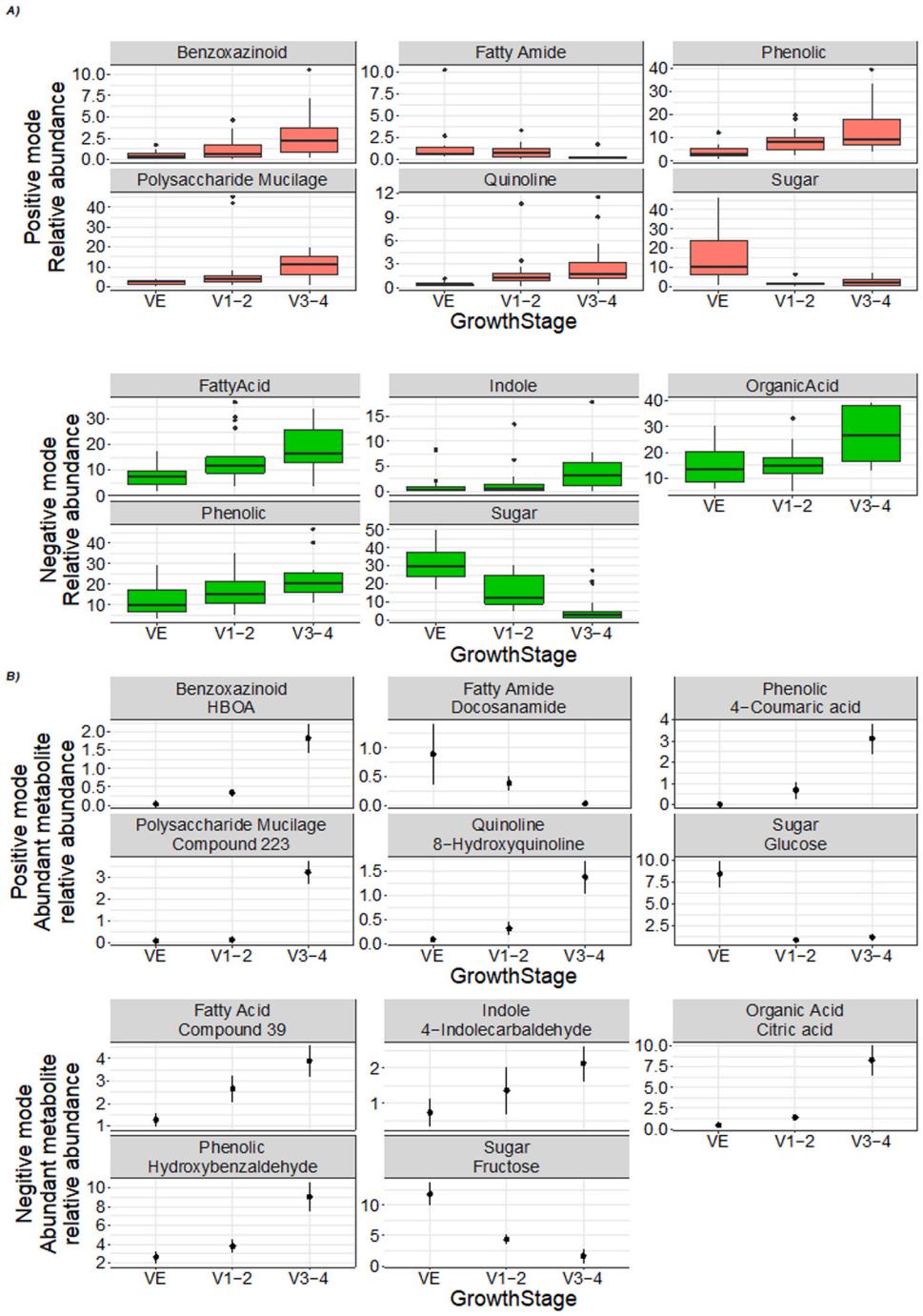

Fig. 5. (A) Relative abundance of functional groups with significant in- or decreases between growth stages. Statistics were performed on auto scaled data. Note the different scales on the y-axes. Negative mode phenolics were not significantly different after adjustment and are shown because of a similar trend to that observed in the positive mode. (B) Specific compounds driving the in- or decrease for each of the functional groups from (A). Mass spectroscopy data for all functional groups with significant differences between growth stages can be found in the supplements (Supplementary Figure 7).

4.2. Root exuded metabolites across development stages

We found that the metabolite richness decreased with plant development, indicating that a more diverse cocktail of metabolites was exuded by the plants in early development stages. During early seedling development, plants face an array of challenges that would benefit from a diverse chemical composition, including taking in nutrients and water while balancing to attract facilitative microbes and defend against antagonistic microorganisms in the soil environment (Zhalnina et al., 2018; Santangeli et al., 2024). Sugars dominated the root exudation profile of VE stage plants with up to 30 % (Fig. 6) of all exuded compounds captured with our method, indicating that easily available carbon was preferentially released, likely an attractive food source for microbial communities in the rhizosphere of roots. Among the sugars, glucose, and fructose were the strongest predictors of an early development stage (Fig. 5B), both of which are simple but energy rich carbohydrates. Less pronounced but also relatively higher in early development stages were releases of fatty amides such as docosanamide (Fig. 5A & B) which are known to stimulate soil nitrogen decomposition by attracting rhizosphere bacteria and increasing their activity (Sun et al., 2016), supporting our hypothesis that soil priming in order to establish a rhizosphere environment was the primary function of early

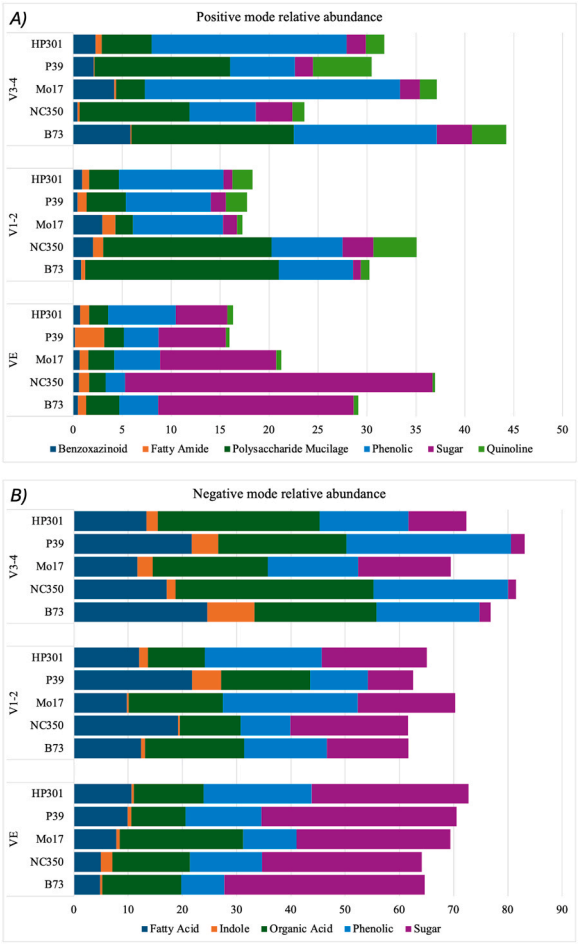

Fig. 6. Relative abundance of functional groups with significant in- or decreases between growth stages, split into the five studied maize genotypes.

root exudation. In contrast, during later development, metabolite richness of exuded compounds was smaller and dominated by more complex metabolites. As the maize plants aged, phenolic compounds became relatively more pronounced in the exudate profiles (up to 30 %, Fig. 6), in particular, coumaric acid and hydroxybenzaldehyde (Fig. 5A & B) which have been linked to plant defense, by their allelopathic or fungicidal function (Lattanzio et al., 2006; Lanoue et al., 2010; Weston et al., 2013; Mehmood et al., 2019). Similar results were reported on arabidopsis, where initially sugars dominated the exudation profile (>50 %) and later decreased (~10 %) while phenolics increased from about 20–30 % (Chaparro et al., 2013). In field grown maize plants, phenolics were relatively less abundant at a similar development stage (V3–4: ca. 10 %; Santangeli et al., 2024). Other compound classes often related to plant defense were also relatively more abundant in the later development stages, including benzoxazinoids (HBOA; allelopathic) (Lattanzio et al., 2006; Rasmann and Turlings, 2016) or quinolines (8-Hydroxyquinoline; phytotoxic) (Kaur et al., 2009; Vives-Peris et al., 2020). Additional compounds more important in later development stages were related to root growth, e.g. indoles (Vives-Peris et al., 2020) and mucilage (Jones et al., 2009). Fatty acids, involved in plant signaling processes (Rasmann and Turlings, 2016; Wang et al., 2021), were also increasingly abundant in later development stages (Fig. 5A). Organic acids that are thought of to be related to nutrient uptake (Carvalhais et al., 2011; Vives-Peris et al., 2020) were also relatively more dominant in later development stages. The trend of exuding relatively more compounds related to defense would likely continue even in later development stages and during flowering (Zhalnina et al., 2018; Santangeli et al., 2024).

4.3. Root exuded metabolites across genotypes

Comparing genotypes, we saw that NC350, which increased both, root (~ 49x) and leaf (~ 70x) biomass the most from the V1–2 to the V3–4 stage (Table 1), had the highest relative abundance of sugars in the VE stage (positive mode), and comparatively high abundance of fatty and organic acids and mucilage in the later development stages (Fig. 6). Exudation of mucilage may be related to facilitate root growth into the soil and maintain (hydraulic) connectivity to the soil particles (Iijima et al., 2004; Zeppenfeld et al., 2017; Nazari, 2021), while fatty and organic acids help to coordinate root-microbe signaling and nutrient uptake. Our indicator species analysis also revealed that sugars and mucilage were among the compounds that were indicative for NC350. It appears plausible that this genotype, with faster root system development as the plant developed compared to other genotypes, would have more compounds that facilitate root growth and rhizosphere development. In turn, Mo17, that had the lowest development of roots between V1–2 and V3–4 (~ 5x), had relatively less exudation of fatty and organic acids as well as mucilage, however higher abundance of phenolic compounds in the V3–4 development stage (Fig. 6), which may indicate that for genotypes with slower developing root systems, maintenance of existing roots via exudation of defensive compounds is relatively more important. Additionally, the average root diameter was higher in Mo17 than in NC350, especially during early development (Table 1). It has been postulated that plants that develop bigger root diameters may focus on building long living roots but also depend more on mycorrhizal or microbial symbionts for resource acquisition while plants with thinner roots rely more on their own root system for uptake of water and nutrients (Bergmann et al., 2020). Our findings add to this theory, suggesting that also the exuded compounds support either greater root expansion (NC350) or maintenance of less extending root systems (Mo17). Therefore, root exudates appear to be a viable parameter for genotypic profiling of different maize lines with the potential to determine spatiotemporal patterns and strategies of growth, defense or reaction to environmental stress.

5. Conclusion

Overall, we found a significant change of root exudation in very young and developing plants that aligns with the plant's ability to develop and maintain an optimum environment around their root system. Our results suggest that root development rate influences the composition of root exuded metabolites. The genotype that developed more root biomass per development stage and had relatively thin average root diameters exuded relatively more compounds that support root establishment (growth and rhizosphere development), like mucilage or organic and fatty acids, and genotypes with less root development and thicker average root diameters exuded relatively more protective compounds, especially phenolics. This concept should be tested on different species on more contrasting sides of the root economics space (Bergmann et al., 2020) to determine how closely root exudation is linked to root structure and development. Additionally, exudation patterns can be influenced by the soil physical and chemical environment (Santangeli et al., 2024), which still makes it hard to compare root exudation between different studies. Future experiments should consider a wider range of soil variables to further understand root-soil feedback.

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Author contributions

BDH and TLB designed the research; OP, BDH and JBS performed the research; WLK, JBS and BDH analyzed the data; BDH and OP wrote the first draft of the paper, and all authors read, commented and approved the manuscript in final form prior to submission.

CRediT authorship contribution statement

Bauerle Taryn L.: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Scharfetter Jacob B.:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation. **Hafner Benjamin D.:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **King William L.:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis. **Pietz Olivia:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2025.112439.

Data availability

Data will be made available on request.

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