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Insights into Fusarium Basal Rot Resistance through Saponin Profiling of Basal Plate Tissue of Short-day Onion Cultivars

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Abstract. **Fusarium basal rot (FBR), caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *cepae* (FOC), can be a substantial threat to onion crops by affecting bulb yield adversely. With an objective to increase FBR resistance in onions, investigating the role of plant metabolites in defense mechanisms has the potential to make breeding efforts more accurate. Saponins, a type of secondary metabolite, have demonstrated in vitro antifungal properties in both onion and shallot against *Fusarium* pathogens. However, additional research is needed through in vivo studies to investigate the role of saponins in FBR resistance. Our study sought to identify saponins associated with FBR resistance and to quantify fluctuations in saponin levels in the onion basal plate tissue before and after inoculation with FOC. Basal plate tissue was sampled at 0, 5, 10, and 15 days postinoculation (dpi) from mature onion bulbs of three open-pollinated, short-day onion cultivar populations and two check cultivars (FBR partially resistant and FBR susceptible). Extracted saponins from the sampled basal plate tissues were analyzed using ultra-high-performance liquid chromatography coupled to an Orbitrap fusion mass spectrometer, which led to the identification of 19 putative saponins. The mean relative abundance of *Allium cepa* saponin 14 (ACS-14) differed between the two check cultivars and across cultivar populations over time after inoculation. An ~10-fold increase in the levels of ACS-14 was detected in the partially resistant check compared with the susceptible check at 5 dpi. ACS-14 showed 8- to 24-fold increased levels in basal plates of the advanced selected populations at 5 dpi compared with 0 dpi (uninoculated bulbs). Additional research can be conducted to confirm the involvement of this and other putative saponins detected in FBR resistance through in vivo and in vitro studies, and to explore the biosynthetic pathway of these saponins, if demonstrated conclusively to be involved in resistance.**

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The common bulb onion (*Allium cepa* L.) is one of the most commonly grown vegetables in the world. Various diseases present substantial challenges to onion cultivation by reducing bulb yield (Khar et al. 2022; Sharma et al. 2024a). Fusarium basal rot (FBR) is a devastating disease caused by the soil-borne fungus *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *cepae* (H.N. Hans.) W.C. Snyder & H. N. Hans (FOC). FOC can result in substantial yield losses by rotting the basal stem plates of onion bulbs (Cramer 2000; Sharma et al. 2024b). Although exact losses to FBR are not well documented, observations in a shallot (*A. cepa* L. var. Aggregatum group) study revealed a considerable economic loss of ~45% in bulb yield, in addition to postharvest storage loss (12%–30%) (Sintayehu et al. 2011).

Different approaches have been used to control FBR, such as crop rotation, soil solarization, applications of fungicides or fumigants, and biocontrol agents (Cramer 2000; Cramer et al. 2021; Sharma and Cramer 2023). These methods are not equally effective, and each has limitations in controlling FBR, as factors such as environmental conditions, disease pressure, efficacy of the treatment, and timing and/or method of application can influence the efficacy significantly of these management practices. The development of FBR-resistant cultivars is potentially the most sustainable approach for reducing the impact of this disease; however, no resistant cultivar is available yet for short-day onions (Sharma and Cramer 2023). A reliable phenotypic screening method is a prerequisite for developing FBR-resistant cultivars. Onion researchers have used different traditional breeding approaches to increase the levels of FBR resistance in germplasm, such as a seedling screening (Caligiore-Gei et al. 2020; Mandal et al. 2020; Taylor et al. 2013) and a mature bulb screening assay (Mandal and Cramer 2020, 2021; Sharma and Cramer 2023). The availability of an effective biochemical screen for aspects of biochemical pathways involved in resistance responses to FOC would potentially improve the accuracy of breeding efforts to select FBR-resistant onion germplasm. In the "-omics" era, the field of metabolomics could play an important role in exploring plant natural defense systems to biotic and abiotic stresses. Metabolomics methods can potentially be integrated to speed breeding and hasten crop improvement (Razzaq et al. 2022). In addition, delving into biosynthetic pathways could potentially be used to identify the genes responsible for disease resistance.

Plants synthesize diverse metabolites with different structures and concentrations (Arbona et al. 2013; Hong et al. 2016). The metabolites are classified into two major types: primary and secondary metabolites. Both help plants survive; however, an important function of primary metabolites is to support plant growth and development, whereas secondary metabolites play an essential role in plant protection from different types of stresses (Abdelrahman et al. 2019; Fujii et al. 2015). Saponins are a group of secondary metabolites with a distinct chemical structure and unique biological activities (Abdelrahman et al. 2014; Mostafa et al. 2013). Plants produce saponins as a normal activity of growth and development, contributing to resistance mechanisms (Abdelrahman et al. 2017). Several saponins have been recognized as effective antifungal compounds against various plant pathogenic fungi (Osbourn 1996). Saponins are also known for other beneficial properties such as insecticidal and molluscicidal effects, showcasing their versatility as compounds with multiple potential applications (Heng et al. 2006; Kitagawa 2002; Sparg et al. 2004). The structure of saponins is comprised of two components: aglycone (sapogenin) and a glycone (sugar) moieties. Saponins can be either steroidal or have triterpenoid attachment to sugar (oligosaccharide) moieties (Abdelrahman et al. 2017;

Abe et al. 1993; Lanzotti et al. 2012). The glycone moieties attached to the aglycone core consist of chains of various sugars such as glucose, xylose, rhamnose, galactose, and arabinose (Abdelrahman et al. 2017).

Allium species are known to produce steroidal saponins with antifungal properties (Adão et al. 2011). This attribute of saponins largely depends on their core aglycone and the number, structure, and types of sugar units in the glycone moiety (Abdelrahman et al. 2017; Yang et al. 2006). The antifungal mode of action of saponins is primarily a result of their ability to form complexes with fungal sterols, which increase fungal cell membrane permeability, and leads to leakage of cell contents (Morrissey and Osbourn 1999). Steroidal saponins are further classified into three categories based on their chemical structure: 1) furostanols, 2) spirostanols, and 3) open-chain cholestane saponins (Challinor and De Voss 2013). Different plant parts have been studied to determine which part produces the highest levels of saponins. In *Allium* species, the highest accumulation has been observed in the root and basal stem plate tissues compared with leaf and bulb tissues (Abdelrahman et al. 2014, 2017; Mostafa et al. 2013). These saponins could be responsible for protecting onion bulbs from soil-borne pathogens.

More than 200 saponins and sapogenins are known in *Allium* species (Wang et al. 2023). Several studies have been conducted to explore the antifungal activities of isolated *Allium* saponins in vitro. Saponins have showed varying levels of antifungal activities against several fungi, including *F. oxysporum* (Barile et al. 2007; Lanzotti et al. 2012). One specific saponin, aginoside, isolated from *Allium nigrum* L., demonstrated partial inhibition of FOC (Mostafa et al. 2013). Antifungal properties of the saponin alliospiroside A were presented against several fungi pathogenic to different crops, including an FOC isolate from Welsh onion (*Allium fistulosum* L.) (Teshima et al. 2013). A more recent investigation also identified alliospiroside A as having in vitro antifungal activity against FOC in Welsh onion (Abdelrahman et al. 2017).

Saponins produced at different time points after infection of the onion plant by FOC could be involved in inhibiting FBR development in onion. However, no research has demonstrated how the levels of saponin production change over time after infection by a virulent FOC isolate, particularly using in vivo studies. We hypothesize that the production of different saponins in the basal plate tissue of onion bulbs varies depending on cultivar susceptibility to FOC. In addition, we hypothesize that saponins involved in resistance are more abundant in advanced breeding populations selected for resistance to FBR compared with the initial population of each cultivar. The objectives of this study were 1) to investigate fluctuations in the mean relative abundance of putative saponins detected in the onion basal plate tissue of FBR-partially resistant and FBR-susceptible (check) cultivars over time following FOC inoculation, 2) to assess differences in the mean relative abundance of saponins among cultivar populations during the first 15 d postinoculation (dpi) relative to the two FBR check cultivars, and 3) to determine whether the more advanced, selected FBR-resistant populations of each cultivar show a greater increase in production of some saponins relative to the initial/first population of each cultivar.

Materials and Methods

Location of study and plant material. The fieldwork, inoculation, and rating of the bulbs for our study were conducted at the Fabian Garcia Research Center, New Mexico State University, Las Cruces, NM, USA (lat. 32.2799°N, long. 106.7725°W), and the saponin extraction and identification were conducted in the Crop Quality and the Chemical Analysis and Instrumentation laboratories in Las Cruces, NM, USA, for the 2022–23 growing season. Populations of three autumn-sown, open-pollinated New Mexican short-day onion cultivars selected for resistance to FBR, and belonging to three maturity groups, were planted in the field in a randomized complete block design with four replications. The cultivars were NuMex Camino (early maturing, mid-May), NuMex Mesa (intermediate maturing, late May), and NuMex Luna; (late maturing, late June) (Corgan 1995, 1996; Cramer and Corgan 2003a). These cultivars have different origins and genetic makeup. For each cultivar, bulbs of the first/initial [E1 (NuMex Camino), I1 (NuMex Mesa), and L1 (NuMex Luna)] and the advanced selected (E5 and E6 for NuMex Camino, I5 and I6 for NuMex Mesa, and L5 and L6 for NuMex Luna) populations were included in the study. The advanced selected populations were generated by advancing the selected FBR-resistant bulbs to the next generation in every selection cycle using inoculation of mature bulbs as described by Sharma and Cramer (2023). Bulbs of the two check cultivars, an FBR-partially resistant cultivar, Serrana (Black et al. 2022) (Monsanto Vegetable Seeds, Woodland, CA, USA), and an FBR-susceptible cultivar, NuMex Crimson (Cramer and Corgan 2003b), were also included to evaluate the reliability of the inoculation method and to compare the levels of different saponins detected across the cultivar populations and check cultivars.

Sampling and disease scoring. After harvesting, the mature onion bulbs were stored for 2 to 3 weeks to allow any latent infection to show up. Only healthy bulbs were selected for inoculation using a local, highly virulent FOC isolate, CSC 515 (Saxena and Cramer 2009), to differentiate between bulbs of FBR-resistant and FBR-susceptible cultivars (Mandal and Cramer 2020, 2021; Sharma and Cramer 2023). The day before inoculation of the bulbs, a spore suspension was prepared by adding autoclaved, distilled water to plates of acidified potato dextrose agar (PDA) colonized by CSC 515, scraping the 2-week-old actively growing mycelia with a spatula and filtering the suspension through cheese cloth to collect spores. A measured amount of the spore suspension was then added to molten PDA after autoclaving when the medium had cooled to $<40\,^{\circ}C$ (to avoid killing the FOC spores), but had not yet solidified, to achieve a final concentration of 3.0×10^4 spores/mL (Sharma and Cramer 2023). Inoculum plates were then prepared by pouring the spore-suspended PDA into petri plates (90 × 15 mm). For inoculation, a 1-cm-diameter plug (~1200 spores) from a spore-suspended PDA plate was placed onto a transversely cut basal plate of an onion bulb. Subsequently, the inoculated bulbs from the population were placed in a plastic crate ($58 \times 37 \times 22$ cm^3) (Bekuplast GmbH, Ringe, Germany) with the basal plate facing up. The plastic crates were covered with a polyethylene bag ($83.8 \times 104 \times 0.00254$ cm^3) to maintain high humidity (~85%), and the crates were removed from the bags after 24 h of incubation at room temperature during summer. For the advanced selected populations of each cultivar, all available healthy bulbs were inoculated for advancing the resistant bulbs to the next generation, and 20 bulbs per replication were selected randomly for disease severity assessment. For the initial populations, only 40 bulbs per replication were inoculated to observe the progress of selection in the advanced populations compared with the initial population. In addition, at each time point, three bulbs were selected randomly from each cultivar or population for saponin study.

Samples for saponin extraction were collected by cutting a thin slice (~0.25–0.30 mm) of the basal plate where the pathogen was inoculated. Basal plate tissue samples were collected for saponin extraction at four different dpi (i.e., 0 dpi, wounded uninoculated control; and 5, 10, and 15 dpi). Three basal plate tissue samples for each treatment combination (9 cultivar populations and 2 check cultivars × 4 sampling points × 3 replicates) were frozen at $-20\,^{\circ}C$ until they were freeze-dried. Average disease severity was calculated by rating 20 random bulbs from each replication of each cultivar population by recutting the basal plates of onion bulbs transversely and using a severity rating scale of 1 point (no symptoms of FBR) to 9 points (70% or more of the cut surface area of the basal plate with symptoms of FBR) at 20 dpi when the disease was fully established in the susceptible check (Gutierrez and Cramer 2005).

Extraction of saponins. Freeze-dried basal plate tissue for each bulb was ground into a fine powder using a sterilized pestle and mortar. After filling one third of the volume of a 2 mL extraction tube with 2-mm-diameter zirconia beads (BioSpec Products Inc., Bartlesville, OK, USA), all the tubes were subjected to an autoclave cycle for sterilization, including a dry cycle. The tubes were dried thoroughly overnight, then 10 mg of ground basal plate tissue from each sample was added to a tube. The extraction buffer with 80% methanol (80 parts methanol:20 parts distilled water) was prepared by adding 18 $\mu g \cdot mL^{-1}$ umbelliferone (7-hydroxycoumarin) (Sigma-Aldrich Corp., St. Louis, MO, USA) as an internal standard. Then, 1 mL of the extraction buffer solution was transferred to each tube containing basal plate tissue powder, and

the tubes were prepared for bead-beating extraction using a Precellys® 24 bead-beater (Bertin Corp., Rockville, MD, USA). The bead-beating cycle was set to 5500 rpm for 2 cycles of 15 s each, with a 120 s pause between the two cycles. After bead-beating, the tubes were immediately placed in ice water to cool. Following this, the tubes were subjected to centrifugation at 4000 g_n for 30 min at 4 °C. The resulting supernatant was filtered using a 0.2 µm syringe nylon filter, and samples were kept at –20 °C until they could be processed for chromatographic separation.

Identification of saponins. Separation and identification of putative saponins were performed using ultra-high-performance liquid chromatography (M class Acquity; Waters, Milford, MA, USA) coupled to an Orbitrap Fusion tandem mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic separation was achieved using a NanoEase M/Z high strength silica (HSS) C18 column (300 µm × 150 mm, 1.8 µm) from Waters. The mobile phases were comprised of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The initial gradient started at 1% B for 1 min and then ramped up to 95% over 60 min, followed by a wash step at 95% B, and re-equilibration at 5% A for 15 min. The total run time was 82 min. The column oven was set to 35 °C, and the sample injection volume was 1 µL. The samples were ionized in the negative mode using a heated electrospray ionization probe (Thermo Fisher Scientific). The ion source spray voltage was 3600 V, with sheath, auxiliary, and sweep gases set at 6, 4, and 2 a.u., respectively. The temperature in the ion transfer tube was 275 °C. The mass spectrometer was operated on full mass spectrometry (MS)-data dependent MS^2 scan mode. The MS^1 scan range was a mass-to-charge ratio (m/z) of 150 to 1500, with resolutions of 60,000. For data-dependent MS^2, the automatic gain control was set to 5×10^4, with an isolation window of m/z 1.5. The normalized high-collision dissection energy was set to 35%, 50%, and 65%. Nitrogen was used as a drying, nebulizing, and collision gas. *Allium cepa* saponins (ACSs) were putatively identified based on their elemental composition, determined by mass accuracy, and supported further by their MS^2 mass spectra neutral loss patterns (Supplemental Figs. 1–19).

Data analysis. For FBR severity data, the statistical analysis was done using SAS Studio within SAS® OnDemand for Academics, a web-based platform (SAS Institute Inc., Cary, NC, USA). Average FBR severity at 20 dpi was determined for each cultivar population based on data collected from 20 bulbs per replication per plot. Proc MEANS was used to determine the plot means and Proc GLM was used to conduct analysis of variance (ANOVA) to determine the level of variation among the cultivar populations and check cultivars. Pairwise comparisons were also performed for the populations of each cultivar to evaluate the reduction in mean FBR severity in the advanced selected populations compared with the initial population by using CONTRAST statement.

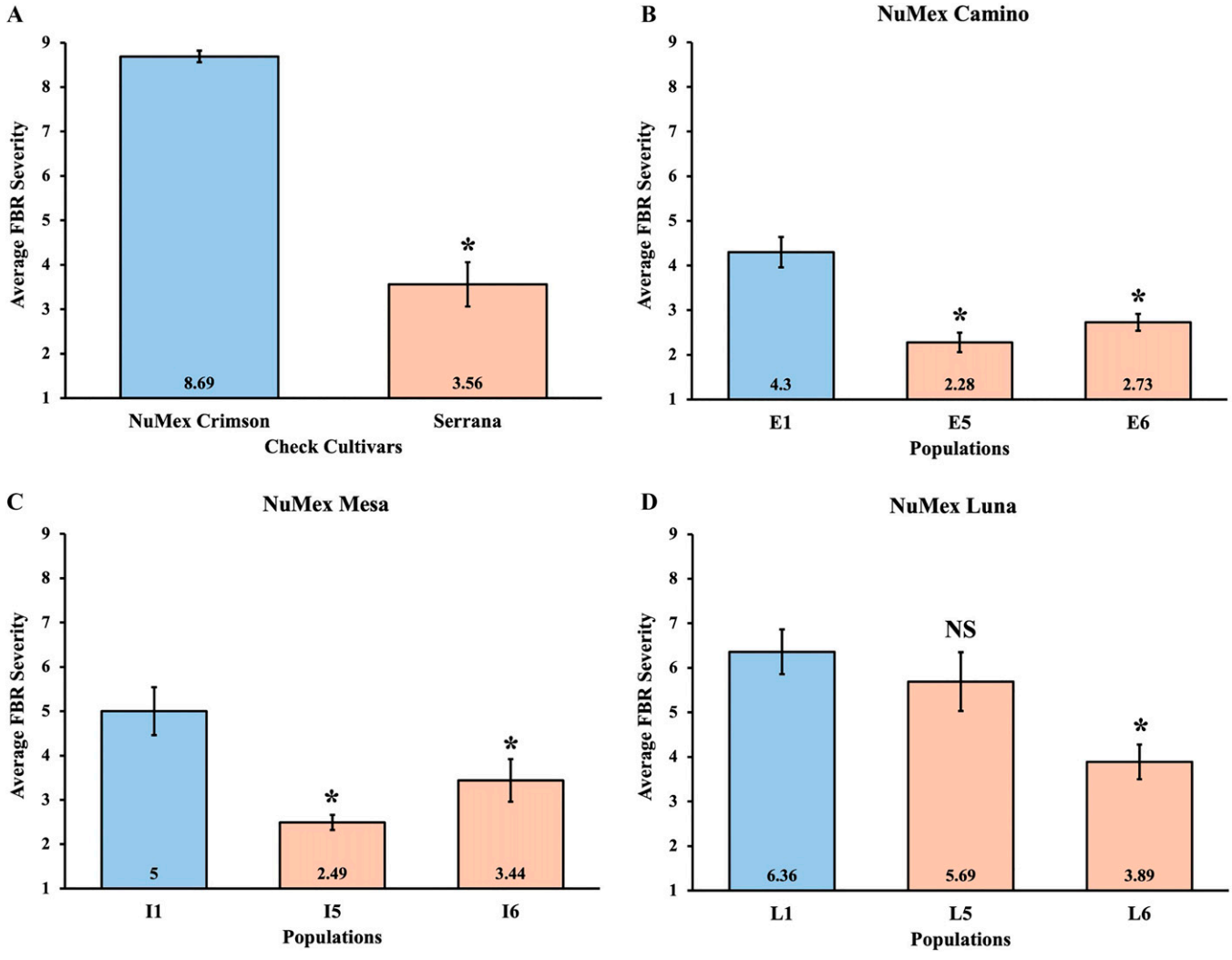

Fig. 1. Average severity of Fusarium basal rot (FBR) in different onion cultivar populations. (**A**) Check cultivars NuMex Crimson (FBR susceptible) and Serrana (FBR partially resistant). Initial and advanced populations selected for resistance to FBR: 'NuMex Camino' (**B**), 'NuMex Mesa' (**C**), and 'NuMex Luna' (**D**). E1 is the initial population, and E5 and E6 are advanced selected populations of 'NuMex Camino'. I1 is the initial population, and I5 and I6 are advanced selected populations of 'NuMex Mesa'. L1 is the initial population, and L5 and L6 are advanced selected populations of 'NuMex Luna'. Asterisks indicate that the mean severity of FBR for bulbs of that cultivar or population is significantly different from the mean bulb rating for the cultivar or population for which the bar is blue ($P < 0.05$), whereas NS means no significant difference.

Table 1. List of putatively identified *Allium cepa* saponins (ACSs; ACS-1 to ACS-19) detected in the basal plate tissue of bulbs of short-day onion cultivar populations following inoculation of the basal plate of each bulb with the Fusarium basal rot pathogen, *Fusarium oxysporum* f. sp. *cepae*.

Putative saponin identifier	Putative saponin structure based on neutral loss	RT[i]	Experimental $[M-H]^{-}$[ii]	Theoretical $[M-H]^{-}$[iii]	Error (ppm)[iv]	Chemical formula
ACS-1	Spirostane base +2O, CO_2, O-Hex	17.09	679.36987	679.3699	–0.05	$C_{36}H_{56}O_{12}$
ACS-2	Furostane base, dHex, dGlcA-H_2O	20.68	707.40057	707.4012	–0.89	$C_{38}H_{60}O_{12}$
ACS-3	Furostane base, dHex, CO_2, O-Hex	19.15	737.41119	737.4118	–0.78	$C_{39}H_{62}O_{13}$
ACS-4	Furostane base, COOH, dHex, dGlcA-H_2O	20.70	753.40613	753.4067	–0.73	$C_{39}H_{62}O_{14}$
ACS-5	Spirostane base –4H, +2O, COOH, O-Hex, dHex	24.75	781.40155	781.4016	–0.06	$C_{40}H_{62}O_{15}$
ACS-6	Spirostane base +3O, COOH, O-Pen, O-Hex	10.88	787.41205	787.4122	–0.14	$C_{39}H_{64}O_{16}$
ACS-7	Spirostane base +3O, COOH, dHex, O-Hex	11.48	801.4267	801.4278	–1.38	$C_{40}H_{66}O_{16}$
ACS-8	Spirostane base +2O, COOH, dPen, $C_6H_8O_3$	26.40	881.49023	881.4904	–0.20	$C_{46}H_{74}O_{16}$
ACS-9	Spirostane base –2H, +1O, COOH, dHex, O-Pen	18.66	885.44873	885.4489	–0.24	$C_{44}H_{70}O_{18}$
ACS-10	Spirostane base +3O, dHex, O-Pen, O-Hex	10.73	887.46393	887.4646	–0.74	$C_{44}H_{72}O_{18}$
ACS-11	Spirostane base –2H, +2O, COOH, dHex, O-Pen, O-Hex	14.10	915.45941	915.4595	–0.10	$C_{45}H_{72}O_{19}$
ACS-12	Spirostane base +3O, dHex, O-Hex, O-Hex	10.35	917.47491	917.4752	–0.27	$C_{45}H_{74}O_{19}$
ACS-13	Spirostane base +3O, COOH, dHex, O-Pen, O-Hex	10.72	933.47021	933.4701	–0.15	$C_{45}H_{74}O_{20}$
ACS-14	Spirostane base +3O, COOH, dHex, dHex	14.86	947.48492	947.4857	–0.84	$C_{46}H_{76}O_{20}$
ACS-15	Spirostane base +3O, COOH, dHex, O-Hex, O-Hex	10.35	963.47949	963.4806	–1.19	$C_{46}H_{76}O_{21}$
ACS-16	Spirostane base +3O, dHex, dHex, O-Pen, O-Hex	10.99	1033.55713	1033.5589	–1.70	$C_{51}H_{86}O_{21}$
ACS-17	Spirostane base +2O, O-Hex, dHex, O-Hex, O-Hex	12.59	1063.53162	1063.5331	–1.36	$C_{51}H_{84}O_{23}$
ACS-18	Spirostane base –2H, +2O, COOH, dHex, O-Pen, O-Hex	10.03	1065.51123	1065.5123	–1.03	$C_{50}H_{82}O_{24}$
ACS-19	Spirostane base +3O, COOH, O-Hex, dHex, O-Pen	9.80	1095.52075	1095.5229	–1.95	$C_{51}H_{84}O_{25}$

[i] RT = retention time, the time required for a compound to elute from the ultra-high-performance liquid chromatographic column after injection of the basal plate tissue extract.
[ii] Theoretical $[M-H]^{-}$ is the expected mass-to-charge ratio.
[iii] Experimental $[M-H]^{-}$ is the observed ratio from the experiment.
[iv] Error, or mass accuracy in parts per million, is an indication of the deviation of the observed mass-to-charge ratio from the theoretical mass-to-charge ratio.

MS-DIAL version 5.1 software (RIKEN Center for Sustainable Resource Science, Yokohama, Japan; National Institute of Genetics, Mishima, Japan; and University of California, Davis, CA, USA) (Tsugawa et al. 2020) was used for data preprocessing, including baseline correction and noise filtering to ensure the quality and reliability of data analysis. MS-DIAL's peak alignment helped to match peaks accurately across multiple samples by addressing retention time shifts and other variations in the MS data. The acceptable range for mass accuracy or error was kept within ± 2 ppm for greater accuracy of the metabolite identification. Saponin data interpretation and visualization for metabolomics were conducted using the online software MetaboAnalyst version 6.0 (Wishart Research Group, University of Alberta, Edmonton, Alberta, Canada; Xia Lab, McGill University, Montreal, Quebec, Canada) (Pang et al. 2024). Saponin data were normalized using an internal standard (umbelliferone, 7-hydroxycoumarin), present in the extraction solvent, as a reference for tracking and adjusting for any changes or errors in the samples during the process. Peak areas were used for calculating fold increases and converted to relative abundances using log-transformation (base 10) in MetaboAnalyst version 6.0. A heatmap was generated to illustrate changes in the mean relative abundance of putatively identified saponins between the partially resistant and the susceptible check cultivars. A two-sample *t* test was performed for the check cultivars to assess differences in the relative abundance of saponins at different sampling points (0, 5, 10, and 15 dpi). To assess differences in the saponin's relative abundance among cultivar populations and across sampling times, a two-way ANOVA was performed to test for the main effects of population, sampling time point, and their interaction. Where significant interactions were detected, simple main effects were analyzed to compare populations within each time point and time points within each population using the least significant difference test for pairwise comparisons ($P < 0.05$). Replicates were treated as independent samples for different sampling time points. These analyses were performed in R version 4.2.3 (R Foundation for Statistical Computing, Vienna, Austria) using the package agricolae (R version 1.3-7, R Foundation for Statistical Computing). Log-transformed values were used to generate dot plots to show mean relative abundance in cultivar populations and check cultivars for the saponins for which there were significant differences in the relative abundance detected. Line graphs for each cultivar with standard error bars were also generated for the relative abundance of the particular saponin detected. A correlation heatmap was generated using Pearson's correlation coefficient to assess the relationship between average FBR severity and saponin relative abundance across the sampling time points using R version 4.2.3 (R Foundation for Statistical Computing) using the ggplot2 package (Wickham 2016).

Results

Variability in cultivar responses to FBR susceptibility. The efficacy of inoculation screening method was evident in the performance of the two check cultivars. Bulbs of the partially resistant cultivar Serrana had less severe FBR compared with bulbs of the susceptible cultivar NuMex Crimson (Fig. 1A). In terms of selection progress for resistance to FBR, the advanced selected populations from three cultivars—NuMex Camino, NuMex Mesa, and NuMex Luna—demonstrated less severe FBR compared with the initial population of each (Fig. 1B–D) ($P < 0.05$), with one exception: L5, an advanced selected population of 'NuMex Luna', did not show a significant difference in average FBR severity compared with the initial population, L1 (Fig. 1D).

Putative identification of saponins in onion basal plate tissue. A total of 19 putative saponins, labeled ACS for *A. cepa* saponin, were identified based on their elemental composition and mass accuracy. Table 1 presents information on the putative saponin identifier, retention time for each putative saponin, and chemical formula, along with their structures based on the neutral loss of sugars. Because this was an untargeted metabolomics study, information on the relative abundance of each putative saponin at different dpi has been included in Supplemental Table 1. Among the 19 putative saponins detected in basal plates of all cultivar populations, ACS-4 (2.89–3.70) was the most abundant across all populations, including the two check cultivars (Supplemental Table 1). In addition, ACS-8 and ACS-16 were the least abundant saponins in the partially resistant check Serrana and across cultivar populations. The relative abundance of ACS-8 was the lowest in the initial population of NuMex Camino at 0 dpi (E1; relative abundance, −1.66), followed by the advanced population of NuMex Mesa at 5 dpi (I6; relative abundance, −1.41). ACS-16 showed the lowest abundance in the initial population at 5 dpi (E1; relative abundance, −1.54), followed by the advanced population at 0 dpi (E5; relative abundance, −1.13) for NuMex Camino (Supplemental Table 1).

Pre- and postinfection comparison of the relative abundance of saponins detected in bulbs of the two check cultivars. Putatively identified saponins differed in constitutive levels before infection (0 dpi) between the

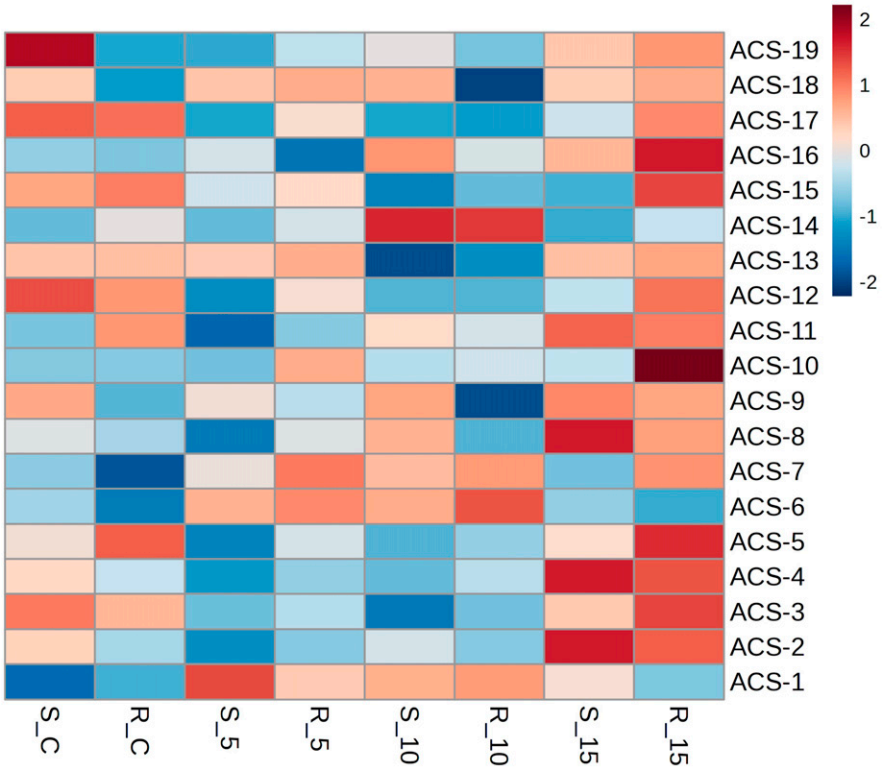

Fig. 2. A heatmap illustrating changes in the mean relative abundance of 19 putative *Allium cepa* saponins (ACSs; ACS-1 to ACS-19) detected in the basal plate tissue of onion bulbs before and after inoculation with the Fusarium basal rot pathogen, *Fusarium oxysporum* f. sp. *cepae* in two check cultivars. S_C, S_5, S_10, and S_15 represent 0, 5, 10, and 15 d postinoculation (dpi), respectively, for the susceptible check cultivar NuMex Crimson. R_C, R_5, R_10, and R_15 represent 0, 5, 10, and 15 dpi, respectively, for the partially resistant check cultivar Serrana. Darker blue shades (–2) represent lower relative abundance of the putative saponin detected, whereas darker reds (+2) represent higher relative abundance. Autoscaling of saponins after log-transformation was done to standardize each saponin by centering on its mean and scaling by its standard deviation, enabling fair comparison across saponins and helping reveal true patterns and clusters in the heatmap.

two check cultivars (Fig. 2). Specifically, ACS-5, ACS-11, and ACS-14 were detected at a higher mean relative abundance in basal plates of the partially resistant check compared with the susceptible check. In addition, five saponins (ACS-3, ACS-12, ACS-13, ACS-15, and ACS-17) were detected at higher relative abundance in both check cultivars at 0 dpi compared with other saponins.

As infection progressed, fluctuations in the levels of different saponins were observed compared with the levels detected in uninoculated bulbs at 0 dpi. At 5 dpi, nine saponins (ACS-5, ACS-7, ACS-8, ACS-10, ACS-12, ACS-14, ACS-15, ACS-17, and ACS-19) showed a higher relative abundance in bulbs of the partially resistant check compared with the susceptible check (Fig. 2). Four other saponins (ACS-1, ACS-6, ACS-13, and ACS-18) were detected at a higher relative abundance in both check cultivars at 5 dpi compared with other saponins. At 10 dpi, ACS-1, ACS-6, ACS-7, and ACS-14 had a higher relative abundance in bulbs of both check cultivars, and ACS-14 was the most abundant saponin (Fig. 2). By 15 dpi, most saponins were detected at a greater relative abundance in bulbs of both check cultivars, with a greater number of saponins showing elevated levels in the partially resistant check than the susceptible check (16 vs. 12 of the 19 saponins, respectively) (Fig. 2). In particular, increased levels of saponins for ACS-3, ACS-5, ACS-7, ACS-10, ACS-12, ACS-15, ACS-16, and ACS-17 were observed in bulbs of the partially resistant check compared with the susceptible check, with ACS-10 being the most abundant saponin detected at 15 dpi, followed by ACS-16.

In addition, saponins with a significantly higher relative abundance in the partially resistant check compared with the susceptible check were recognized. Before infection (0 dpi), the constitutive levels of two saponins, ACS-5 and ACS-14, were detected at higher levels in basal plates of the partially resistant check 'Serrana' compared with the susceptible check 'NuMex Crimson' ($P < 0.05$) (Fig. 3A and B). At 5 dpi, levels of two saponins, ACS-14 and ACS-17, were elevated in basal plates of the partially resistant check 'Serrana' compared with the susceptible check 'NuMex Crimson' ($P < 0.05$) (Fig. 3C and D). No saponins were detected at significantly different levels at 10 dpi between the two check cultivars. However, at 15 dpi, ACS-14 and ACS-17 were detected in the basal plates at increased levels in the partially resistant check bulbs relative to the susceptible check bulbs ($P < 0.05$) (Fig. 3E and F).

Pre- and postinfection dynamics in the relative abundance of the saponin across cultivar populations. Saponins showing significant changes in their relative abundance over time after inoculation were identified across cultivar populations, especially the saponins detected at higher relative abundance in the partially resistant check at various time points. Only one saponin, ACS-14, showed a unique pattern of abundance across the selected cultivar populations. Baseline saponin levels (0 dpi) across populations of the three selected cultivars along with check cultivars showed no significant differences in relative abundance ($P = 0.32$). For the partially resistant check cultivar, baseline levels of ACS-14 (relative abundance, 0.64) detected in the basal plates of bulbs before inoculation were slightly greater than in the basal plates of the other populations (relative abundance, –0.63 to 0.24) (Fig. 4, Supplemental Table 1). ACS-14 levels at 0 and 5 dpi were ~17-fold and 10-fold higher, respectively, in the partially resistant check compared with the susceptible check. ACS-14 abundance was lower in the susceptible check compared with those of 'NuMex Camino' and 'NuMex Luna' initial and advanced selections at 0 and 5 dpi (Fig. 4A and C), but not less than in the basal plates of the I1 and I5 populations of 'NuMex Mesa' at 0 dpi, nor of I1 bulbs at 5 dpi ($P < 0.05$). However, an approximate 8- to 24-fold increase from 0 to 5 dpi was observed in the advanced selected populations of three cultivars: NuMex Camino (E6) (Fig. 4A), NuMex Mesa (I5) (Fig. 4B), and NuMex Luna (L5 and L6) (Fig. 4C). The greatest increase in ACS-14 levels detected in the basal plates was observed in all cultivar populations, including the checks, from 5 to 10 dpi, followed by a sharp decline from 10 to 15 dpi. Interestingly, ACS-14 levels at 10 dpi were similar across all cultivar populations, including the check cultivars ($P = 0.65$) (Fig. 4, Supplemental Table 1).

Mean relative abundance of ACS-14 detected in the basal plates at 5 dpi was ~4-fold higher in the advanced selected populations (E5 mean relative abundance, 1.02; E6 mean relative abundance, 1.36) of 'NuMex Camino', and was ~8-fold higher in 'NuMex Mesa' (I5 mean relative abundance, 0.76) compared with their initial populations (E1 mean relative abundance, 0.52; I1 mean relative abundance, –0.34; $P < 0.05$) (Fig. 5, Supplemental Table 1). However, advanced selected populations (L5 mean relative abundance, 1.00; L6 mean relative abundance, 1.33) of 'NuMex Luna' showed no significant differences in ACS-14 levels detected in the basal plates compared with the initial population (L1 mean relative abundance, 1.21) and L5 also did not differ significantly in mean FBR severity compared with L1, as shown earlier in Fig. 1D. In addition, the relative abundance of ACS-14 correlated negatively with FBR severity at 5 dpi ($r = –0.48$; $P = 0.1$) (Supplemental Fig. 20).

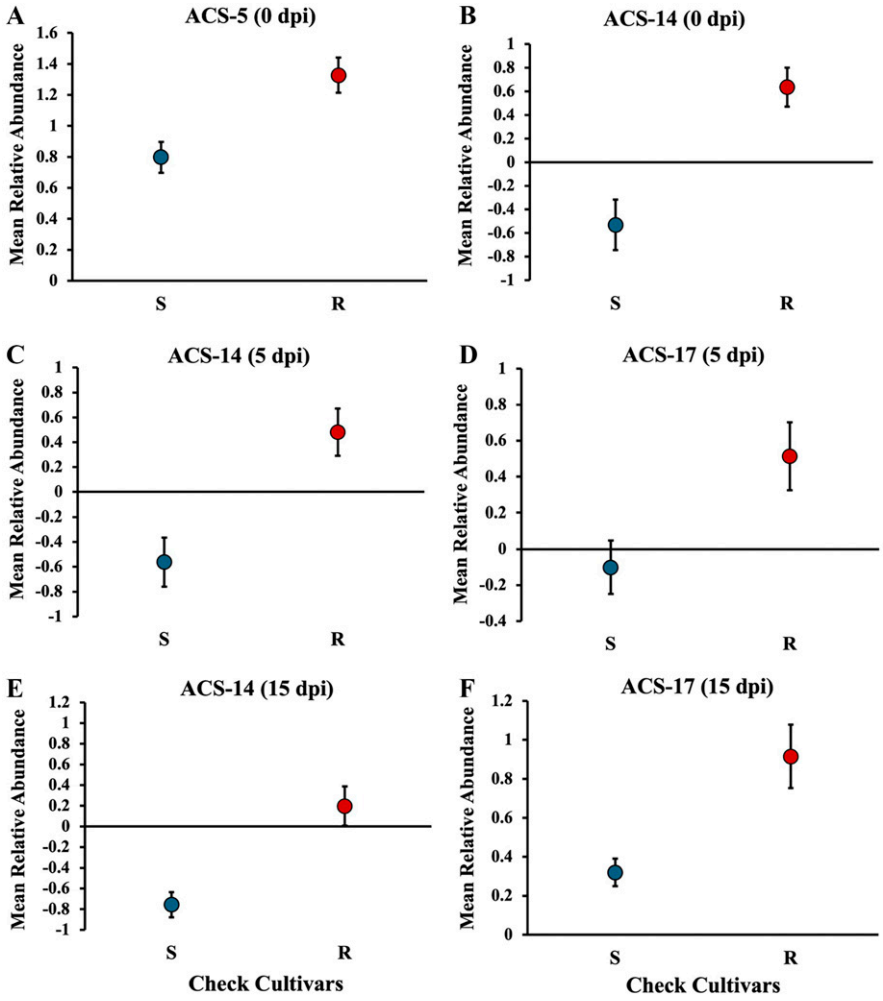

Fig. 3. Mean relative abundance of different saponins detected in the basal plate tissue of two onion check cultivars at different time points postinoculation with the Fusarium basal rot pathogen, *Fusarium oxysporum* f. sp. *cepae*. *Allium cepa* saponin (ACS)-5 (**A**) and ACS-14 (**B**) detected on uninoculated bulbs at 0 d postinoculation (dpi); ACS-14 (**C**) and ACS-17 (**D**) detected at 5 dpi; and ACS-14 (**E**) and ACS-17 (**F**) detected at 15 dpi. S is the susceptible check cultivar NuMex Crimson; R is the partially resistant check cultivar Serrana. Each data point represents the mean ± standard error of saponin relative abundance detected in bulbs of that cultivar population based on log-transformed values corresponding to peak areas detected by ultra-high-performance liquid chromatography coupled to an Orbitrap fusion mass spectrometer after using an internal standard umbelliferone (7-hydroxycoumarin) as a reference. Significant differences in the relative abundance of each saponin were detected between the two check cultivars (t test, $P < 0.05$).

Discussion

Understanding the intricate dynamics of interactions between plants and pathogens is essential for unraveling plant defense mechanisms against pathogens. Saponins are produced abundantly in *Allium* species and have emerged as promising chemical compounds as a result of their antifungal properties (Sobolewska et al. 2016). Although many different saponins are produced by *Allium* species (Lanzotti et al. 2013), the specific roles of these saponins in combating FBR and conferring resistance to onion cultivars have been relatively poorly explored. Because of the dynamic nature of saponins, they undergo substantial changes in production within plant cells during pathogen infection (He et al. 2019). Initially stored within plant vacuoles in an inactive state, saponins transition rapidly to a metabolically active state in response to pathogenic stimuli (Jha and Mohamed 2022). The role of specific saponins in FBR resistance of onions is not well understood. To the best of our knowledge, no experiment has been reported on the association of different saponins with FBR resistance at various time points after FOC inoculation. Our study marks the first report of in vivo fluctuations in the levels of various saponins in the onion basal plate across diverse cultivar populations during the onion–FOC interaction. Our focus on three populations of each of three cultivars in addition to two check cultivars revealed patterns in the levels of 19 putative saponins detected during the first 15 dpi.

Inherent genetic differences in the three onion cultivars and two check cultivars were associated with variations observed in saponins levels. Differences in saponin content among plants can demonstrate a genotype- and cultivar-dependent nature. A systematic analysis of *Centella asiatica* L. accessions revealed significant changes in the levels of saponin, with some accessions showing no detectable levels (Thomas et al. 2010). In addition to the genetic makeup of cultivars, differences in virulence of FOC isolates could further complicate this by influencing FBR severity and the levels of saponins produced among cultivar populations (Kliebenstein et al. 2005). In our study, the basal plate tissue was sampled from different bulbs at each time point for each cultivar population, making them independent samples. Therefore, the observed trend in the levels of saponins may have not only indicated the development of FBR over time, but also could be influenced by genetic variation in each bulb sampled at the different time points. Moreover, degradation of the basal plate tissue may act as a significant confounding factor in saponin analysis, as this degradation disrupts normal metabolic processes, compromises tissue integrity, and potentially leads to variability in saponin concentrations.

The lower FBR severity observed for most of the advanced selected populations compared with the initial population of each of the three cultivars indicates the selection of individuals for FBR resistance was associated with an improved defense response after a few selection cycles. A significant increase in the levels of ACS-14 from 0 to 5 dpi was observed for the advanced selected populations of two of the three cultivars, NuMex Camino and NuMex Mesa, but not for 'NuMex Luna'. These results suggested that several cycles of phenotypic selection for FBR resistance may have resulted in the selection of populations that produce onion bulbs with greater saponin levels associated with the improved resistance to FOC.

A heatmap of the mean relative abundance of 19 putative saponins detected in the basal plates of the check cultivars clearly illustrates that certain saponins were produced at greater levels during the early stages of infection by FOC. Even though the levels of some of these saponins were elevated, these saponins could not confer protection to the onion basal plate in bulbs of the susceptible check from FOC. The results suggest that identifying specific saponins and their roles in defense must be combined with phenotypic screening for FBR susceptibility using a virulent FOC isolates. In our study, the levels of a few saponins increased in basal plates of the partially resistant check at 5 dpi, indicating an early response to infection within the first 5 dpi. However, most saponins were detected at similar or decreased levels at 10 dpi, followed by an increase at 15 dpi for several saponins in both check cultivars, suggesting a dynamic temporal variation in saponin production in response to FOC infection, along with degradation of the basal plate tissue.

ACS-14 was detected at greater preinfection levels (0 dpi) in most of the cultivar populations and the partially resistant check compared with bulbs of the susceptible check, with the exception of the I1, I5, and populations of 'NuMex Mesa'. After infection, a significant increase in the levels of ACS-14 detected in the basal plates of most of the cultivar populations

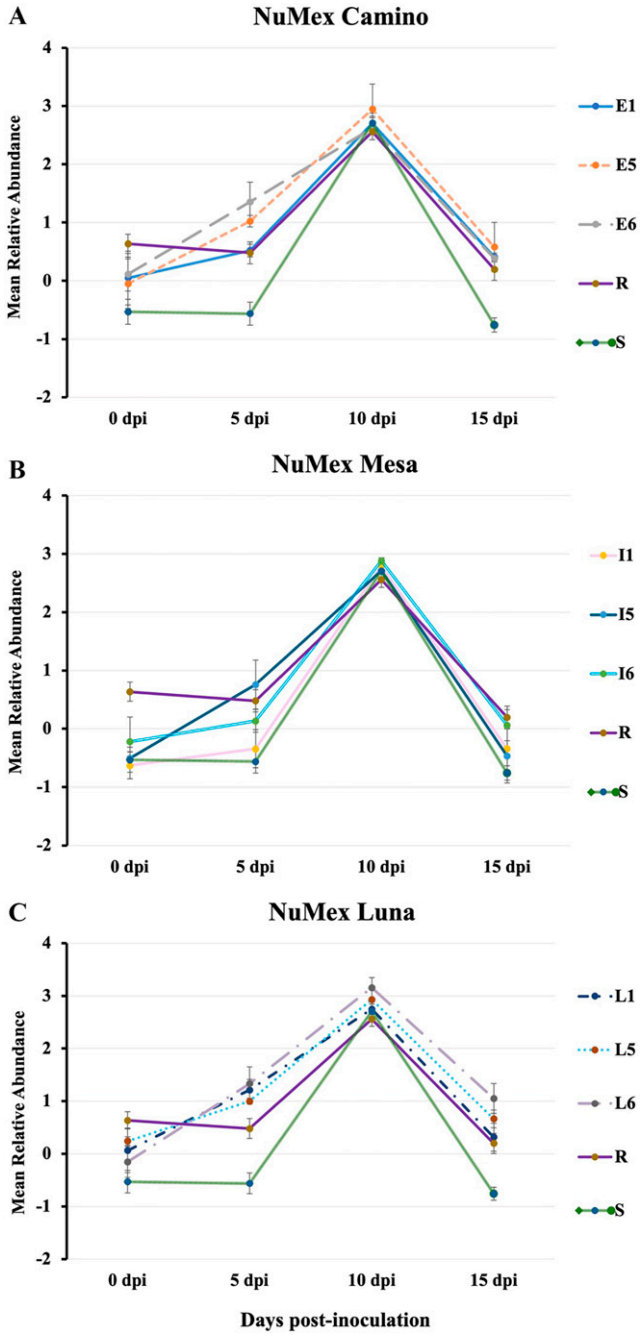

Fig. 4. Mean relative abundance of the putative saponin *Allium cepa* saponin (ACS)-14 detected in the basal plate tissues of bulbs of onion cultivar populations at different time points postinoculation with the Fusarium basal rot pathogen, *Fusarium oxysporum* f. sp. *cepae*, compared with bulbs of the two check cultivars Serrana and NuMex Crimson, 'NuMex Camino' populations (**A**), 'NuMex Mesa' populations (**B**), and 'NuMex Luna' populations (**C**). E1 is the initial population and E5 and E6 are two advanced selected populations for 'NuMex Camino'. I1 is the initial population and I5 and I6 are two advanced selected populations for 'NuMex Mesa'. L1 is the initial population and L5 and L6 are two advanced selected populations for 'NuMex Luna'. R is the partially resistant check cultivar Serrana and S is the susceptible check cultivar NuMex Crimson. Each data point represents the mean ± standard error of saponin relative abundance detected in bulbs of that cultivar population based on log-transformed values corresponding to peak areas detected by ultra-high-performance liquid chromatography coupled to an Orbitrap fusion mass spectrometer after using an internal standard umbelliferone (7-hydroxycoumarin) as a reference. The main effects of time, population, and their interaction were significant (two-way analysis of variance: time, $P < 0.05$; population, $P < 0.05$; interaction, $P = 0.03$). Pairwise differences between populations at each sampling time point and between sampling times for each population were tested using the least significant difference test ($P < 0.05$).

suggests the potential involvement of ACS-14 in conferring FBR resistance. Also, a significant negative correlation between ACS-14 abundance and FBR severity was observed only at 5 dpi, suggesting that early accumulation of this saponin may play a role in imparting resistance to FOC. Surprisingly, the drastic increase in the levels of ACS-14 at 10 dpi did not differ across cultivar populations and both the resistant and susceptible check cultivars. The high levels of ACS-14 in basal plates of the susceptible check at 10 dpi could be the result of a delayed response to FOC infection. The monoisotopic mass of this saponin matches that of a saponin identified previously in *Allium macrostemon*, known as macrostemonoside H (Peng et al. 1995). Because both share the same monoisotopic mass, ACS-14 may correspond to an analog of macrostemonoside H or may be a new saponin with a distinct chemical structure.

Saponins may exhibit specificity in activation pattern, as some were induced at earlier time points in response to the FOC inoculation, whereas others were upregulated later, some of which may inhibit basal plate tissue degradation, although this remains to be determined. In addition, the increase in levels of certain saponins in both check cultivars indicates they could be involved in general plant defense and may not be associated directly with FBR resistance. There is also a possibility of involvement of other saponins in the defense response to FOC, even if their levels did not increase significantly after infection. In our study, levels of three specific saponins (ACS-2, ACS-3, and ACS-4) showed a significant increase in the levels detected at 15 dpi in both check cultivars. The monoisotopic masses of these three saponins match those of alliospirosides A, B, and D, respectively, which have been identified in shallots (Teshima et al. 2013). In addition, ACS-10 has the same monoisotopic mass as ceposide A/B (Lanzotti et al. 2012), and was detected at increased levels at 5 and 15 dpi in the partially resistant check compared with the susceptible check. These saponins have previously showed in vitro antifungal activities against different FOC isolates (Abdelrahman et al. 2017; Lanzotti et al. 2012; Teshima et al. 2013).

The early stages of disease development are critical as fungi attempt to break plant tissues, triggering the activation of plant defense mechanisms. After inoculation, FOC can initiate germination and penetration within 1 to 2 d under conditions of high humidity, leading to the dissolution of cell walls in the onion basal plate. This may stimulate a response from the onion basal plate, resulting in increased production of different saponins. Saponins susceptible to fungal enzymatic detoxification decline during the early stages of plant infection, whereas others involved in disease resistance continue to increase until complete fungal eradication is achieved. Several fungal saponin hydrolases can break down specific saponins by cleaving their sugar chains, making them ineffective against fungi (Westrick et al. 2021). Observations on melon roots showed that *F. oxysporum* began root colonization within 1 to 2 d postinoculation, with mycelial growth extending into the cortex and endodermis by the fourth day (Zvirin et al. 2010). A 2- to 4-fold difference in the constitutive transcript levels of three defense-related genes was evident between the resistant and the susceptible genotypes, and the levels increased in the resistant genotype relative to

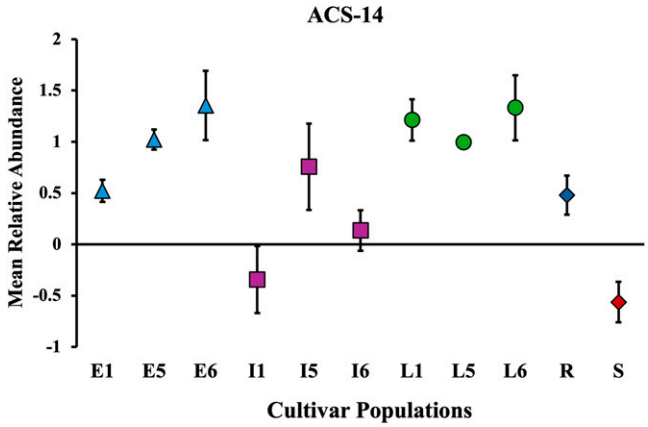

Fig. 5. Mean relative abundance of the putative saponin *Allium cepa* saponin (ACS)-14 detected 5 d postinoculation (dpi) in the basal plate tissue of onion cultivar populations inoculated with the Fusarium basal rot pathogen, *Fusarium oxysporum* f. sp. *cepae*. E1 is the initial population and E5 and E6 are two advanced selected populations for 'NuMex Camino'. I1 is the initial population and I5 and I6 are two advanced selected populations for 'NuMex Mesa'. L1 is the initial population and L5 and L6 are two advanced selected populations for 'NuMex Luna'. R is the partially resistant check cultivar Serrana and S is the susceptible check cultivar NuMex Crimson. Each data point represents the mean ± standard error of saponin relative abundance detected in bulbs of that cultivar population based on log-transformed values corresponding to peak areas detected by ultra-high-performance liquid chromatography coupled to an Orbitrap fusion mass spectrometer after using an internal standard umbelliferone (7-hydroxycoumarin) as a reference. Cultivar populations differ significantly ($P < 0.05$) and pairwise differences between populations were tested by the least significant difference test ($P < 0.05$).

the susceptible genotype after infection. These findings suggest that both constitutive (preinfection) and induced defense responses can contribute to reduced fungal colonization in the resistant genotype, as observed with higher baseline levels of ACS-14 in the partially resistant check cultivar Serrana in our study and induced levels in other cultivar populations compared with the susceptible check 'NuMex Crimson' at 5 dpi. In a shallot study (Vu et al. 2012), total saponin content in the roots of shallot plants was observed to increase significantly 3 d after inoculation compared with uninoculated plants.

An increase in the production of saponins is just one component of a successful strategy to limit pathogen infection. Plants are known to produce a wide array of antimicrobial secondary metabolites, including alkaloids and flavonoids to terpenes and organic acids, in addition to saponins (Pusztahelyi et al. 2015). For instance, onion bulbs that were inoculated with *F. oxysporum* revealed significant fluctuations in flavonoid levels over a 9 d postinoculation period (Lee et al. 2012). Initially, one flavonoid gradually declined over the first 3 d, whereas three others decreased over the first 4 d. These four flavonoids increased continuously in concentration, reaching a peak 6 d postinfection before gradually declining again. These findings suggest a dynamic activation of other metabolites in the plant defense response to pathogen invasion besides saponins.

Conclusion

Our study highlighted the potential role of ACS-14 in the early defense of the onion basal plate to infection by FOC, warranting additional investigation into its role in FBR resistance. Future studies could involve the purification of this and other potential resistance-related compounds to obtain concentrated forms, followed by in vitro testing of their antifungal activities against virulent FOC isolates. Moreover, advanced biochemical techniques such as nuclear magnetic resonance spectroscopy can be used to study their chemical structures. Understanding the biosynthetic pathways and genetic regulation of different saponins could have important implications for the development of onion cultivars with improved FBR resistance. By integrating metabolomics with other "-omics" tools such as genomics and transcriptomics, intricate gene–metabolite networks governing FBR resistance in onions could be revealed.

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