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Biochemical, Histochemical and Enzymatic Studies in Relation to Sorghum Downy Mildew Infection of Resistant and Susceptible Genotypes of Maize

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ABSTRACT

Biochemical, histochemical and enzymatic studies were conducted on indigenous susceptible, CM-500, NAI-127 and resistant, NAI-129, SKV-10 maize leaves infected with *Peronosclerospora sorghi* and sampling was done at 15, 30, 45, 60 and 75 days after infection with appropriate controls. Biochemical observations revealed high total sugars, starch and amino-acid content in resistant lines compared to susceptible in both inoculated and uninoculated leaves at all times whereas the total phenol and ortho dihydroxy phenols decreased during the first sampling, then later increased consistently in inoculated tissues at all the days. Further evidence, from leaf histochemical analysis draws the same trend as described in the above biochemical analysis. Upon pathogen attack, peroxidase (POX) and phenylalanine ammonia-lyase (PAL) activity increased drastically and high levels were maintained at all times. Higher levels of carbohydrates, amino-acids, different phenols and also elevated activity of POX and PAL in NAI-129, SKV-10 may play a role in inhibiting *P. sorghi*.

Keywords: amino-acids, carbohydrates, *Peronosclerospora sorghi*, phenols, resistant/susceptible genotypes

INTRODUCTION

In nature the crop plants are constantly challenged by a diverse array of pathogenic microorganisms resulting in heavy losses in yields, maize (*Zea mays* L.) is not exception to this. Maize is an important cereal crop and it ranks third in world production (458 metric tons from 126 million ha) after wheat and rice, however, considering its productivity, this crop ranks first (3.5 t/ha) followed by rice (3.3 t/ha) and wheat (2.3 t/ha) (Anon 2004) and thus it is rightly designnated as "King of grain crops". *Zea mays* is subjected to as many as 112 diseases on a global basis, in India alone recorded of about 35 of them (ikisan.com). Sorghum downy mildew (*Peronosclerospora sorghi* Weston and Uppal Shaw.) of maize an exciting group of fungi which infect larger number of crops and the yield losses of maize exceeds 70% in tropical Asia (Thakur and Mathur 2002).

The host-pathogen interaction is very complex and diverse in nature. Resistance in higher plants against microbial pathogens is the result of constitutive and inducible defense mechanism. The metabolic changes occurring in diseased plants frequently leads to accumulation of aromatic compounds especially phenolic compounds which are generally more pronounced in resistant varieties than susceptible (Bashan *et al.* 1986; Sindhu *et al.* 1995). During infection the resistant host plant defends itself against potential pathogens by means of a number of physical and chemical factors which may already be present in the host, or may be produced in response to the infection (Singh and Bhatnagar 1983). The accumulation of such defence related compounds was discussed in tea infected by various diseases (Ponmurugan *et al.* 2006), rust infected *Populus cathayana* (Zhang *et al.* 2009), yellow vein mosaic infected mesta (Chatterjee and Ghosh 2008). Banana plants infected with *Banana bract mosaic virus* (BBrMV) (Dhanya *et al.* 2006) and *Fusarium oxysporum* (Kumar *et al.* 2010) exhibited isozyme variability as defence mechanism. The physical characteristics are mechanical barriers which prevent the entry and spread of the pathogen. Chemical factors, which are toxic to the pathogen, inhibit its growth and activity in the host. More is known about the basis of mechanism of resistance and the causes for susceptibility but the probability of predicting its stability in different indigenous genotypes is still inadequate. Therefore, biochemical, histochemical and enzymatic studies on different indigenous resistance and susceptible maize genotypes were carried out in this study.

MATERIALS AND METHODS

Inoculation and maintenance of cultures

Downy mildew resistant (NAI-129, SKV-10) and susceptible (CM-500, NAI-127) maize genotypes were collected from Downy Mildew Research Center, Regional Research Station, VC farm, Mandya, India. The selected seed varieties were grown in pots (12") in greenhouse, at Department of Botany, UAS, GKVK, Bangalore, India. The 7-day-old seedlings were inoculated with *P. sorghi* conidial suspension of 2×10^4 conidial spores/ml. The inoculation was continued for three continuous nights in glasshouse and the inoculated seedlings were covered with polythene to maintain the humidity (85 ± 5°C) which is required for conidial germination and the pots were kept in moist, dark condition for next 12 hrs. The uninoculated control plants of all the genotypes were covered with 'spore proof polythene bags' separately and were maintained under the same conditions as inoculated plants.

Sampling was done from both inoculated and uninoculated plants by harvesting third nodal leaf (from the top). Leaves were harvested at every 15 days interval starting from 15 to 75 days after inoculation (DAI), for biochemical and enzymatic studies the samples were freezed in liquid nitrogen whereas, fresh samples

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were fixed for histochemical studies in freshly prepared Carnoy's B fixative (ethyl alcohol: chloroform: glacial acetic acid; 6: 3: 1).

Preparation of downy mildew conidial spore solution

The lower leaves of maize infected by downy mildew, *P. sorghi* were freshly collected from the unsprayed (fungicide) control plots of an experimental field at G.K.V.K, UAS, Bangalore. Later the lower sporulated surfaces of leaves were washed with 50ml of sterile water and conidial spores load was adjusted to 2×10^4/ml (Kalpana Reddy *et al.* 2009).

Biochemical, enzymatic and histochemical studies

1. Extraction and estimation of metabolites

Metabolites were extracted by a modified method of Barnett and Naylor (1966). Leaf tissue (500 mg) was ground into fine powder in liquid nitrogen and homogenized in 80% ethanol (v/v) and centrifuged for 10 min at 10,000 rpm. The homogenate was refluxed thrice for 15 min on a water bath at 60°C. The supernatants were pooled together and a final volume was made up to 25 ml with ethanol and used for estimation of total soluble sugars (Yemm and Willis 1954), starch (Hassid and Neufeld 1966), amino acids (ninhydrin method - Moore and Stein 1958), total phenolics (Amorim *et al.* 1977) and ortho-dihydroxy phenolics (Johnson and Schaal 1957). The enzyme assay was carried out using 200 mg of acetone powder (prepared using 80% chilled acetone) (Chang and Beevers 1968) for POX (peroxidase) (Chance and Maehly 1952) and PAL (phenylalanine ammonia lyase) (Koukol and Conn 1961) assays.

Inoculated and uninoculated fresh leaf samples were washed in distilled water to remove dirt and then cut into 1 cm^2 pieces and fixed in Carnoy's B fixative for 24 hrs. The leaf samples were processed according to Jensen (1962) for microtome sectioning. The 7-µm thick sections taken in ERMA rotary microtome (Coslab^{TM}, India, Model RA-303) were stained for total polysaccharides (Periodic Schiff's reagent; Jensen 1962) and total proteins (mercuric bromophenol blue; Mazia *et al.* 1953).

The experiment was conducted in a completely randomized design (CRD). Each experiment had 5 replications and the experiment was repeated to ensure reproducibility. Total sugars, starch, amino acids, total phenols and *O*-dihydroxy phenols were expressed in mg/g. Histochemical assessment was made through visual intensity, POX and PAL were quantified in U/g.

RESULTS AND DISCUSSION

From our earlier work it is evident that the inbread maize genotypes CM-500, NAI-127, NAI-129 and SKV-10 differ in their percent disease index against downy mildew categorizing into highly susceptible, moderately susceptible, moderately resistant and resistant types respectively (Kalpana Reddy *et al.* 2009). The present work is focused on biochemical, enzymatic and histochemical variation against downy mildew infection in these indigenous maize genotypes.

The involvement of carbohydrates i.e. total sugars and starch during the pathogenicity, serves as constant carbon source for the growing pathogen (Angra and Mandahar 1991, 1993). Depending on downy mildew resistant levels of selected maize genotypes varied the net carbohydrate content. In susceptible varieties, CM-500 and NAI-127, the total sugars were optimum i.e. 19.2 to 21.5 mg/g in uninoculated (healthy controls) leaves and slight fall was observed in inoculated (with downy mildew) leaves at 15 DAI. But, gradually the total sugars content in inoculated leaves decreased with progress in infection and time. Very low levels of total sugars i.e., 8.37 mg/g in CM-500 and 8.85 mg/g in NAI-127 were estimated at 75 DAI in inoculated leaves however, no such drastic fall was observed in unioculated samples (**Fig. 1**). The resistant varieties NAI-129 and SKV-10 showed a reverse trend i.e., the amount of total sugars increased with time in inoculated samples (**Fig. 1A**). Starting from 15 DAI, the quantity of sugars gradually increased up to 75 DAI from 30.7 to 34.95 mg/g in NAI-129 and 41.9 to 43.4 mg/g in SKV-10. Whereas, uninoculated NAI-129 recorded slight increase in total sugars up to 45 DAI followed by sudden fall in subsequent 60 and 75 DAI due to the slight disease progress in moderately resistant genotype. No such significant oscillation was observed in uninoculated resistant SKV-10 at all times (**Fig. 1A**). Overall, the total sugar content is high in resistant lines compared with susceptible once in both inoculated and unioculated plants at all times. Angra-Sharma and Sharma (1994) observed the rate of increase in carbohydrates in the infected susceptible host was less than in the infected resistant host when maize infected with *Helminthosporium mayd*. The observations of Bhat Tanmai (1997) in groundnut infected with late leaf spot and Reeti Singh *et al.* (1993) in safflower are in agreement with the present findings.

Similar trend was noticed in starch content of both inoculated and uninoculated leaves (**Fig. 1B**). In susceptible CM-500 and NAI-127 the starch content was optimum (10 and 6 mg/g) in uninoculated leaves and slight fall was observed in inoculated samples at 15 DAI. With the increase of disease and time the starch content in inoculated leaves decreased. Very low levels of starch (5 and 4 mg/g respectively in CM-500 and NAI-127 were estimated at 75 DAI in inoculated leaves however, no such drastic fall was observed in uninoculated samples (**Fig. 1B**). While, the resistant varieties NAI-129 and SKV-10 showed the reverse trend i.e., the amount of starch increased with time in inoculated samples (**Fig. 1B**), starting from 15 DAI, the quantity of starch gradually increased up to 75 DAI from 13.02 to 14.01 mg/g in NAI-129 and 5.2 to 15.15 mg/g in SKV-10. Whereas, uninoculated NAI-129 recorded slight increase in starch amount gradually up to 75 DAI however, resistant SKV-10 was recorded no such significant oscillations of starch content in uninoculated samples (**Fig. 1B**).

Hydroxyproline-rich plant cell-wall glycoproteins have been implicated in the resistance of plants to pathogens (Esquerre-Tugaye *et al.* 1979). The amino acid content in susceptible lines, CM-500 and NAI-127 recorded less in uninoculated samples than inoculated at all days (**Fig. 1C**). At 15 DAI amino acid content was 0.04 and 0.69 mg/g in CM-500 and NAI-127 in uninoculated and inoculated samples recorded 0.04 and 0.79 mg/g, respectively. The amino acid content progressively increased in both inoculated and uninoculated plants at all observable times and at 75 DAI the CM-500 recorded 0.17 and 0.16 mg/g and NAI-127 recorded 0.91 and 0.92 mg/g, respectively in inoculated and uninoculated samples. Similar trend was observed in resistant varieties, except that the net amino acid content in resistant lines was higher than susceptible. NAI-129 and SKV-10 showed 0.7 and 0.71 mg/g in uninoculated, 1.13 and 1.3 mg/g in inoculated samples at 15 DAI. The trend continued at all the observable days and highest amino acid content of 1.6 and 1.77 mg/g in uninoculated and 1.7 and 1.9 mg/g in inoculated NAI-129 and SKV-10, respectively at 75 DAI (**Fig. 1C**). Overall, the amino acid content of both susceptible and resistant varieties increased during infection compared with the uninoculated tissues. The resistant host showed high amino acid content compared with the susceptible variety. Sharma *et al.* (1992) and Angra and Sharma (1994) observed the similar trend while studying the leaf blight disease of maize.

The data from **Fig. 1D** it is evident that the total phenol content was also highest in resistant lines than susceptible varieties at all days. The phenol content consistently increased in inoculated tissues at all the days starting from 15 to 75 DAI the susceptible CM-500 recorded 0.04 to 0.12 mg/g and 0.09 to 0.41 mg/g in NAI-127, respectively. In uninoculated tissues the total phenol content was gradually increased up to 45 DAI and declined later in both susceptible and resistant lines. In contrast, the total phenol content in inoculated tissues of resistant NAI-129 and SKV-10 recorded higher and it gradually increased at all the days starting from 30 DAI to 75 DAI. And the values ranged from 3.77 to 4.5 mg/g in NAI-129 and 4.5 to 5.62 mg/g in SKV-

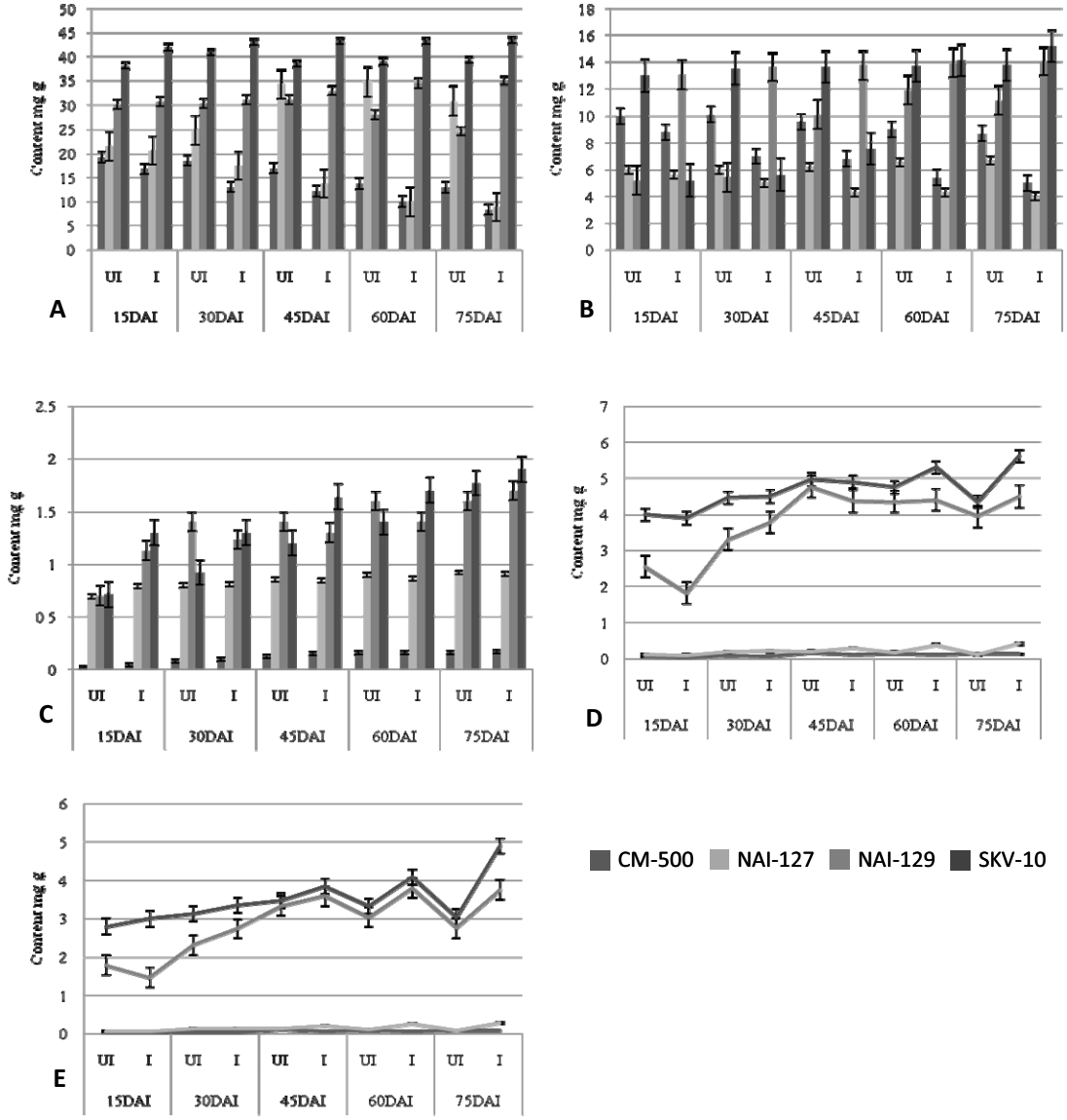

Fig. 1 Biochemical analysis of downy mildew infected resistant (NAI-129 and SKV-10) and susceptible (CM-500 and NAI-127) maize and its healthy controls. Third nodal leaf samples were collected at 15, 30, 45, 60 and 75 DAI along with the appropriate healthy (uninoculated) controls and assay was conducted to quantify: (**A**) Total sugars, (**B**) starch, (**C**) amino acids, (**D**) total phenols, (**E**) O, dihydroxy phenols. DAI, days after inoculation; UI, uninoculated; I, inoculated.

10, respectively for 30 and 75 DAI. Whereas, slight fall in phenol content was observed at the first sampling (15 DAI) irrespective of resistant and susceptibility (**Fig. 1D**). Contents of total sugars, nitrogen, amino acids, proteins, polyphenols, and catechin were reduced in diseased plant leaves compared with healthy leaves (Kalappanavar 1996; Mitter *et al.* 1997; Mali *et al.* 2000; Ponmurugan *et al*. 2007). The presence of higher levels of phenolics in resistant lines could be responsible for the delay and decline in number of germinations, appressoria, penetrations and colonisations at the onset of infection (Angra 1989).

O-dihydroxy phenols were highest in both inoculated and uninoculated resistant varieties at all days. It increased gradually in inoculate tissues starting from 3-4.9 mg/g in SKV-10 and 1.47-3.75 mg/g in NAI-129, respectively for 15 to 75 DAI. Similarly, in uninoculated tissues the values are 2.8-3.1and 1.8-2.8 mg/g, respectively in SKV-10 and NAI-129. Very low and not much variation was observed in inoculate and unioculated CM-500 and NAI-127 tissues (**Fig. 1E**). The results were in confirmation with Reeti Singh *et al.* (1998) and Rathi *et al.* (1998) while studying the samples of rust infected safflower and powdery mildew infected pea respectively.

The inoculated and uninoculated tissues of highly susceptible (CM-500) and resistant (SKV-10) leaf samples were fixed respectively in Periodic Schiff's reagent and mercuric bromophenol blue to stain polysaccharides and proteins. The transverse sections of the leaf samples reveals the same trend as described in the above biochemical analysis (**Table 1**, **Fig. 2A-E**). The polysaccharide content in uninoculated samples was always high in CM-500 (**Fig. 2A**), in contrary low in SKV-10 (**Fig. 2C**). Whereas, in inoculated samples they are low in CM-500 (**Fig. 2B**) and high in SKV-10 (**Fig. 2D**) at all the time indicating the increase of pathogen load decrease the net carbon content in susceptible tissues, since no such disease progress occurs in highly resistant SKV-10 the polysaccharides were high at all the times. Further the cross sections of proteins reveals that the resistant SKV-10 showed high protein content (**Fig. 2G, 2H**) in inoculated tissues than uninoculated in contrary, they are very low in susceptible CM-500 at all the times in both inoculated and uninoculated tissues (**Fig. 2E, 2F**). These findings further supports that the high and low protein contents in resistant and susceptible genotypes associate in resistance and susceptibility to pathogens (Esquere-Tugaye *et al.* 1979).

Plant POXs are involved in a verity of physiological processes including lignin formation, and its activity increases in response to pathogen attack (Golubenko *et al.* 2007). Lignin is also involved in cell wall building pro-

Table 1 Cytochemical assessment of different histochemical components.

Genotypes	Status	Type of stain	Different cell types of leaf		
			Epidermis	Parenchyma	Bundle sheath cells
CM-500	Uninoculated	MBB	+	+++	++
		PAS	++	++++	++
	Inoculated	MBB	+	+++	++
		PAS	+	++	++
NAI-127	Uninoculated	MBB	+	+++	+++
		PAS	++	++++	++++
	Inoculated	MBB	-	+	+
		PAS	+	+++	+++
NAI-129	Uninoculated	MBB	+	++	++++
		PAS	+++	++++	++++
	Inoculated	MBB	+	++	++++
		PAS	++	++++	++++
SKV-10	Uninoculated	MBB	+	+++	++++
		PAS	++	++	++++
	Inoculated	MBB	++	+++	++++
		PAS	++	++	++++

Note: MBB- Mercuric Bromophenol Blue; PAS- Periodic schiff's reagent. Very high ++++ ; High +++ ; Medium ++ ; Low + ; Poor/Absent –

Table 2 The specific activities of peroxidase in downy mildew infected and resistant maize genotypes.

Genotypes	Total activity (Units/g)									
	15 DAI		30 DAI		45 DAI		60 DAI		75 DAI	
	UI	I	UI	I	UI	I	UI	I	UI	I
CM-500	8.4	6.24	12.4	12.03	12.1	10.12	12.0	9.84	11.8	8.4
NAI-127	10.01	7.12	14.9	14.4	14.16	13.08	13.36	9.12	13.0	8.9
NAI-129	18.6	17.9	22.2	34.8	24.28	33.04	20.4	30.2	20.02	28.5
SKV-10	22.7	21.06	28.4	43.2	28.92	44.4	28.32	42.12	26.08	39.0

cesses, which are considered a primary defense against pathogens. In the present study the POX activity of susceptible and resistant lines varied with downy mildew infection (**Table 2**). In inoculated susceptible CM-500 and NAI-127 the POX activity gradually increased from 6.24, 7.12 to 12.03, 14.4 U/g, respectively for 15 and 30 DAI and started declining. The measured activity at 75 DAI was 8.4 and 8.9 U/g in CM-500 and NAI-127, respectively. Similarly, in uninoculated samples the activity increased up to 30 DAI and further it was maintained more or less same up to 75 DAI. Whereas, very high POX activity was observed in resistant NAI-129 and SKV-10 starting from 17.9 and 20.06 U/g on 15 DAI, 34.8 and 43.2 U/g on 30 DAI in inoculated tissues, respectively. Further the activity declined in NAI-129 for all the days but, the activity increased further in SKV-10 up to 45 DAI. In uninoculated lines the activity remained between 18.6-24.28 U/g in NAI-129 and 22.7–28.92 U/g in SKV-10 (**Table 2**). Similar observations such as, high POX activity was observed in male *Populus cathayana* when infected with *Melamprosa larici-populina* than female (Zang *et al*. 2009). Increased POX activity was increased in susceptible cultivars of pigeonpea leaf and root when inoculated with *Fusarium udum* (Prasad *et al.* 2003).

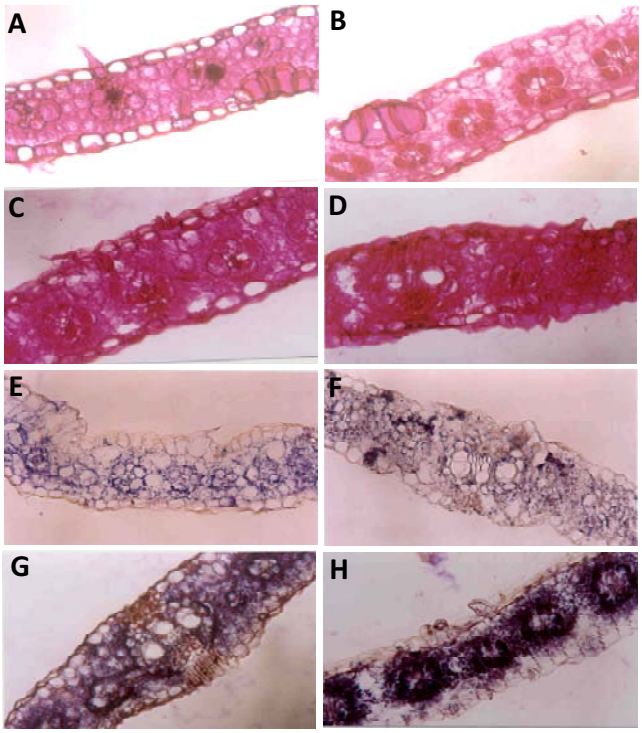

Fig. 2 Light microscopic images of histochemical analysis. The cross sections (7 µm thick) of fresh leaf samples were stained for polysaccharides and proteins. Maize CM-500 and SKV-10 was selected as a representative for susceptible and resistant genotypes. Stained for polysaccharides: (**A**) Uninoculated CM-500, (**B**) Inoculated CM-500, (**C**) Uninoculated SKV-10, (**D**) Inoculated SKV-10, Stained for proteins: (**E**) Uninoculated CM-500, (**F**) Inoculated CM-500, (**G**) Uninoculated SKV-10, (**H**) inoculated SKV-10.

Phenylalanine ammonia lyase catalyzes the deamination of L-phenylalanine to *trans*-cinnamic acid, which is the first step in phenylpropanoid pathway which supplies the precursors for phenolics, lignin and furanocoumarin, phytoalexins (Lincoln and Zeiger 2006). In the present study, PAL activity exhibited the similar trend as POXs. With the increase of downy mildew in susceptible lines the PAL activity decreased and uninoculated plant behaved normally (**Table 3**). In contrast, very high amount of PAL was recorded in both inoculated and uninoculated tissues. Inoculated samples of NAI-129 recorded gradual increase of PAL from 39.01 U/g on 15 DAI to 55.44 U/g on 75 DAI and in SKV-10, 44 U/g on 15 DAI to 78.32 U/g on 75DAI. In uninoculated tissues 40.97 to 51.12 U/g and 46.31 to 71.12 U/g in NAI-129 and SKV-10, respectively. The present findings were in line with Umesha (2006), who reported a high PAL at 21 h in resistant genotypes of tomato infected with *Clavibacter michiganensis* than susceptible lines.

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Table 3 The specific activities of phenylalanine ammonia lyase in downy mildew infected and resistant maize genotypes.

Genotypes	Total activity (Units/g)									
	15 DAI		30 DAI		45 DAI		60 DAI		75 DAI	
	UI	I	UI	I	UI	I	UI	I	UI	I
CM-500	20.9	17.92	23.7	22.14	27.36	18.9	27.36	16.0	26.16	12.48
NAI-127	39.76	36.11	42.9	41.0	44.8	36.08	44.24	32.64	41.04	28.19
NAI-129	40.97	39.01	43.1	43.7	43.44	47.76	46.08	51.6	51.12	55.44
SKV-10	46.31	44.0	49.42	51.63	51.6	64.0	69.84	74.0	71.12	78.32

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