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CHANGES IN ESTERASES IN RESPONSE TO BLAST INFECTION IN FINGERMILLET SEEDLINGS

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Key Word Index—*Eleusine coracana*; Gramineae; fingermillet; esterase; isoenzyme; *Pyricularia grisea*; fungus blast infection.

Abstract—The esterases of susceptible and resistant fingermillet seedlings were studied during infection. Infected susceptible genotype showed higher esterase activity compared with the resistant genotype. Isoenzyme 12 was found in all the developmental stages. On infection, isoenzyme 11 was suppressed in susceptible genotype but isoenzymes 3 and 4 appeared in the resistant genotype. Based on the inhibitor studies, isoenzyme 12 can be classified as a general esterase, and isoenzymes 3 and 4 were classified as carboxyl- and aryl-esterases, respectively. Isoenzyme 12 was relatively stable to almost all treatments. The carboxylesterase (IE3) was resistant to Zn^{2+} ions, 80° and pH 4 treatments. Arylesterase (IE4) was stable to Mg^{2+} only. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Fingermillet (*Eleusine coracana* Gaertn) is one of the important millets grown in Karnataka. Blast, a major fungal disease of this crop, causes extensive loss of yield. Esterases are ubiquitous and present in all of the developmental stages of this plant in isoforms that hydrolyse the ester linkage of different metabolites. Previous studies in our laboratory showed an increase in esterase activity in blast-inoculated fingermillet seedlings [1]. An increase in the isoesterase intensity was noted in bean and barley leaves upon infection with different pathogens [2, 3]. Appearance of a new isoesterase in the diseased stems of pepper plants infected with *Phytophthora capsici*, has also been reported [4]. Involvement of carboxylesterase in conferring resistance to cultivars of tomato to the nematode *Meloidogyne incognita* has been reported by Melillo *et al.* [5]. The present investigation was initiated to study the esterase activity, isoesterase patterns and characterization of the isoenzymes in the resistant (I8IE) and susceptible (Indaf-5) fingermillet genotypes during development and on infection with blast fungus.

RESULTS AND DISCUSSION

Esterase activity assayed during different developmental stages is presented in Table 1. An increase in esterase activity was noted in the susceptible genotype over the resistant one. Greater esterase activity was observed in the infected seedlings when compared with the uninfected controls of both genotypes. The maximum increase in esterase activity in both susceptible (160%) and in resistant (113%) genotypes was observed in the 22-day-old seedlings.

Polyacrylamide gel electrophoresis showed changes in the isoesterase pattern of the resistant and susceptible seedlings (Fig. 1). Isoesterase (IE), IE1, IE6 and IE7 were present only in I8IE, while IE2 and IE5 were found in the Indaf-5 seedlings. The presence of IE3 and

Table 1. Esterase activity of blast infected finger millet seedlings at different developmental stages

Variety	Age of seedlings (days)	Uninfected 1	Uninfected 2	Infected 1	Infected 2	Percent increase/decrease 1	Percent increase/decrease 2
Indaf-5	12	11.70	0.26	16.20	0.61	38	135
	17	5.12	0.38	9.85	0.33	92	− 13
	22	3.00	0.27	7.80	0.36	160	33
	27	7.93	0.51	10.53	0.67	33	31
I8IE	12	4.62	0.15	5.26	0.27	14	80
	17	3.66	0.23	6.30	0.29	72	23
	22	2.14	0.17	4.56	0.35	113	106
	27	6.18	0.49	8.08	0.58	31	18
'F' test		1 45.040**		2 13.698**			
SEM		0.356		0.015			
CD at 5%		1.487		0.063			

1: Total activity expressed as units g^{-1} AP.
2: Specific activity expressed as units mg^{-1} protein.

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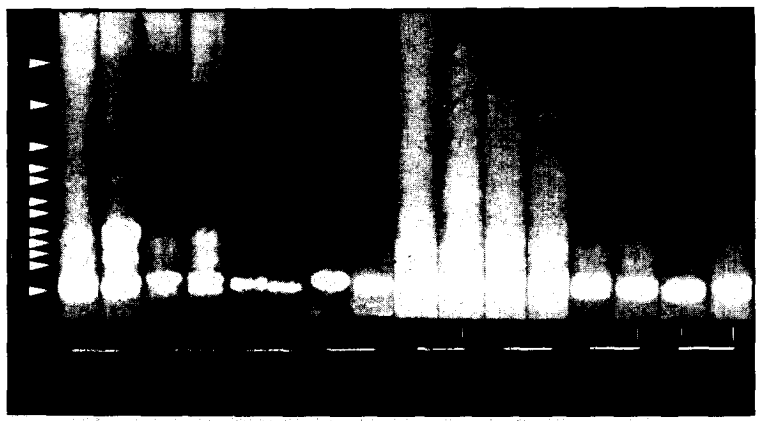

Fig. 1. Esterase isoenzyme patterns at different developmental stages of I8IE and Indaf-5. U, Uninfected; I, infected; a, a', 12-day-old seedlings; b, b', 17-day-old seedlings; c, c', 22-day-old seedlings; d, d', 27-day-old seedlings; 1, I8IE (resistant genotype); 2, Indaf-5 (susceptible genotype).

IE4 was observed in the resistant genotype only after infection. IE8 was present in both susceptible and resistant genotypes, and was intensified in the 12th day of infected I8IE seedlings. IE12 was present as a major band in all the samples analysed and in the susceptible genotype this band was intensified upon infection.

Table 2. Effect of various inhibitors (all at 5 mM) and their treatments on the esterase activity in the 17-day-old finger-millet seedlings (U = uninfected; I = infected)

	Indaf 5 (percent inhibition)		I8IE (percent inhibition)	
Treatments	U	I	U	I
ES	20	40	43	63
pCMB	50	64	22	38
PMSF	35	55	26	42
$HgCl_2$	68	75	72	94
$MgCl_2$	28	58	17	20
$ZnCl_2$	53	42	29	42
Heated to 60°	48	25	39	33
Heated to 80°	58	25	100	38
pH 4.0	00	28	00	25

Hwang *et al.* [4] have also reported the appearance of new isoesterase bands in pepper plants infected with *P. capsici*. An increase in the esterase isoesterase intensity was noted in barley leaves infected with powdery mildew [3].

Characterization of isoesterases

The maximum changes in the isoesterase patterns were noted in the 17-day-old seedlings. The effects of enzyme inhibitors, metal ions, heat- and acid-treatments on the esterase activity and isoesterase were investigated. (Tables 2–4).

Although the enzyme inhibitors, eserine sulphate (ES), *p*-chloromercuric benzoate (*p*CMB), and phenyl methyl sulphonyl fluoride (PMSF), suppressed the esterase activity, the extent of inhibition was greatest in the infected controls over the uninfected samples of both genotypes. In the infected seedlings, maximum inhibition of esterase activity was observed in the susceptible genotype in the presence of all inhibitors used. In the infected resistant seedlings, a notable increase in the inhibition was observed only in the presence of eserine sulphate.

Table 3. Comparative isoenzyme patterns of Indaf-5 17-day-seedlings with different treatments (U = uninfected, I = infected)

			Inhibitors*						Metal ions*						Heat treatment					
Esterase isoenzyme	Control		ES		*p*CMB		PMSF		$HgCl_2$		$MgCl_2$		$ZnCl_2$		60°		80°		Acid treatment	
band	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I
IE2	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IE5	+	+	−	−	−	−	−	−	−	−	+	−	−	++	−	+	−	−	+	+
IE8	+	+	−	−	−	−	+	−	−	−	+	−	−	−	+	++	−	+	+	+
IE10	++	++	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	+	−
IE11	++	++	−	−	−	−	−	−	++	+	−	−	−	−	+	++	+	++	+	−
IE12	+++	+++	+++	++	+++	++	+++	+	+++	++	+++	+	+	++	+++	+++	+++	+++	+++	+++

*Gels were preincubated at 5 mM concentration for 30 min.

−, No band; +, light band; + +, medium band; + + +, dark band.

Note: IE1, IE3, IE4, IE6, IE7 and IE9 were absent in the 17-day-old seedling.

Table 4. Comparative isoenzyme patterns of 18IE 17-day-seedlings with different treatments (U = uninfected, I = infected)

Esterase isoenzyme band	Control		Inhibitors*						Metal ions*						Heat treatment				Acid treatment	
			ES		*p*CMB		PMSF		$HgCl_2$		$MgCl_2$		$ZnCl_2$		60°		80°			
	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I
IE1	+	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
IE3	NP	+	−	++	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	+
IE4	NP	+	−	−	−	−	+	−	−	−	−	++	−	++	−	−	−	−	−	−
IE6	++	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
IE7	+	++	+	+	−	−	−	−	−	−	−	−	−	−	−	++	−	+	+	++
IE10	++	++	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−
IE11	++	++	−	−	+	−	+	+	−	−	−	−	+	−	+	++	−	+	+	+
IE12	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++	+++	+++	++	+++	++	+++	+++	+++

*Gels were preincubated at 5 mM concentration for 30 min.

NP, Not present; −, no band; +, light band; + +, medium band; + + +, dark band.

Note: IE2, IE5, IE8 and IE9 were absent in the 17-day-old seedling.

Earlier studies have indicated that both carboxylesterases (EC 3.1.1.1) and acetylesterases (EC 3.1.1.6) are not inhibited by ES, but cholinester hydrolase (EC 3.1.7–3.1.8) is inhibited. Acetylesterase and cholinester hydrolase are reported to be inhibited by the sulphydryl reagent, *p*CMB, while arylesterases (EC 3.1.1.2) are resistant [6]. Inhibition of esterase activity by PMSF suggests the presence of serine at the active centre [7]. Earlier studies reported that different types of esterases, such as cholinester hydrolase, and carboxyl- and arylesterases, are present in plants [8, 9]. Based on the results of the present studies IE12 present in both resistant and susceptible seedlings can be classified as a general esterase. IE3 and IE7 of resistant seedlings could be carboxylesterases as they were stable to ES. IE10 and IE11 of resistant seedlings could be acetylesterases because they were not inhibited by *p*CMB. All the other isoesterases may be classified either as arylesterases or cholinester hydrolase. PMSF was not inhibitory to IE4 and IE11 of resistant seedlings and IE8 of the susceptible genotype, indicating the possibility that these isoesterases may not have serine at their active sites [7].

Greater inhibition of the esterase activity was observed in the infected samples as compared with the uninfected samples of both genotypes in the presence of the divalent metal ions tested. However, the esterase activity of the infected susceptible seedlings showed less inhibition in the presence of Zn^{2+}. Maximum inhibition of esterase activity was noticed in all the samples in the presence of Hg^{2+}, which inhibited all the isoesterases, except IE12 and IE11 of susceptible seedlings and IE12 of resistant seedlings. The inhibition pattern was found to be similar to that of *p*CMB, indicating that -SH groups may be required for the enzyme activity. Partial stability of IE12 to Mg^{2+} was noted in both susceptible and resistant genotypes. IE4, an arylesterase, found only in the infected sample, was stable to Mg^{2+} in the resistant genotype. IE12 was inhibited to a greater extent by Zn^{2+} in the susceptible genotype compared with the resistant one. Carboxylesterase (IE3) and arylesterase (IE4) of the infected resistant seedlings were stable to Zn^{2+}. These results agree with those obtained by Sugihara *et al.* [7] in which the esterase purified from *Pseudomonas* sp. KWI-56 was inhibited by Hg^{2+} but not by Zn^{2+} and Mg^{2+}.

Treating the enzyme at 60° and 80° decreased the specific activity (Table 2) and the number of isoesterases (Tables 3 and 4) in both genotypes. IE10, IE11 and IE12 were stable at 60°, while partial stability was noted for IE12 at 80°. In the susceptible genotype, IE11 was stable at 80° and IE8 was stable only in the infected seedlings. In the resistant genotype the carboxylesterases (IE3 and IE7) were stable at 80°. The present studies show that esterases of infected seedlings of susceptible and resistant genotypes were more stable at high temperatures. Such thermal stability in the esterases has also been reported in *Arthrobactor globiformis* [10].

Treating the samples at pH 4 also showed some changes in the specific activity (Table 2) and in the isoesterase patterns (Tables 3 and 4) of both the genotypes. IE12 was less active in the pH 4-treated samples of the infected seedlings. IE10 and IE11 of the uninfected susceptible and resistant genotypes showed tolerance to pH 4 treatment. The carboxylesterase (IE3) of the resistant genotype showed tolerance to pH 4 treatment, but the arylesterase (IE4) of the infected samples of the resistant genotype was unstable at pH 4. In the susceptible genotype, except for IE2 of the uninfected and infected seedlings and IE11 of the infected samples, all other isoesterases were stable at pH 4.

The role of esterases in plant pathogen interaction is not well understood. Infection-induced carboxylesterase in the resistant genotype of fingermillet is resistant to Zn^{2+}, high temperatures and pH 4 treatments (Fig. 2). In a similar study, carboxylesterase of a resistant cultivar of tomato promoted resistance against the nematode, *M. incognita*, probably by the release of phenolic components [5]. Melillo *et al.* [11] also reported that the carboxylesterases and peroxidases localized near *Heterodera goettingiana*-infected pea roots, possibly by catalysing the formation and deposition of suberin, may help in the defence of plants by

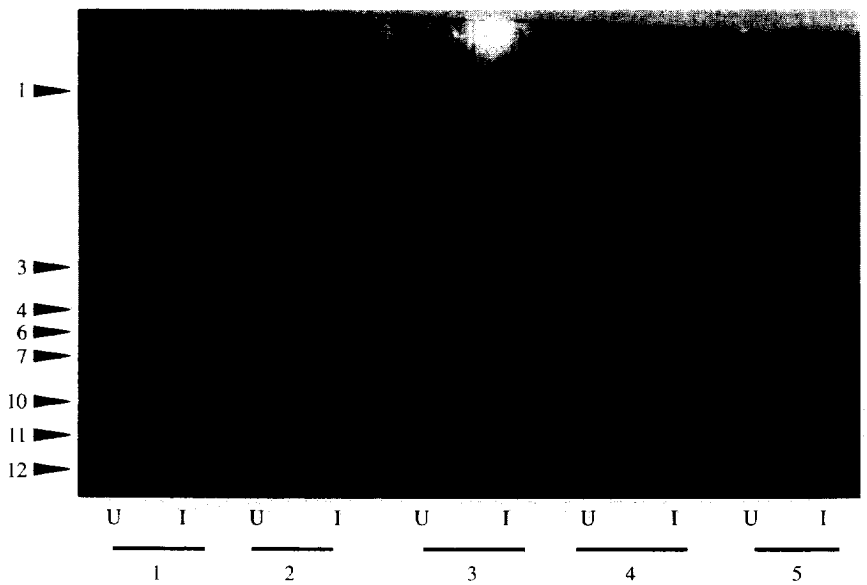

Fig. 2. Comparison of the different treatments on the isoenzymes of infected and uninfected 17-day-old I8IE (resistant to blast) seedlings. U, Uninfected; I, infected; 1, control; 2, Zn^{2+}; 3, 4, enzyme extract was treated at 60 and 80°; 5, enzyme extract was treated at pH 4.

forming a barrier that blocks the pathogens [11]. Thus, the carboxylesterases may have a positive role in the release of more phenolics and in conferring resistance.

EXPERIMENTAL

Fingermillet plants and blast infection. Two genotypes of finger millet seeds (*E. coracana* Gaertn.) I8IE and Indaf-5, resistant and susceptible for blast disease, respectively, were from the Millet Scheme, UAS, Bangalore. The seeds were grown in 1 m square plots under controlled conditions and inoculated with blast fungus, *Pyricularia grisea* L. on 5-day-old seedlings. The samples were collected at different stages of development, i.e. 12-, 17-, 22- and 27-day-old seedlings and the respective uninoculated controls. An Me_2CO powder (AP) was prepared from these samples and stored at $-20°$.

Biochemical analysis. The AP was extracted in 50 mM Na Pi buffer, pH 7 to give the required protein content for the enzyme assay and isoesterase analysis. Soluble protein content was assayed by the method of ref. [12]. Esterase was assayed using α-naphthyl acetate as substrate [13]. One enzyme unit of esterase was defined as the amount of enzyme that liberates 1 μmol min^{-1} α-naphthol.

Isoenzyme analysis. Disc electrophoresis was carried out using 7.5% polyacrylamide gel at 4° using Tris-glycine buffer, pH 8.2 at 100 V (constant) [14]. About 0.2 mg of protein sample was applied to each tube. The gels were then incubated at room temp. in 100 mM Na Pi buffer, pH 7.5 for 30 min with two changes, and then in the same buffer containing α-naphthyl acetate (2 mg/10 ml initially dissolved in Me_2CO) and fast blue RR salt (7.5 mg ml^{-1}) until the bands appeared [15].

Characterization of esterase enzymes. Treatment with inhibitors and metal ions. Three enzyme inhibitors, ES, *p*CMB and PMSF were dissolved in 1 ml dimethyl formamide and then made up with 100 mM NaPi buffer, pH 7.5. Solns of $HgCl_2$, $MgCl_2$ and $ZnCl_2$ in H_2O were used for the metal ion studies. Esterase enzyme assay was conducted in the presence of these inhibitors and metal ions (5 mM) using α-naphthyl acetate as substrate (25 mM). For isoesterase analysis, after electrophoresis gels were incubated with inhibitors/metal ions (5 mM) dissolved in 100 mM Na Pi buffer, pH 7.5 for 30 min at room temp. At the end of the incubation period, the gels were thoroughly washed with H_2O and stained for esterase enzyme activity using α-naphthyl acetate. *Heat treatment.* The enzyme extracts were treated at 60° and 80° for 30 min and immediately chilled to 0° and centrifuged for 15 min at 7500 *g* at 5°. *Acid treatment.* The pH of the enzyme extracts was adjusted to 4 using 200 mM HCl and centrifuged for 15 min at 7500 *g* at 5°. The pH of the supernatants was adjusted back to 7 by dialysing against 0.1M Na Pi buffer, pH 7 and centrifuged for 15 min at 7500 *g* at 5°. Enzyme activity and isoenzyme patterns were carried out for these supernatants by the method described earlier.

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