Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Supporting information

Chitosan nanocarriers loaded with salicylic acid for controlling fall armyworm (*Spodoptera frugiperda*) and alleviating oxidative stress in maize plants

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Supplementary method

Text S1. Measurement of oxidative stress and antioxidant enzyme activity

Superoxide radicals ($O_2^{\cdot-}$) was estimated according to Liu et al. (2021) [1] with slight modification. In brief, 50 mg of maize fresh leaf were ground in liquid nitrogen and added with 0.1 mL 65 mM potassium phosphate buffer (pH = 7.8) and 20 μL 10 mM hydroxylamine hydrochloride. After incubating in 25 ℃ water bath for 20 min, the sample centrifuged at 4 ℃, 12,000 rpm for 10 min to obtain supernatant. Subsequently, the supernatant was mixed with 58 mM sulfanilic acid (0.2 mL) and 7 mM naphthyl ethylene diamine dihydrochloride (0.2 mL). After 20 min of reaction, trichloromethane was mixed to the same reaction mixture gently and centrifuged at 2000 rpm for 5 min. The absorbance of the pink aqueous phase was recorded at 530 nm with a multifunctional microplate reader (Varioskan Lux, Thermo Scientific, Finland). A standard curve was prepared with $NaNO_2$ and used to calculate the production of $O_2^{\cdot-}$. The production of NO_2^- is equivalent to generation of $O_2^{\cdot-}$.

Hydrogen peroxide (H_2O_2) was estimated based on Sheteiwy et al. (2021) [2] Briefly, 100 mg of maize fresh leaf were ground into powder with liquid nitrogen and added 1.5 mL of pre-cooled 0.1% trichloroacetic acid (TCA). After centrifuging at 4 ℃, 10,000 rpm for 10 min, the supernatant (0.5 mL) mixed with 0.5 mL 10 mM potassium phosphate buffer (pH = 7.0) and 1 mL 1M potassium iodide. The mixture incubated for 1h in dark. The absorbance of reaction mixture was recorded at 390 nm with a multifunctional microplate reader (Varioskan Lux, Thermo Scientific, Finland). Hydrogen peroxide concentration was calculated by using a standard curve prepared

with H_2O_2.

Malondialdehyde (MDA) was estimated according to a previous study. [3] Briefly, 100 mg of maize fresh leaf were ground into powder with liquid nitrogen and added 1.5 mL of pre-cooled 0.1% trichloroacetic acid (TCA). After centrifuging at 4 ℃, 10,000 rpm for 10min, the supernatant (0.25 mL) reacted with 0.5 mL of 20% TCA and 0.5 mL of 0.5% thiobarbituric acid (TBA) at 95℃ for 30 min. Subsequently, the mixture was cooled down on ice before measuring absorbance at 450 nm, 532 nm and 600 nm with a multifunctional microplate reader (Varioskan Lux, Thermo Scientific, Finland). MDA content was determined using the following equation: $C_{MDA}(\mu mol/L)=6.45*(A\ bsorbance_{532}-Absorbance_{600})-0.56*Absorbance_{450}$.

The antioxidant enzyme activity of leaves was determined according to the method of Luo et al. [4] and slightly modified. Firstly, for the extraction of enzyme solution, 200 mg of plant samples were homogenized in 1.6 mL of pre-cooled phosphate buffer (pH=7.8), and the supernatant was used as the enzyme solution after vortex at 4 ℃ and centrifugation at 12000 rpm for 20 min.

For SOD activity, the crude enzyme was mixed with L-methionine, nitroblue tetrazole, riboflavin, and EDTA-Na_2. The mixture was exposed under a fluorescent tube lamp for 20 min, and then determined by a multifunctional microplate at 560 nm. SOD activity takes the amount of enzyme required to inhibit NBT photoreduction by 50% as one unit of SOD.

For POD activity, the crude enzyme was mixed with 200 mM phosphate buffer solution (pH 6.0) containing guaiacol solution and 30% H_2O_2, the mixtures were

immediately measured in the wavelength of 470 nm for 2min each 30s by a multifunctional microplate reader. The increase in OD value of 0.01 per minute was considered as one unit of POD.

For CAT activity, the absorbance of 200 μL of the reaction mixture (15 mM phosphate buffer (pH 7.0), 0.05% H_2O_2, and 6.67 μL enzyme extracts) was recorded for 3 min at 240 nm. The reduction of 0.01 of OD value per min was used as one unit of CAT.

Text S2. Determination of leaf phytohormones by UHPLC-MS/MS

The content of phytohormones (JA and SA) in maize leaves were determined according to a previous method [5] with a slight modification. A 5 μL aliquot of extract solution was then directly injected into a UHPLC-MS/MS system (Vanquish Flex, Thermofisher Scientific, Germany) with a 2.1 × 100 mm × 1.8 μm C18 column (Acquity HSS T3, Waters, USA) coupled to Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific, USA). The mobile phase A (H_2O, 0.01% formic acid) and B (acetonitrile, 0.01% formic acid formic acid in acetonitrile) were used for the elution gradient: 0 min, 5 % B; 1.5 min, 5 % B; 9 min, 70 % B; 10 min, 70 % B; 10.1 min, 5 % B; 15 min, 5 % B. The flow rate was 0.35 mL min^{-1}. The concentration of phytohormones was conducted by using a calibration equation obtained by linear regression from five calibration points for each analysis. The R^2 value for the JA and SA standard was 0.9992, 0.9984, respectively.

Text S3. The parameters of UHPLC-MS/MS for determining BXs

The analysis of benzoxazinoids was performed on an HPLC system (Vanquish Flex, Thermofisher Scientific) with a C_{18} column (2.1 × 100 mm, 1.8 μm, Acquity HSS T3, Waters) coupled to a Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific) with an electrospray ionization (ESI) source. Gradient elution was performed at 5–20% B over 3.0 min, 20–98% B over 3.0 min, holding at 98% B for 1 min, followed by re-equilibration at 5% A for 3.0 min, where A = 0.05% formic acid/water (v/v) and B = 0.05% formic acid/acetonitrile (v/v). The flow rate was 0.3 mL/min. The temperature of the column was maintained at 40 °C, and the injection volume was 5 μl. The ESI mass spectrometer was operated in a positive Q3 scan mode.[6] The abundance of each metabolite was quantified from its peak area normalized by the area of internal stand. The data obtained was used for subsequent analysis

Table S1. Primer sequences used in this study.

Gene	Primer	Sequence	Reference
Pep1	Forward	CAGCCACCGAGGTGTGGG	Gene ID: 103645871
	Reverse	CAGCCACCGAGGTGTGGG	
Pep3	Forward	CCTCCCTGCCGGAAACTAAA	Gene ID: 103645874
	Reverse	CCTCCCTGCCGGAAACTAAA	
JAR1a	Forward	GGCCGGGCCCATGACAATCTGCAGTAGTGAAG	7
	Reverse	CGCGTTTAAACAGAGCCCATACGCAGTGCTGA	
JAR1b	Forward	CGCGGGCCCATGCCGATCTGTAGCTGTGAAG	7
	Reverse	CGCGTTTAAACAGAGCCCATAGGCGGTACTGAA	
JAC1	Forward	CGCGTTTAAACATGGAGAACCCTGCTCCGAGC	7
	Reverse	CGCCCTGCAGGCCCGAGGCCTGCCTGGCACCAC	
AMP	Forward	AAAAGGGCCCATGGAGAGTGGCAGCAAGAAG	7
	Reverse	CGCGTTTAAACATTTCACGCAGAGTTTGTTTTCA	
MPI	Forward	CGCGGGCCCATGAGCTCCACGGAGTGCGGC	7
	Reverse	CGCGTTTAAACAGCCGATGTGGGGCGTCTGGGC	
RIP2	Forward	GTTTTGGATATTGAGCACTACGCAGCGGAGCCAAACCCAGAGTTGAGTG	7
	Reverse	GGATTGGTGAAACACGTCCTCGTTTTGGTCCTTAACGAGCGCAAC	
B73 lox	Forward	GCGACACCATGACCATCAAC	8
	Reverse	GCGACACCATGACCATCAAC	
Cystin-like PI	Forward	CAAGGAGCACAACAGGCAGA	8
	Reverse	GGACATGAGCTGGCGATTTT	

Actin	Forward	CCATGAGGCCACGTACAACT	[9]
	Reverse	GGTAAAACCCCACTGAGGA	

Table S2. Time-dependent changes of the cumulative release of SA under various pH conditions. Data were expressed as means ± standard error (SE, n = 6). Different letters in the same row indicate significant differences among pH gradients (Tukey's HSD, $p < 0.05$).

Time/h	pH = 3	pH = 4	pH = 5	pH = 6	pH = 7	pH = 8
1	27.35 ± 1.50 a	30.81 ± 4.21 a	29.08 ± 10.28 ab	37.43 ± 7.78 ab	26.03 ± 2.42 b	22.10 ± 6.28 b
2	32.93 ± 7.23 a	30.88 ± 1.86 a	29.15 ± 3.68 a	31.88 ± 5.73 a	33.01 ± 3.84 a	26.43 ± 2.01 a
4	65.02 ± 5.08 a	60.08 ± 5.75 b	52.36 ± 1.85 b	51.39 ± 1.86 b	48.43 ± 2.54 b	44.23 ± 3.53 b
8	70.53 ± 10.54 a	58.86 ± 2.64 ab	55.29 ± 4.46 ab	57.84 ± 6.34 b	57.61 ± 3.53 ab	51.30 ± 4.82 ab
12	92.90 ± 7.09 a	88.14 ± 10.78 ab	79.19 ± 7.02 ab	79.54 ± 2.68 b	74.56 ± 6.01 b	69.74 ± 5.17 b
24	96.83 ± 14.04 a	86.35 ± 9.78 a	79.11 ± 5.63 a	84.06 ± 1.43 a	80.87 ± 2.66 a	79.32 ± 5.04 a
36	96.99 ± 9.67 a	86.51 ± 8.11 ab	79.27 ± 1.47 ab	84.22 ± 3.19 b	81.04 ± 7.14 ab	79.49 ± 4.42 ab
48	96.83 ± 14.04 a	86.35 ± 9.78 a	80.04 ± 7.24 a	85.59 ± 2.42 a	79.80 ± 10.45 a	82.99 ± 3.82 a
60	97.36 ± 10.86 a	86.88 ± 7.80 ab	80.57 ± 4.07 ab	86.79 ± 1.58 b	83.60 ± 6.26 ab	79.89 ± 5.71 ab
72	98.83 ± 5.21 a	88.35 ± 7.01 a	81.74 ± 2.13 a	87.29 ± 6.95 a	85.07 ± 11.74 a	81.35 ± 8.91 a

Figure S1

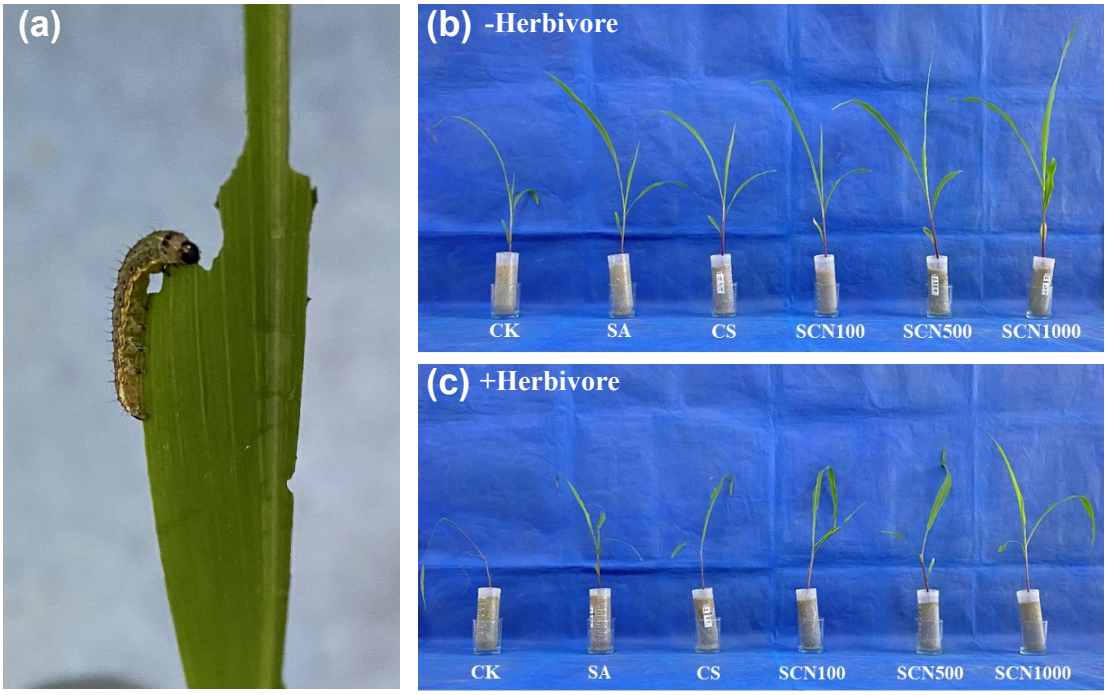

Fig. S1. (a) Photograph of maize leaves damaged by fall armyworm (*Spodoptera frugiperda*); Pot experiment results for testing the effect of SCNs on the growth of maize without (-Herbivore, b) and with (+Herbivore, c) the presence of *S. frugiperda*.

Figure S2

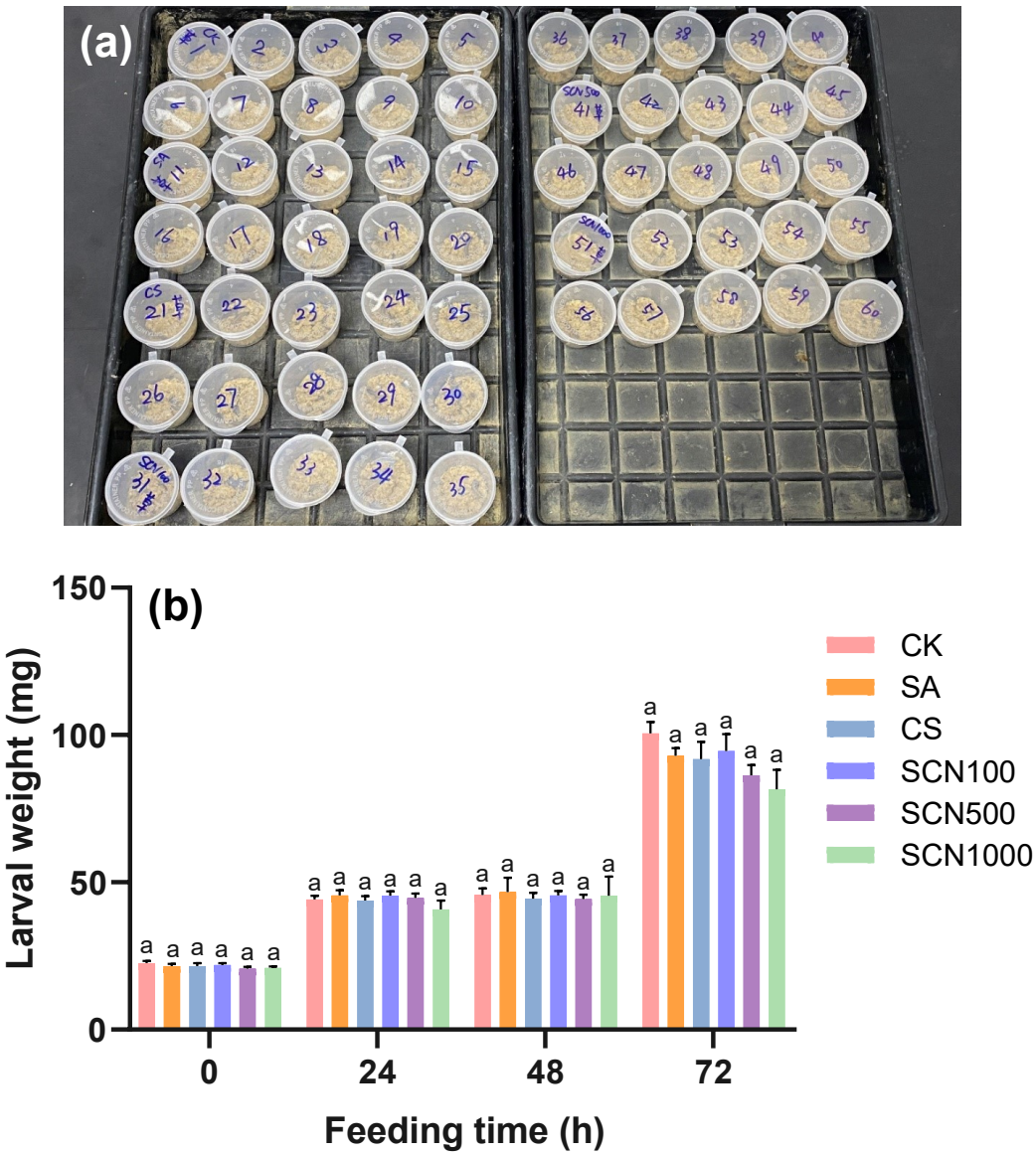

Fig. S2. *In vitro* feeding experiment with artificial diet testing the direct insecticidal effect of SA, CS, SCN100, SCN500 and SCN1000 in the boxes within 72 h feeding period (a). Larval weight (b). Data are means ± SE (n = 10). Different letters indicate significant difference among treatments at each feeding time ($p < 0.05$).

Figure S3

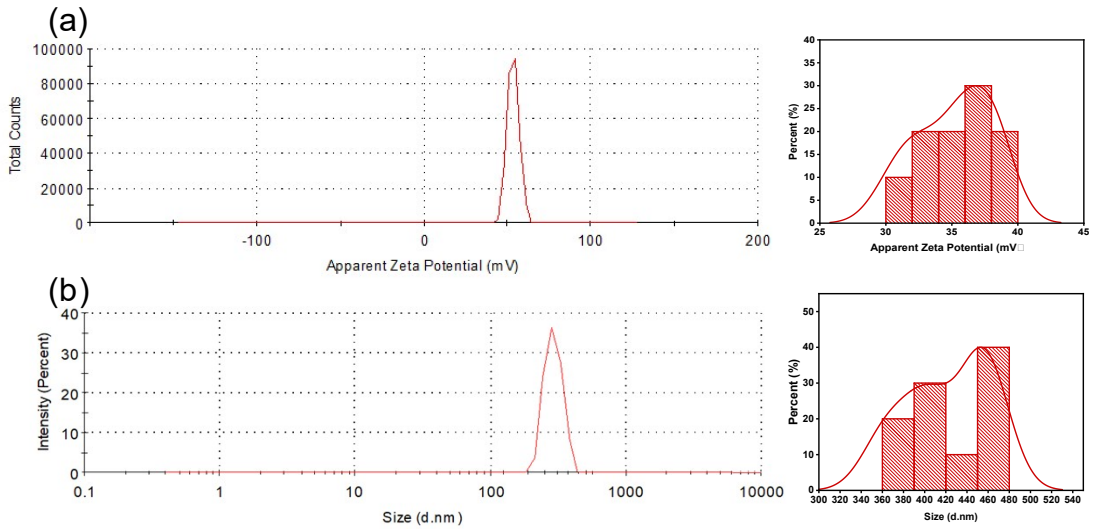

Fig. S3. DLS analysis for the (a) size distribution, and (b) zeta-potential of SCN.

Figure S4

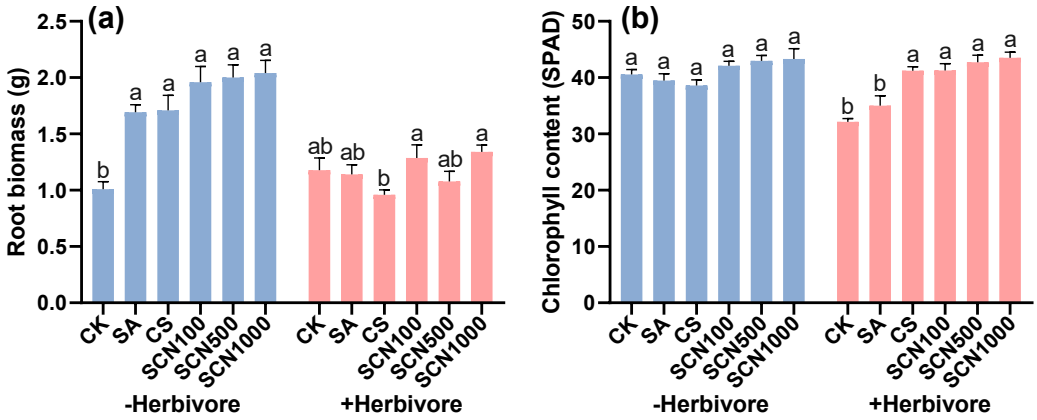

Fig. S4. Effect of SCNs on maize growth functional traits in the absence (−Herbivore) and presence (+Herbivore) of fall armyworm: Root biomass (a) and leaf chlorophyll (b). Significant differences among treatments are identified by different lowercase letters in the absence and presence of fall armyworm, respectively ($p < 0.05$).

Figure S5

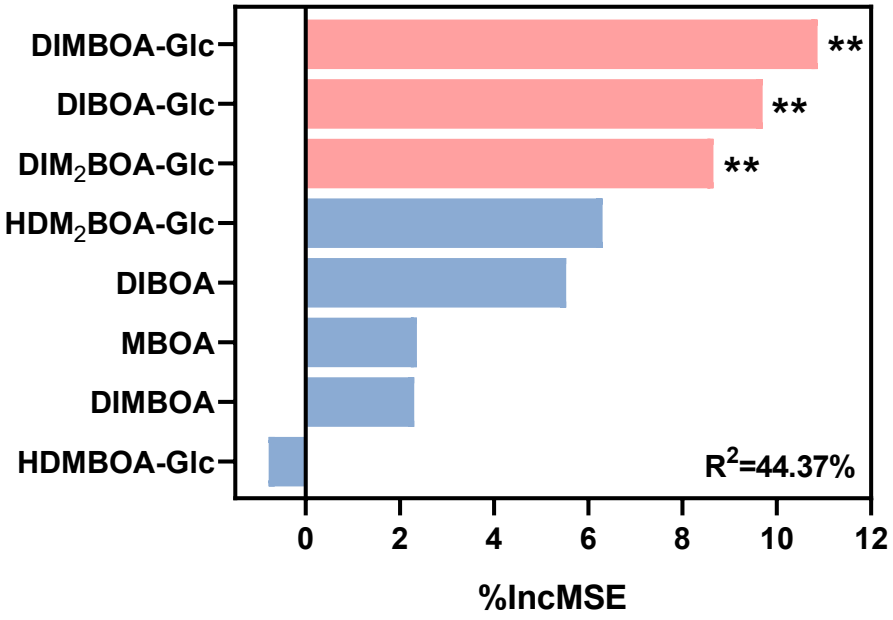

Fig. S5. Relative contribution of main benzoxazinoids to larval growth in terms of the weight gain. Shown is the mean predictor importance (%IncMSE) from the random forest analyses. Significance level of predictor is $^{**}p < 0.01$.

Figure S6

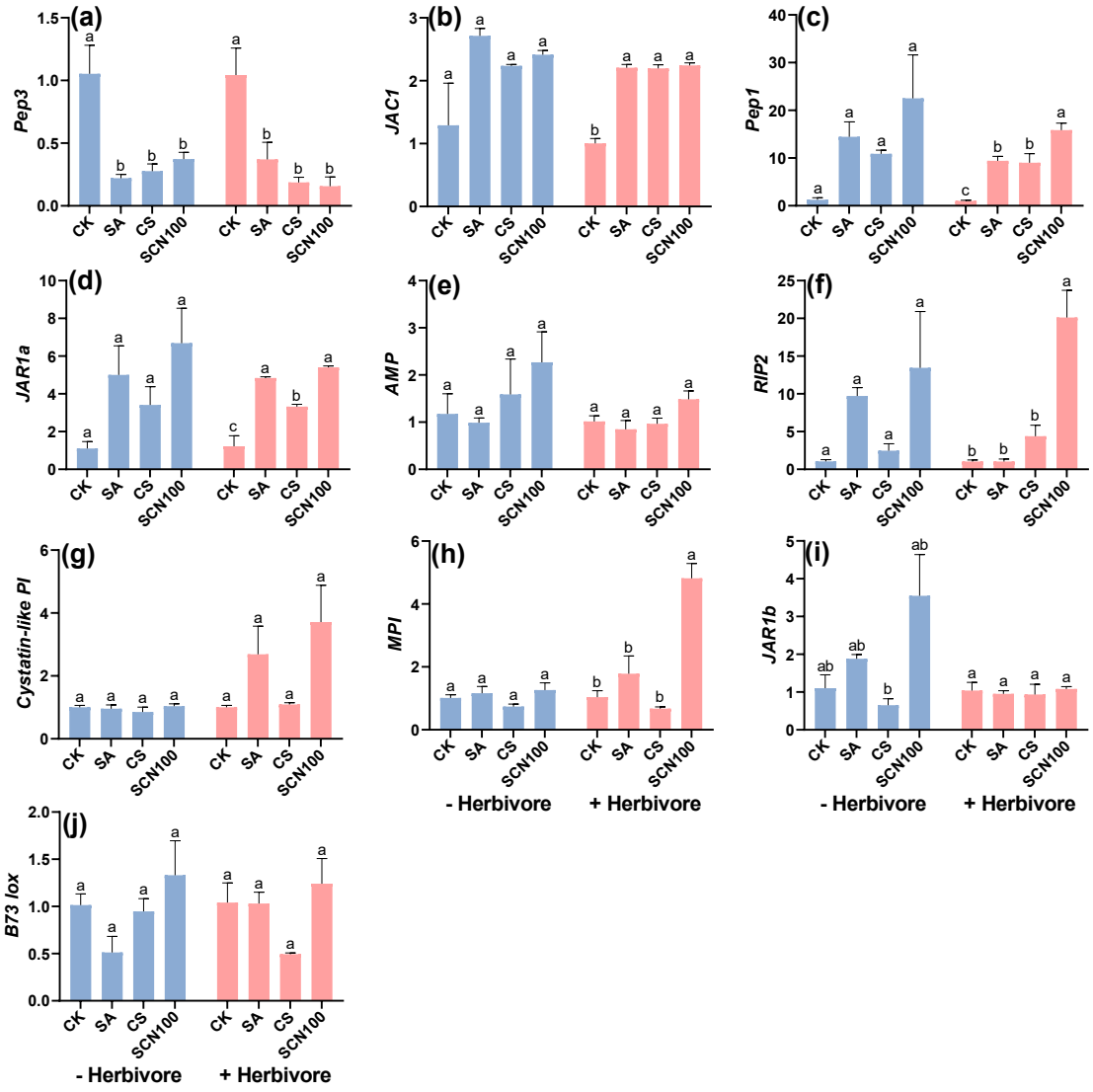

Fig. S6. Effect of SCN on maize leaf anti-insect gene expression in the absence (−Herbivore) and presence (+Herbivore) of fall armyworm: *Pep3* (a), *JAC1* (b), *Pep1* (c), *JAR1a* (d), *AMP* (e), *RIP2* (f), *Cystatin-like PI* (g), *MPI* (h), *JAR1b* (i), *B73 lox* (j). Significant differences among treatments are identified by different lowercase letters in the absence and presence of fall armyworm, respectively ($p < 0.05$).

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