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Role of soluble, cell wall bound phenolics, tannin and flavonoid contents in maize resistance to pink stem borer *Sesamia inferens* Walker

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Abstract

The pink stem borer *Sesamia inferens* Walker is an important pest of winter maize which causes significant yield losses. In an attempt to identify biochemical basis of resistance against *S. inferens*, total soluble phenolics, bound phenolics, cell wall bound hydroxycinnamic acids-p-coumaric acid (*p*-CA), ferulic acid (FA), total tannin content and total flavonoid contents, were measured in leaf at 10, 20 days after germination (DAG); stem at 20, 40 DAG; pith and rind tissues at 60 DAG (stem differentiated). From the present study, it was found that bound phenolics, *p*-CA, ferulic acid and total tannin contentscontribute to the maize defense mechanism against *S. inferens*. Total bound phenolic content showed negative correlation with Leaf Injury Rating (LIR). Highly significant strong positive correlation (+0.9750) was observed between LIR and total soluble phenolics in leaf tissue at 20 DAG. Similarly highly significant strong positive correlation between LIR and total tannins (+0.9354**) and flavonoids (+0.9582**) in pith at 60 DAG was observed. Further, strong significant positive correlation was also observed between LIR and *p*-CA (+0.9199*) in pith at 60 DAG and total ferulic acid (+0.9051*) in rind at 60 DAG. Significant strong negative correlation between LIR and *p*-CA (-0.8441*) in stem at 40 DAG was observed. The total bound phenolics in rind at 60 DAG (0.756), in leaf at 20 DAG (0.681) and total soluble phenolics in stem at 20 DAG (0.685) showed higher loadings with PC1, PC2 and PC3, respectively. Genotype-by-biochemical factor biplot showed that the data of biochemical parameters measured in different tissues and stages could be able to group the genotypes according to their reaction to *S. inferens*.

Introduction

The key insect pest limiting rabi maize yield in India is pink stem borer (PSB), *Sesamiainferens* Walker (Lepidoptera: Noctuidae). The early instar larvae of *S. inferens* upon hatching moves into the leaf whorl and feeds on unopened leaves resulting in oblong holes. The later instar larvae attacks stem, tender tassel and immature ears; severe infestation results in stunted growth of the plant (Reddy et al, 2003). The climatic conditions of tropical region favours rapid development of *S. inferens* and cause significant yield losses (25.7 to 78.9 per cent) in maize (Rao 1983). In order to reduce the losses caused by *S. inferens* several insect pest management approaches such as inter-cropping, trap-cropping, release of natural enemies (biocontrol) and use of synthetic insecticides have been employed. However, the effective management of the PSB pest remains a challenge as it feeds inside the whorl and stem. Under these circumstances, host plant resistance (HPR) could become one of the promising and effective means to reduce the losses due to *S. inferens* infestation. Further, HPR controls the insect pest without environmental hazards and is also compatible with other insect pest management approaches (Morais and Pinheiro, 2012). The search for HPR against *S. inferens* has led to identification of several sources of resistance in maize germplasm. Several workers have reported the biochemical basis of resistance to different corn borers (Bergvinson et al, 1995; Santiago et al, 2011). Among several biochemical compounds, phenolics have been reported as an important secondary metabolites involved in insect resistance in maize. Further, phenolic compounds also provide structural support, pigmentation, signaling, and defense against biotic and abiotic stresses in plants (Malvar et

al, 2017). In immature tissue, phenolic acids are mainly present as monomers esterified to cell wall hemicellulose but later form phenolic dimers linked to lignin (Dhillon and Chaudhary, 2015; Douglas, 1996). The major hydroxycinnamic acids, a widely distributed phenolic acid compounds which impart resistance against stem borers of maize are p-coumaric acid (*p*-CA) and ferulic acid (FA)(Bergvinson et al, 1995). It was reported that both *p*-CA and FA could participate in borer resistance by increasing the levels of cell wall cross linkage and lignin deposition (Ralph et al, 1994). However, the resistance to spotted stem borer Chilopartellus was due to the interaction of ferulic and *p*-CA with other biochemical constitutents (Dhillon and Chaudhary, 2015). Apart from hydroxycinnamic acids, various other biochemical constituents such as acid detergent lignin, acid detergent fiber (Santiago et al, 2011), DIMBOA, diferulates, polysaccharides (Jaime et al, 2011), surface wax (Bergvinson et al, 1995) etc. have also been reported to be associated with resistance/susceptibility to insect pests in maize. Even though several resistance sources in maize against *S. inferens* infestation have been identified (Sekhar et al, 2008, 2016a, 2016b), the biochemical basis of resistance has not yet been reported. In this context,the present study was carried out to investigate the role of phenolics in S. inferens resistance by using maize genotypes, which have already been identified as resistant or susceptible to the target pest.

Materials and Methods

Plant materials and experimental design

Five maize inbreds differing in pink stem borer (PSB) response viz., DMR E63 (early maturity, resistant); WNZPBTL 8 and WNZ ExoticPool (medium maturity, moderately resistant); CM 202 and BML 6 (late Maturity, susceptible) were used in the study. These lines were selected after multiple years of evaluations under artificial infested conditions (ICAR-DMR 2014; ICAR-IIMR 2015; ICAR-IIMR 2017) against *S. inferens*. These lines were further evaluated in large plots (7.5 m2) during rabi 2017-18 in a randomized complete block design (RCBD) replicated four times at Winter Nursery Centre (Latitude-17.3254; Longitude 78.4004; Sea level-527m), ICAR-Indian Institute of Maize Research, Rajendranagar, Hyderabad, Telangana, India. Each experimental unit or plot was of four rows of 2.5 m long with 75 × 20 cm spacing between rows and plants within row, respectively. The crop was raised by following recommended agronomic practices for inbred lines.

Infestation with S. inferens

The second generation neonate larvae of *S. inferens*, reared from field population were released into the whorls of 12 day old plants @ 10 larvae/plant with the help of camel hair brush (Tantawi et al. 1989). The ideal plant population of 12/row was maintained before artificial infestation and only the middle two rows were infested. Visual rating of ten plants damage was recorded by following 1-9 leaf injury rating (LIR) scale (Table 1) at 35 days after infestation (Reddy et al, 2003).The resistant, moderately resistant and susceptible lines are classified by LIR 1-3, >3.1-6 and >6.1-9 respectively.

Table 1 - Leaf Injury Rating scale (1-9) for *Sesamia inferens*

Rating	Description
1	Apparently healthy plant
2	Plant with parallel, oval or oblong holes, slightly bigger than pin sized (2-3 mm) on 1-2 leaves
3	Plant with more elongated holes (4-5 mm or match stick head sized) or shot holes on 1-2 leaves
4	Plant with injury (oval holes, shot holes and slits of 1-4 cm) in about 1/3 of total number of leaves and midrib damage on 1-2 leaves
5	Plants with about 50% leaf damage, oblong holes, shot holes, slits and streaks of 5-10 cms and midrib damage on leaves
6	Plants with a variety of leaf injuries to about two thirds of the total number of leaves (ragged appearance) or one or two holes or slits at the base of the stem (> 10 cms streaks are observed)
7	Plants with every type of leaf injury and almost all the leaves damaged (ragged or crimpled appearance), with tassel stalk boring or circular dark ring at the base of stem
8	Plants with stunted growth in which all the leaves are damaged
9	Plants with dead heart

Sampling for biochemical analysis

Fifteen seeds of each genotype were sown in plastic pots (30 cm height, 30 cm top diameter, and 19 cm bottom diameter) filled with black soil. Leaf samples were collected from three plants of each germplasm at 10 and 20 days after germination (DAG). Stem samples were also obtained in the same way at 20, 40 and 60 DAG. However, the stem samples at 60 DAG were manually separated into rind and pith tissues for independent analyses of the two single tissues. The samples

Table 2 - Leaf Injury Rating of maize genotypes under study based on 1-9 scale by *Sesamia inferens*

Inbreds	Pedigree	Pest Reaction	LIR (1-9 Scale)
DMRE 63	CM 500 SEL	Resistant	2.46±0.03^{e}
WNZPBTL 8	MIRTC4AmF150-B-1-3-B	Moderately Resistant	3.55±0.03^{d}
WNZ Exotic Pool	WNZPBTL1/2/3/4/5/6/7/8/9####	Moderately Resistant	4.15±0.08^{c}
CM 202	C121E	Susceptible	7.85±0.20^{a}
BML 6	SRRL 65-B96-1-1-2-#-2-2-1-0-1-1-0b-0b	Susceptible	6.62±0.04^{b}
LSD (P=0.05)			0.30

Each value represents the mean ± SEm of 4 replications. Means within a column followed by different letters are significantly different (LSDTest p =0.05)

were finely grounded in liquid nitrogen immediately after sampling and used for extraction of various components.

Extraction of cell wall bound hydroxycinnamate contents

The cell wall bound hydroxycinnamate contents were estimated according to Hung et al (2009).

Determination of p-CA and FA contents

The analysis was carried out using Shimadzu Ultra Fast Liquid Chromatograph (UFLC) equipped with SPD-M20A Prominence photo diode array detector. The HPLC pumps, auto sampler, column temperature and diode array system were monitored and controlled using LC Solution Chromatography data software program. *p*-CA and FA separation was performed on C18-

Table 3 - Amount of total soluble and bound phenolics in maize genotyps on fresh weight at different Days After Germination (DAG)

Total soluble phenolics						
	Leaf (mg/g)				Stem (mg/g)	
Inbreds	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
DMRE 63	2.98± 0.23^{b}	1.96±0.05^{a}	1.93±0.48^{ab}	1.17±0.07^{b}	1.63±0.21^{b}	1.27±0.21^{a}
WNZPBTL 8	1.69±0.05^{c}	2.22±0.20^{a}	1.45±0.48^{abc}	1.14±0.12^{b}	1.18±0.22^{b}	0.75±0.04^{a}
WNZ Exotic Pool	3.66±0.13^{a}	2.43±0.52^{a}	0.33±0.22^{c}	1.28±0.12^{ab}	2.77±0.36^{a}	0.87±0.23^{a}
CM 202	2.93±0.18^{b}	2.85±0.35^{a}	1.11±0.60^{bc}	1.39±0.13^{ab}	1.43±0.61^{b}	0.79±0.18^{a}
BML 6	3.14±0.12^{b}	2.78±0.09^{a}	2.78±0.44^{a}	1.58±0.10^{a}	1.47±0.17^{b}	1.02±0.40^{a}
LSD (P=0.05)	0.48	0.94	1.44	0.34	1.10	0.75
Total bound phenolics						
Inbreds	Leaf (mg/g)				Stem (mg/g)	
	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
DMRE 63	2.89±0.50^{a}	5.32±0.93^{a}	2.07±0.13^{a}	1.58±0.14^{a}	2.28±0.10^{d}	2.25±0.10^{b}
WNZPBTL 8	1.87±0.09^{b}	2.77±0.06^{c}	1.67±0.04^{b}	1.50±0.01^{a}	4.24±0.24^{c}	2.24±0.04^{b}
WNZ Exotic Pool	2.69±0.09^{a}	4.45±0.22^{ab}	0.60±0.01^{d}	1.64±0.12^{a}	7.69±0.21^{a}	3.19±0.39^{a}
CM 202	1.71±0.03^{b}	2.97±0.35^{c}	1.31±0.17^{c}	1.04±0.05^{b}	5.81±0.34^{b}	2.31±0.15^{ab}
BML 6	1.73±0.05^{b}	3.04±0.12^{bc}	1.04±0.03^{c}	1.46±0.26^{ab}	5.64±0.10^{b}	2.99±0.10^{a}
LSD (P=0.05)	0.73	1.44	0.30	0.45	0.68	0.61

Each value represents the mean ± SEm of 3 replications. Means within a column followed by different letters are significantly different (LSDTest p =0.05)

Table 4 - Amount of p-Coumaric and ferulic acids in maize genotypes at different Days After Germination (DAG)

p-CA Content						
	Leaf (mg/g FW)				Stem (mg/g FW)	
Inbreds	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
DMRE 63	0.57±0.01 ^{c}	1.22±0.03^{a}	0.79±0.01^{a}	1.01±0.02^{a}	0.93±0.01^{e}	0.80±0.01^{c}
WNZ PBTL 8	0.42±0.01^{d}	1.00±0.21^{ab}	0.59±0.01^{b}	0.82±0.03^{ab}	1.34±0.07^{d}	0.89±0.01^{c}
WNZExotic Pool	0.83±0.01^{a}	0.92±0.03^{ab}	0.10±0.03^{d}	0.87±0.15^{ab}	3.65±0.05^{a}	1.16±0.02^{a}
CM 202	0.70±0.02^{b}	0.70±0.03^{b}	0.61±0.09^{ab}	0.59±0.13^{b}	2.22±0.06^{b}	1.14±0.03^{ab}
BML 6	0.41±0.02^{d}	1.04±0.01^{a}	0.31±0.09^{c}	0.84±0.03^{ab}	1.68±0.07^{c}	0.96±0.13^{bc}
LSD (P=0.05)	0.04	0.30	1.18	0.28	0.17	0.19
Ferulic acid content						
Inbreds	Leaf (mg/g FW)				Stem (mg/g FW)	
	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
DMRE 63	0.43±0.01^{a}	0.61±0.01^{a}	0.34±0.01^{a}	0.27±0.01^{a}	0.25±0.01^{e}	0.24±0.01^{c}
WNZ PBTL 8	0.23±0.02^{d}	0.27±0.01^{c}	0.27±0.01^{b}	0.19±0.01^{b}	0.49±0.01^{d}	0.29±0.01^{a}
WNZ Exotic Pool	0.28±0.01^{b}	0.36±0.01^{b}	0.19±0.01^{c}	0.27±0.02^{a}	0.58±0.01^{c}	0.29±0.01^{a}
CM 202	0.26±0.01^{c}	0.35±0.04^{b}	0.26±0.02^{b}	0.24±0.02^{a}	0.68±0.01^{a}	0.30±0.02^{a}
BML 6	0.23±0.01^{d}	0.38±0.01^{b}	0.25±0.01^{c}	0.25±0.01^{a}	0.63±0.02^{b}	0.27±0.01^{b}
LSD (P=0.05)	0.01	0.05	0.03	0.04	0.03	0.01

Each value represents the mean ± SEm of 3 replications. Means within a column followed by different letters are significantly different (LSDTest p =0.05)

Phenomenex column (250 × 4.6 mm). The column was held at 35°C and the flow rate was set at 1.0 ml per minutes. The solvent system consisted of 2 per cent glacial acetic acid (A) and 100 per cent Acetonitrile (B). A gradient program of 15 per cent B and 85 per cent A for 20 minutes was followed with 20 µl sample injection. All samples were prepared and analyzed in three replications. The peaks of *p*-CA and FA were identified by standards at 280 nm with retention time 12.40 and 13.84 minutes respectively. The amount of *p*-CA and FA in maize leaf and stem samples were quantified by calibration curve of respective standards with the help of Shimadzu LC Solution Software.

Determination of total soluble and bound phenolic contents

Total soluble and bound phenolics were estimated by quantifying the extracted phenolic compounds through Folin-Ciocalteu assay (Singleton et al, 1999). Data were expressed in milligram per gram fresh weight. All tests were carried out in triplicate.

Determination of total tannin content

The tannins were determined by the method of Duval and Shetty (2001). The estimation of the tannin content was carried out in triplicate. The tannin content was expressed in terms of mg of tannic acid equivalents/ g of fresh weight.

Determination of total flavonoid content

Total flavonoids content (TFC) was determined by aluminium trichloride method using catechin as reference compound (Zhishen et al, 1999). TFC was expressed as catechin equivalents (CE)/g of fresh weight.

Statistical Analysis

Leaf injury rating and biochemical parameters were subjected to analysis of variance (ANOVA) by using general linear model (PROC GLM), performed with SAS version 9.3(SAS Institute 2011). Further, the data were subjected to Pearson correlation, Principal Component analysis (PCA) using PROC CORR, PRIN COMP, procedures of SAS, respectively to understand the association of biochemical parameters with leaf injury rating.

Table 5 -Amount of total tannin and flavonoid contents in maize genotypes at different Days After Germination (DAG)

Total Tannin Content						
	Leaf (mg/g FW)				Stem (mg/g FW)	
Inbreds	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
DMRE 63	1.05±0.09^{a}	2.52±0.19^{a}	1.19±0.15^{a}	1.35±0.15^{a}	0.88±0.22^{b}	0.96±0.11^{b}
WNZ PBTL 8	0.74±0.10^{b}	1.87±0.07^{b}	1.34±0.12^{a}	0.88±0.05^{b}	1.72±0.15^{ab}	0.84±0.03^{b}
WNZExotic Pool	0.71±0.14^{b}	2.27±0.10^{a}	0.29±0.04^{c}	1.12±0.09^{ab}	2.78±0.73^{a}	0.95±0.06^{b}
CM 202	0.77±0.08^{ab}	1.81±0.10^{bc}	0.89±0.01^{b}	1.16±0.09^{ab}	2.35±0.14^{a}	1.53±0.07^{a}
BML 6	0.10±0.02^{c}	1.47±0.05^{c}	0.44±0.06^{c}	1.03±0.15^{ab}	2.22±0.39^{a}	1.35±0.08^{a}
LSD (P=0.05)	0.29	0.35	0.29	0.35	1.23	0.22
Total Flavonoid content						
Inbreds	Leaf (mg/g FW)				Stem (mg/g FW)	
	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
DMRE 63	0.04±0.01^{b}	0.11±0.01^{c}	0.08±0.01^{ab}	0.05±0.01^{b}	0.07±0.01^{a}	0.09±0.01^{b}
WNZPBTL 8	0.05±0.01^{b}	0.09±0.01^{c}	0.07±0.01^{b}	0.04±0.01^{b}	0.08±0.01^{a}	0.08±0.01^{b}
WNZExotic Pool	0.12±0.02^{a}	0.19±0.01^{b}	0.05±0.02^{b}	0.10±0.02^{a}	0.10±0.01^{a}	0.09±0.01^{b}
CM 202	0.11±0.01^{a}	0.25±0.02^{a}	0.11±0.01^{a}	0.09±0.01^{a}	0.09±0.04^{a}	0.14±0.03^{a}
BML 6	0.12±0.01^{a}	0.16±0.01^{b}	0.07±0.01^{b}	0.08±0.01^{ab}	0.13±0.05^{a}	0.11±0.01^{ab}
LSD (P=0.05)	0.04	0.02	0.03	0.03	0.08	0.04

Each value represents the mean ± SEm of 3 replications. Means within a column followed by different letters are significantly different (LSDTest p =0.05)

The significance of differences between the genotype means were judged by least significant differences (LSD) at P = 0.05. Further, the biplot analysis using GGEBiplotGUI package was done in R to understand genotypes-trait relationship [R version 3.4.0 (2017-04-21) -- "You Stupid Darkness" Copyright (C) 2017 The R Foundation for Statistical Computing Platform: i386-w64-mingw32/i386 (32-bit)].

Results and discussion

Response of lines to pink stem borer infestation

Data on the leaf injury rating (LIR) of five genotypes screened against S. inferens are presented in Table 2. Among the genotypes, minimum mean LIR was recorded in DMRE 63 (2.46). Based on this, DMRE 63 is rated as resistant and WNZPBTL8 (3.55) and WNZ Exotic Pool (4.15) as moderately resistant. The genotypes CM 202 and BML 6 were found to be susceptible to pink stem borer with LIR score of 7.85 and 6.62, respectively.

Total soluble phenolics

The concentration of total soluble phenolics in leaf at 10 and 20 DAG; stem at 20, 40 DAG and in rind and pith at 60 DAG are given in Table 3. The total soluble phenolics content in leaf tissues at 10 DAG differed significantly between genotypes. The highest concentration of total soluble phenolics at 10 DAG was observed in WNZ Exotic Pool (3.66 mg/g). But there were no significant differences among the genotypes with respect to total soluble phenolics in leaf tissues at 20 DAG. The concentration at 10 DAG ranged from 1.96 mg/g in DMRE 63 to 2.85 mg/g in CM 202.In contrast to leaf, the concentration of total soluble phenolics in stem tissue at 20 DAG differed significantly among genotypes. BML 6 had highest concentrations of soluble phenolics (2.78 mg/g) while WNZ ExoticPool contained lowest quantities (0.33 mg/g). There was no statistically significant difference between BML 6, DMRE 63 and WNZPBTL 8. The level of total soluble phenolic acids in stem samples at 40 DAG was higher again in BML 6 (1.58 mg/g). But, the genotypes DMRE 63 and WNZPBTL 8 which did not show statistically significant

Table 6 - Pearson's correlation coefficients for various biochemical traits in maize genotypes under study with damage parameter (LIR) caused by S. inferens at different Days After Germination (DAG)

Biochemical Parameters	Leaf (mg/g FW)		Stem (mg/g FW)			
	10 DAG	20 DAG	20 DAG	40 DAG	60 DAG	
					Rind	Pith
Total soluble phenolics	0.2115	0.9750**	0.1138	0.7967	-0.1989	-0.4344
Total bound phenolics	-0.7612	-0.6537	-0.4539	-0.8129	0.5069	0.1744
p-Coumaric acid	0.0624	-0.7449	-0.2233	-0.8441*	0.2530	0.9199*
Ferulic acid	-0.5785	-0.4083	-0.3762	-0.0025	0.9051*	0.2494
Tannins	-0.5632	-0.7519	-0.3982	-0.1959	0.6081	0.9354**
Flavonoids	0.6833	0.8117	0.4215	0.6549	0.7398	0.9582**

** Significant at 0.01% level * Significant at 0.05% level

difference with BML 6 for total soluble phenolic acids in stem sample at 20 DAG differed significantly with BML 6 at 40 DAG. Both DMRE 63 (1.17 mg/g) and WNZPBTL 8 (1.14 mg/g) had lower-levels of total soluble phenolics with no statistical significant difference among them and with other two genotypes WNZ ExoticPool (1.28 mg/g) and CM 202 (1.39 mg/g).The amount of total soluble phenolic acids in rind and pith tissues at 60 DAG varied from 1.18 to 2.77 mg/g and 0.87 to 1.27 mg/g, respectively. However, significant differences among genotypes wereobserved only in rind. Among all genotypes, WNZ ExoticPool had higher concentration of total soluble phenolic acids (2.77 mg/g) in rind, which was statistically significantly different from rest of the genotypes, which did not differ statistically among them.

The results of the present study indicate that various biochemical constituents, their combination, levels of expression in different tissues and stage of expression together determine the resistance reaction to S. inferens in maize. Bergvinson (1993) reported that soluble phenolics did not make a major contribution to host plant resistance but used for host plant recognition by European stem borer Ostrinianubilalis. In another study, Santiago et al(2006) reported positive correlation between total soluble phenolics and LIR caused by S. inferens indicating no role in imparting resistance to S. inferens. However in the present study, there was no statistically significant difference between resistant and susceptible genotypes for total soluble phenolics content. Thus, it corroborates the earlier findings that, soluble phenolics do not play a role in imparting resistance at least to pink stem borer of maize.

Total bound phenolics

The amount of total bound phenolics in leaf (at 10, 20 DAG), stem (at 20, 40 DAG) and rind and pith at 60 DAG differed significantly among the genotypes (Table 3). It ranged from 1.71 to 2.89 and 2.77 to 5.32 mg/g in leaf tissue at 10 and 20 DAG, respectively; whereas in stem tissue it ranged from 0.60 to 2.07 and 1.04 to 1.64 at 20 and 40 DAG, respectively. At 60 DAG, it ranged from 2.28 to 7.69 in rind and 2.24 to 3.19 in pith. The amount of bound phenolic acids was consistently higher in DMRE 63 in leaf at 10 DAG (2.89 mg/g) and 20 DAG (5.32 mg/g) and also in stem at 20 DAG (2.07 mg/g). In rind and pith, the amount of bound phenolic acids in DMRE 63 was lowest i.e. 2.28 and 2.25 mg/g, respectively. Among all the other genotype WNZ Exotic Pool also recorded consistently highest bound phenolic acids across different tissues (leaf, stem, rind and pith) and stages (10, 20, 40, 60 DAG) except in stem at 20 DAG (0.60 mg/g). The amount of bound phenolic acids in WNZ Exotic Pool was statistically comparable to DMRE 63 in leaf at 10 (2.69 and 2.89 mg/g) and 20 (4.45 and 5.32 mg/g) DAG and also in stem at 40 DAG (1.64 and 1.58 mg/g). It recorded highest bound phenolics in both pith and rind tissues at 60 DAG. In pink stem borer susceptible genotypes CM 202 and BML 6 did not show statistically significant difference with respect to total bound phenolics in any of the tissues and stages. In general they had significantly lower values than DMRE 63 (resistant to *S. inferens*) in leaf tissue (at 10 and 20 DAG) and stem at 20 DAG.The level of bound phenolic acids in WNZPBTL 8 varied differentially based on stage and type of tissue. Anew and concrete evidence was shown that the cell-wall bound phenolics could have a significant role in resistance to S. nonagrioides (Santiago et al, 2013). In the present study also total bound phenolics were consistently higher in the resistant (DMRE 63) and in one of the moderately resistant (WNZ ExoticPool) genotypes (Table 2) in leaf and stem at different stage. Further, total bound phenolic content showed negative correlation with LIR (Table 5 and Fig. 1) suggesting possible role of bound phenolics in resistance to *S. inferens*.

Table 7 - Contribution of Principal component axis (PCA) to the variation of biochemical traits in different tissues of maize genotypes under study

Biochemical Traits		PC1	PC2	PC3	PC4
		Total Soluble Phenolics			
Leaf at 10 DAG-X1	1	0.14960	0.25842	0.41083	0.43206
Leaf at 20 DAG-X2	2	0.08837	-0.1408	0.10949	0.30543
Stem at 20 DAG-X3	3	-0.19183	-0.24608	0.68525	-0.14152
Stem at 40 DAG-X4	4	0.02896	-0.0511	0.13929	0.09243
Rind at 60 DAG-X5	5	0.1573	0.28654	0.03321	-0.15930
Pith at 60 DAG-X6	6	-0.04476	0.07348	0.13732	0.03025
		Total Bound Phenolics			
Leaf at 10 DAG-X7	7	-0.03203	0.35137	-0.00116	-0.15029
Leaf at 20 DAG-X8	8	-0.09800	0.68108	0.18544	0.06470
Stem at 20 DAG-X9	9	-0.20764	0.00586	-0.11990	0.12326
Stem at 40 DAG-X10	10	-0.00753	0.08448	0.04673	-0.36132
Rind at 60 DAG-X11	11	0.75680	-0.09228	0.02137	-0.08129
Pith at 60 DAG-X12	12	0.13347	0.05717	0.24665	-0.27814
		***p*-CA**			
Leaf at 10 DAG-X13	13	0.04174	0.07150	-0.05363	0.13870
Leaf at 20 DAG-X14	14	-0.04673	0.04743	0.07910	-0.18445
Stem at 20 DAG-X15	15	-0.09122	-0.01214	-0.07605	0.19649
Stem at 40 DAG-X16	16	-0.02458	0.06025	0.05202	-0.16232
Rind at 60 DAG-X17	17	0.37786	0.19164	-0.13738	0.02864
Pith at 60 DAG-X18	18	0.05461	-0.00230	-0.02748	0.10383
		Ferulic acid			
Leaf at 10 DAG-X19	19	-0.01794	0.04114	0.00822	0.03775
Leaf at 20 DAG-X20	20	-0.02717	0.05381	0.05450	0.06792
Stem at 20 DAG-X21	21	-0.01961	0.00247	0.00273	0.02310
Stem at 40 DAG-X22	22	0.00259	0.01527	0.01926	0.02077
Rind at 60 DAG-X23	23	0.04994	-0.06286	0.00766	0.06901
Pith at 60 DAG-X24	24	0.00630	-0.00715	-0.01332	0.00396
		Total Tannin Content			
Leaf at 10 DAG-X25	25	-0.05227	0.13077	-0.24026	0.16919
Leaf at 20 DAG-X26	26	-0.03963	0.24010	-0.12688	0.01161
Stem at 20 DAG-X27	27	-0.14292	-0.04488	-0.25988	0.01946
Stem at 40 DAG-X28	28	-0.01758	0.07877	0.04468	0.19535
Rind at 60 DAG-X29	29	0.26831	-0.09296	-0.00352	0.04376
Pith at 60 DAG-X30	30	0.02885	-0.08916	0.11459	0.43159
		Total Flavonoid Content			
Leaf at 10 DAG-X31	31	0.01316	-0.00456	0.01569	0.01708
Leaf at 20 DAG-X32	32	0.01634	-0.00324	0.00237	0.08948
Stem at 20 DAG-X33	33	-0.00253	-0.00520	-0.00379	0.03599
Stem at 40 DAG-X34	34	0.00857	0.00211	0.00689	0.01911
Rind at 60 DAG-X35	35	0.00491	-0.00584	0.01718	-0.00606
Pith at 60 DAG-X36	36	0.00238	-0.00588	0.00405	0.03958
Eigen Value		7.02	2.49	0.89	0.27
Proportion		65.74	23.31	8.39	2.56

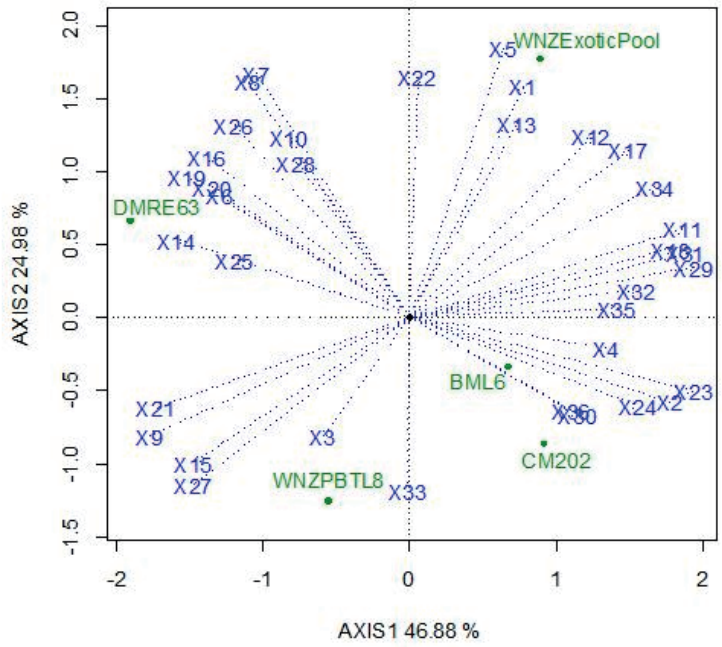

Fig. 1 - Biplot analysis for biochemical traits in different maize genotypes under study

PC1=46.88%, PC2=24.98%; PC1+PC2=73.86%

Scaled (divided) by: 1-Standard Deviation (SD)

Centered by: 2-Tester -Centred G+GE

S.V.P.: GH-(Column Metric Preserving)

Total soluble phenolics:Leaf at 10 DAG-X1, Leaf at 20 DAG-X2, Stem at 20 DAG-X3, Stem at 40 DAG-X4, Rind at 60 DAG-X5, Pith at 60 DAG-X6

Total bound phenolics: Leaf at 10 DAG-X7, Leaf at 20 DAG-X8, Stem at 20 DAG-X9, Stem at 40 DAG-X10, Rind at 60 DAG-X11, Pith at 60 DAG-X12

***p*-CA content**: Leaf at 10 DAG-X13, Leaf at 20 DAG-X14, Stem at 20 DAG-X15, Stem at 40 DAG-X16, Rind at 60 DAG-X17, Pith at 60 DAG-X18

Ferulic acid content: Leaf at 10 DAG-X19, Leaf at 20 DAG-X20, Stem at 20 DAG-X21, Stem at 40 DAG-X22, Rind at 60 DAG-X23, Pith at 60 DAG-X24

Total Tannin Content: Leaf at 10 DAG-X25, Leaf at 20 DAG-X26, Stem at 20 DAG-X27, Stem at 40 DAG-X28, Rind at 60 DAG-X29, Pith at 60 DAG-X30

Total Flavonoid content: Leaf at 10 DAG-X31, Leaf at 20 DAG-X32, Stem at 20 DAG-X33, Stem at 40 DAG-X34, Rind at 60 DAG-X35, Pith at 60 DAG-X36

***p*- CA and FA content**

It was observed that *p*-CA and FA content in leaf (at 10 and 20 DAG), stem (at 20 and 40 DAG) and rind and pith at 60 DAG differed significantly among genotypes (Table 4). The amount of *p*-CA in leaf varied from 0.41 to 0.83 and 0.70 to 1.22 mg/g FW in different maize inbred lines at 10 and 20 DAG respectively; whereas in stem it varied from 0.10 to 0.79 and 0.59 to 1.01 mg/g at 20 and 40 DAG respectively. Similarly in rind and pith it varied from 0.93 to 3.65 and 0.80 to 1.16 respectively. The genotype WNZ ExoticPool showed consistently higher level of *p*-CA in leaf (0.83 and 0.92 mg/g at 10 and 20 DAG), stem (0.87 mg/g at 40 DAG) and rind (3.65 mg/g) and pith (1.16 mg/g) at different stages except in stem at 20 DAG (0.10 mg/g). In fact in WNZ ExoticPool, consistently higher levels of total soluble and bound phenolic acids and *p*-CA was observed in leaf, stem, rind and pith tissue except stem at 20 DAG where they were consistently lowest (Table 2&3). DMRE 63 had higher levels of *p*-CA in leaf at 20 DAG

(1.22 mg/g) and also in stem tissues at 20 (0.79 mg/g) and 40 DAG (1.01 mg/g); on the contrary in rind (0.93 mg/g) and pith (0.80 mg/g) it showed the lowest *p*-CA among all the tested genotypes. The inbred CM 202 showed lowest levels of *p*-CA both in leaf at 20 DAG (0.70 mg/g) and also in stem tissues at 40 DAG (0.59 mg/g); it also had lowest total soluble and bound phenolics as well in leaf and stem. In WNZPBTL 8 and BML 6, the level of p-CA varied differentially depending on type of tissue and stage. DMRE 63 had consistently higher FA in leaf (0.43 and 0.61 mg/g) and stem (0.34 and 0.27 mg/g) (Table 3); the genotype also had consistently higher total bound phenolics in leaf and stem. WNZPBTL 8 has consistently lowest level of FA in leaf at 10 and 20 DAG and in stem at 40 DAG. The ferulic acid content in rind and pith tissues was higher in CM 202 (0.68 mg/g, 0.30 mg/g) while lower concentrations were found in DMRE 63 (0.25 mg/g, 0.24 mg/g), at 60 DAG respectively.

In the present study considerable variation in the content of p-CA and ferulic acid among the genotypes at different plant age was observed, which is in agreement with Orsaket al.(2001) who reported that the variation in secondary metabolites is attributed to the genetic makeup of cultivar. In general *p*-CA was found to be the main hydroxycinnamic acid in stalks of cereals(Sun et al. 2001). It was observed that in almost all the samples analyzed using contrasting maize genotypes in different tissues and stages; *p*-CA indeed is the dominant phenolic acid as compared to ferulic acid. Both *p*-CA and FA involved in cross-linkage with lignin; in cells, *p*-CA is mainly esterified to the γ -position of phenyl propanoid side chains of S units in lignin (Lu and Ralph, 1999). The higher concentrations of cell wall bound *p*-CA and FA in leaf tissues at 10 DAG were found in moderately resistant (WNZ ExoticPool) and resistant (DMRE 63) lines respectively as compared to the susceptible lines (CM 202 and BML 6). The ferulic acid content was significantly higher in resistant genotype DMRE 63 in leaf at 10 and 20 DAG and stem at 20 DAG as compared to susceptible genotypes. The amount of bound phenolic acids was low in the leaf tissues at 10 DAG, however the concentrations increased rapidly in leaf tissues at 20 DAG. The possible reason might be phenolic acids cross linking is associated with tissue maturation which is evident from the low phenolics content in 10 DAG and its higher levels in 20 DAG. In fact 10-20 DAG is the most critical period for stem borer attack on the host plants (Sekhar et al, 2015).

The amount of hydroxycinnamic acids (*p*-CA and FA) was higher in rind tissues compared to pith tissues at 60 DAG. The results are in agreement with Santiago et al.(2011) who reported greater concentrations of *p*-CA and ferulic acid in the rind than in the pith tissues of maize. This is because rind vascular tissues lignify to a greater extent to support the conductive and supportive tissues of the internode(Morrison et al, 1998). This might be one of the reasons that the second generation larvae of pink stem borer feed on pith tissues compared to rind. However, such striking difference in *p*-CA and FA content in rind and pith is absent in resistant genotype DMRE 63. The results showed that the amount of FA is consistently and significantly higher in leaf at 10 and 20 DAG and in stem at 20 DAG in resistant (DMRE 63) genotype over susceptible (CM 202 and BML 6) genotypes which indicates the plausible role in resistance. Further, significant difference in *p*-CA and FA content in pith and rind tissues at 60 DAG between susceptible and resistant lines indicates probably the differences in maturity.

Total tannin content

The results on total tannin content of PSB resistant and PSB susceptible maize tested genotypes were presented in Table 5. DMRE 63 (resistant genotype) showed significantly higher total tannins in leaf (1.05 and 2.52 mg/g) and stem (1.19 and 1.35 mg/g) tissue and significantly lower total tannins in rind (0.88 mg/g) and pith (0.96 mg/g). On the contrary, BML 6 (susceptible genotype) showed significantly lower total tannins in leaf (0.10 mg/g, 1.47 mg/g), and stem (0.44 and 1.03 mg/g) and significantly higher total tannins in rind (2.22 mg/g) and pith (1.35 mg/g) In addition to BML 6, higher concentration of total tannins in rind (2.35 mg/g) and pith (1.53 mg/g) was also observed in another susceptible genotype CM 202 and both the susceptible genotypes are statistically not different .

Total flavonoid content

The results showed that genotypes differed significantly for total flavanoids content in leaf, stem and pith tissues (Table 5). The significant differences between resistant (DMRE 63) and susceptible (CM 202 and BML 6) genotypes were observed for total flavanoids in leaf tissue, where resistant genotype contained low level (0.04 and 0.11 mg/g) and susceptible genotype contained higher levels (0.11, 0.25 in CM 202 and 0.12, 0.16 in BML 6). However, such clear differences were not found in other tissues like stem, rind and pith. The differences between genotypes in stem at 20 and 40 DAG and rind and pith at 60 DAG differed differentially depending both on tissue and stage. Higher levels of flavonoid content were detected in CM 202 (one of the susceptible genotypes) consistently in leaf (0.11 and 0.25 mg/g) respectively at 10 and 20 DAG, stem (0.11 and 0.09 mg/g) respectively at 20 and 40 DAG, andpith (0.14 mg/g) at 60 DAG. In the present study, other ma-

jor phenolic compounds tannins and flavonoids revealed their role in determining resistance to S. inferens. The significant variation was observed in tannin content among genotypes. Tannin content in resistant genotypes was significantly higher in leaf and stem at 20 DAG as compared to susceptible genotypes. It was reported that flavonoids protect plants against insect pests by influencing the behavior, growth and development of insects. Flavonoids act as strong feeding deterrents and also as stimulants to herbivores (Simmonds, 2001). In the present study, resistant and susceptible genotypes differed significantly for total flavanoids content in leaf at 10 and 20 DAG. On the contrary, no significant differences among genotypes were found for flavonoid content in maize suggesting no role in S. nonagrioides resistance (Malvar et al, 2017).

Correlation between various biochemical parameters and LIR

The Pearson's correlation coefficients (r) between various biochemical traitsat different plant age and leaf injury rating are presented in Table 6. Highly significant strong positive correlation (+0.9750**) was observed between LIR and total soluble phenolics in leaf tissue at 20 DAG. Similarly highly significant strong positive correlation between LIR and total tannins (+0.9354**) and total flavanoids (+0.9582**) in pith at 60 DAG were observed. Further, strong significant positive correlation was also observed between LIR and *p*-CA (+0.9199*) in pith at 60 DAG and total ferulic acid (+0.9051*) in rind at 60 DAG. Significant strong negative correlation between LIR and *p*-CA (-0.8441*) in stem at 40 DAG was observed. A non significant negative correlation was also observed between LIR and *p*-CA (-0.8129) in stem at 40 DAG and total bound phenolics (-0.7612) in leaf at 10 DAG. The genotypes differed as resistant, moderately resistant and susceptible to pink stem borer, similarly, the amount of biochemical constituents' also varied among genotypes indicating specific role of different biochemical constituents as part of defense against S. inferens. The present result is in accordance with Dhillon and Chaudhary (2015) who demonstrated role of biochemical compounds in sorghum against C. partellusin determining resistance reaction. The correlation between hydroxycinnamic acid (*p*-CA) and LIR in leaf at 20 DAG and stem at 20 and 40 DAG was significant, strong and negative. The result is in agreement with Bergvinson (1995) who reported that *p*-CA and ferulic acid content was negatively correlated with leaf damage ratings; *p*-CA and FA are associated with resistance to O. nubilalis. Similarly tannins also showed negative correlation with LIR suggesting possible role of resistance to S. inferens. The present result is in accordance with Sharma and Sujana (2009) who reported that tannins have strong deleterious effect on insect pests and affect their growth and development. In the current evaluation, based on correlations, PCA and biplot analysis, the relationship between total flavonoid content and insect resistance is unclear. The PCA and biplot analysis was done by considering different biochemical constituents in different tissues and stages as independent and separate factor to understand critical biochemical compound responsible for determining resistance. The result indicated that not one factor but the combination of many factors contribute for resistance to *S. inferens*. The PCA results indicated that total bound phenolics in rind at 60 DAG and in leaf at 10 and 20 DAG, p-CA and total tannins content in rind at 60 DAG, total soluble phenolics in leaf at 10 and 20 DAG together explained around 97 per cent of total variation.

Principal Component Analysis

The results of principal component analysis (PCA) of various biochemical traits measured in different tissues at different stages in the different genotypes under study are presented in Table 7. PC1, PC2, PC3 and PC4 accounted for 65.74, 23.31, 8.39 and 2.56 per cent of the total variation respectively and together accounted 97.44 per cent of the total variation. The total bound phenolics in rind at 60 DAG (0.756), in leaf at 20 DAG (0.681) and total soluble phenolics in stem at 20 DAG (0.685) showed higher loadings with PC1, PC2 and PC3, respectively. However, other parameters like *p*-CA in rind at 60 DAG, total tannins in rind at 60 DAG also showed relatively higher correlation coefficient with PC1.

Biplot analysis of genotypes with different biochemical traits

The relationship between genotypes and different biochemical constituents is visualized in the form of Genotype-Trait biplot (Figure 1). The resistant genotype DMRE 63 showed strong correlation with p-CA content present in leaf at 20 DAG and in stem at 40 DAG and total tannin content present in leaf at 10 DAG. In addition it has also showed high correlation with total soluble phenolics present in pith at 60 DAG and ferulic acid content in leaf at 10 and 20 DAG. The genotype WNZPBTL 8 (moderately resistant to *S. inferens*) showed prominent correlation to total soluble phenolics and total flavonoid content present in stem at 20 DAG. WNZ Exotic Pool, another moderately resistant genotype showed strong correlation with total soluble phenolics and *p*-CA content in leaf at 10 DAG, total soluble phenolics present in rind at 60 DAG, total bound phenolics present in pith tissues at 60 DAG and ferulic

acid content present in stem at 40 DAG. Whereas in the susceptible genotypes (CM202 and BML6), the biochemical compounds which resulted highly correlated with either resistant or moderately resistant genotypes were found in low concentration. In the biplot, the susceptible genotypes were exactly in opposite side in relation to resistant genotype DMRE63, while moderate resistant lines were placed in intermediate groups.

DMRE 63, a resistant genotype grouped distinctly as compared to susceptible genotypes CM202 and BML6 through Genotype-by-biochemical factor biplot. The biochemical factors which showed high correlation with DMRE 63 in biplot were also having high mean values. On the contrary, the biochemical parameters which have high correlation with DMRE 63 have significantly lower mean values in both the susceptible lines. The moderately resistant lines namely WNZPBTL 8 and WNZ ExoticPool fell in between resistant and susceptible. The factors responsible for moderate resistance found to be different between themselves. Thus, the biplot analysis could able to explain that the resistance to S. inferens is determined by not any one but different biochemical compounds in different levels, tissues and stages together determines the resistance mechanism. The present study throws light on the changes in levels of biochemical constituents during different plant developmental stages and its role in determining host plant resistance. This is because most of the compounds undergo dramatic changes in chemical constituents during maturation and also due to different weather conditions during their development. In fact interaction between host-plant and insect pest involve two most important factors like time and space (micro- and macro-climate).

Conclusions

The results indicated that bound phenolics, *p*-CA, ferulic acid and total tannin content contribute to the maize defense mechanism against *S. inferens.* The results of the study suggested that increasing the concentration of the phenolic compounds through breeding approaches may improve natural resistance in maize against pink stem borer. However, the underlying mechanism of resistance in different genotypes may differ depending on the combination of biochemical compounds, stage and level of expression. As the two moderately resistant genotypes (WNZPBTL 8 and WNZ ExoticPool) tested in the present study showed contrasting combinations of biochemical compounds, studies on crosses between resistant and susceptible inbreds may further help to understand mechanisms of resistance.

Abbreviations Used

p-CA - p-coumaric acid; FA - Ferulic acid; LIR - Leaf Injury Rating; DAG - days after germination;PSB - pink stem borer

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Conflict of interest

The authors declare that there is no conflict of interest.

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