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Ribonucleases in the seedlings of pearl millet and their involvement in resistance against downy mildew disease

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Abstract

Tissue homogenates of pearl millet seedlings (cultivars HB 3, 843 B, ICMP 451 and IP 18292), with varying degree of resistance to downy mildew disease were tested for ribonuclease (RNase) enzyme activity and the profile of major RNase isozymes by substrate based gel assay. Polyacrylamide gel electrophoresis (PAGE) of the four pearl millet homogenates revealed 15–20 isozymes, varying in size from 6.5 to 121.0 kDa. Most of the RNases were highly active between pH 6 and 8 with maximum activity at pH 7. Tissue specific expression of RNase was observed with more activity in the root, i.e., 38.84, 59.61, 39.90 and 49.23 units in HB 3, 843 B, ICMP 451 and IP 18292, respectively than in shoot 11.54, 9.95, 9.46 and 9.49 units in HB 3, 843 B, ICMP 451 and IP 18292, respectively. Effect of metal ions on the RNase profile indicates Zn^{++} at 2, 20 and 200 μM concentrations to be inhibitory. Ca^{++} and Mg^{++} at 1 mM concentration enhanced the enzyme activity while at 10 mM inhibition of enzyme activity was observed. Inoculation with the downy mildew pathogen *Sclerospora graminicola* reduced RNase activity by 4–13% in compatible interactions while in incompatible combinations, the enzyme activity increased by 10–27%. The significance of RNase in pearl millet–downy mildew interaction and its involvement of in systemic acquired resistance of pearl millet against the downy mildew pathogen are discussed.

Abbreviations: ELISA – enzyme linked immuno sorbent assay; IR – induced resistance; RNase – ribonuclease.

Introduction

RNA degrading enzymes, ribonucleases (RNases), have been reported as stress enzymes showing a common pattern of changes in detached leaves and tissues attacked by pathogens (Barna et al., 1989; Green, 1994; Bariola and Green, 1997). Identification and characterisation of the major RNases have been reported from senescent and nonsenescent wheat leaves (Blank and MeKeon, 1991a), dark-induced senescent wheat leaves (Blank and MeKeon, 1991b) and from leaves of *Arabidopsis* (Bariola et al., 1999). In recent years, diverse functions have been attributed to RNase in plants. They are reported to function in mRNA degradation (Yen and Green, 1991), nutrient remobilisation, gametophytic self-incompatibility (Galiana et al., 1997), post-transcriptional regulation of gene expression in seedlings (Fennoy et al., 1997), recycling of phosphate (Bariola and Green, 1997) and in plant defense (Green, 1994; Galiana et al., 1997; Bariola et al., 1999). High levels of RNase activity in the pistil and the low susceptibility of this tissue to pathogens suggest the involvement of RNase in defense mechanisms of plants (Lee et al., 1992). There is also evidence of the involvement of RNase in hypersensitive disease resistance and programmed cell death (Green, 1994; Bariola and Green, 1997). RNase activity increased in leaves infected with pathogens, especially biotrophs

(for references see Barnes et al., 1988; Green, 1994). Pathogenesis related (PR) proteins have been linked to RNase with the report that a ginseng RNase with non-specific activity has protein sequence homology to two PR proteins from parsley (Moiseyev et al., 1994). The parsley proteins interns are known to be members of a large PR protein family designated as PR-10 (Van Loon et al., 1994).

In recent years, induction of systemic acquired resistance (SAR) has emerged as an eco-friendly strategy for protecting plants against the infection caused by bacteria, fungi and viruses by using both biotic and abiotic agents (Sticher et al., 1997; Jensen et al., 1998; Lucas, 1999). Induction of SAR is correlated with the induction of a well-characterised set of genes that comprise the so-called pathogenesis-related (PR) proteins (Ward et al., 1991; Uknes et al., 1992; Ryals et al., 1996). Increase in RNase upon induction of systemic acquired resistance has been reported in tobacco against TMV infection (Lusso and Kuć, 1995) and *Phytophthora parasitica* (Galiana et al., 1997). This response seems to modulate primary resistance mechanisms, converting a compatible interaction into an incompatible one (Molina et al., 1998).

Pearl millet (*Pennisetum glaucum* (L.) R. Br.), one of the important cereal crops, is grown in the semi-arid tropics of the world and forms food for millions of people. The major biotic constraint in pearl millet production is downy mildew disease caused by the oomycete biotrophic pathogen *Sclerospora graminicola* (Sacc.) Schroet. (Safeeulla, 1976), which causes losses upto 30% amounting to 260 million US dollars annually. Management of pearl millet downy mildew by chemicals is neither economical or ecofriendly and hence use of resistant cultivars is being practiced routinely. Recently SAR, as a possible strategy of pearl millet-downy mildew management was demonstrated (Kumar et al., 1993, 1998). A clear understanding of the biochemical basis of pearl millet resistance to downy mildew is important to obtain a complete picture of pearl millet–downy mildew interactions. Earlier studies identified the involvement of phenylalanine ammonia lyase, lipoxygenase and peroxidase in pearl millet defense against downy mildew disease (Nagarathna et al., 1992, 1993; Kumar et al., 1998). However, no information is available on RNase in pearl millet–downy mildew interactions and their biological and pathological significance. In this paper, the identification of 15–20 RNase isoforms from different pearl millet cultivars with varying resistance to downy mildew pathogen is described. RNase activity has been characterised according to pH optima and ion dependence. Evidence is also presented for the significance of RNase in pearl millet–downy mildew interaction and their involvement in pearl millet plant defense against downy mildew upon induction of systemic acquired resistance.

Materials and methods

Seeds

Seeds of pearl millet cultivars IP 18292 (highly resistant), ICMP 451 (resistant), 843 B (susceptible) and HB 3 (highly susceptible) to pathotype 1 of *S. graminicola* were obtained from the International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India.

Pathogen

Pathotype 1 of *S. graminicola* was isolated from pearl millet cultivar (HB 3) and maintained in the same cultivar under glass house conditions of the Downy Mildew Research Laboratory, University of Mysore, Mysore, India.

Induction of resistance by seed treatment

Pearl millet seeds of HB 3 cultivar were used for induction of resistance studies. Seeds were osmoconditoned in 0.5 mM acetyl salicylic acid (ASA) in 20% aqueous polyethylene glycol (PEG) for 24 h. Seeds soaked in 20% of PEG for 24 h served as control. After seed treatment, seeds were dried at room temperature and plated for germination. Two-day-old seedlings were used for inoculation studies.

Inoculum preparation, inoculation and sampling

Infected pearl millet leaves showing sporulation were collected in the evening, washed in running tap water to remove the remnants of sporangia, dried using tissue paper and kept in moist chamber for sporulation. Fresh sporangia were harvested, in sterile distilled water, early next morning and zoospores were released by incubating them in the dark. Pearl millet seedlings were inoculated with a suspension of zoospores

(4×10^4 zoospores ml^{-1}) by a root-dip technique (Safeeulla, 1976). The inoculated seedlings were incubated at room temperature in the dark. Seedlings incubated with sterile distilled water were used as control. Sampling was carried out after 24 h of inoculation. Seedlings were removed from the Petri plates, dried using absorbent paper and stored immediately at $-80\,^{\circ}C$.

Enzyme extraction

Pearl millet seedlings (1 g fresh wt. of three-day-old seedlings) were extracted with 0.1 M sodium acetate buffer (pH 5.2) at $4\,^{\circ}C$ using a prechilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 min at $4\,^{\circ}C$. The protein content of the supernatant was estimated by the dye-binding method (Bradford, 1976) and used as enzyme source.

RNase enzyme activity assay

RNase assay was carried out spectrophotometrically (Galiana et al., 1997) with slight modification using 0.1 M sodium acetate buffer (pH 5.2) for extraction instead of 50 mM Tris–HCl buffer (pH 7.0). The protein extracts (50 µl) were incubated for 2 h at $37\,^{\circ}C$ in 250 µl of 50 mM Tris–HCl (pH 7.0) buffer containing BSA (0.01%) and yeast RNA (400 µg ml^{-1}). After incubation, the remaining RNA in the reaction mixture was precipitated with ethanol in the presence of 2.5 M ammonium acetate (30 : 1) and resuspended in 100 µl of distilled water. Optic density was measured at 260 and 280 nm (the ratio ranged from 1.7 to 2.0). One enzyme unit is defined as the change in one OD at 260 nm mg^{-1} of protein per hour.

Electrophoresis and RNase activity staining

Detection of RNase activity in gels was performed according to the method of Yen and Green (1991). Protein samples (60 µg) were subjected to native Poly Acrylamide Gel Electrophoresis (PAGE) of 15% separating gel containing yeast RNA (5 mg ml^{-1}) and 5% stacking gel (Laemmli, 1990). Tank buffer contained 0.1% SDS, 0.3% Tris buffer and 1.44% glycine. Electrophoresis was carried out using mini gel electrophoretic unit (Genei, Bangalore). After electrophoresis, the gels were washed twice for 10 min with 25% (v/v) isopropanol in 10 mM Tris–HCl buffer (pH 7.0) and twice for 10 min with Tris–HCl buffer alone. The gels were incubated for 50 min at $50\,^{\circ}C$ in 100 mM Tris–HCl buffer (pH 7.0) and washed for 10 min with 10 mM Tris–HCl buffer (pH 7). The activity of the renatured RNases was observed by staining the gel for RNA (negatively stained for RNases activity) with 0.2% toluidine blue in 10 mM Tris–HCl buffer (pH 7.0) for 10 min. After extensive washing in 10 mM Tris–HCl buffer (pH 7.0) and in 10% v/v glycerol–Tris–HCl buffer (pH 7.0), the gels were documented using a Vilber Lourmat gel documentation unit (France). The apparent molecular masses were estimated based on the mobility of pre-stained low molecular markers (Sigma).

Effect of pH on RNases activity

The effect of pH on the RNases identified from pearl millet was investigated by slicing gel lanes containing plant extracts and treating each slice with buffers (10 mM Tris–HCl) that were adjusted to the pH values of 4, 5, 6, 7, 8, 9 and 10 for 50 min at $50\,^{\circ}C$ and stained for RNases activity.

Effect of divalent cations

Salts of divalent cations, Ca^{++}, Mg^{++} and Zn^{++} were tested for their effect on RNases profile when added to the incubation buffer (10 mM Tris–HCl, pH 7.0). Ca^{++} and Mg^{++} were used at concentrations of 1 and 10 mM; Zn^{++} was used at the concentrations of 2, 20 and 200 µM. The gels were incubated at $50\,^{\circ}C$ for 50 min and gels were stained with toluidine blue dye.

Quantification of in vivo S. graminicola *by enzyme linked immunosorbent assay (ELISA)*

Concentration of *S. graminicola* biomass in the inoculated tissues was estimated using polyclonal antibodies raised against the sporangia of pathogen by an amplified ELISA (Kumar et al., 1993). PBS extracts of the host plants (1 g of plants ground with 7.5 ml of PBS, clear supernatant obtained after centrifugation at 10,000 rpm for 15 min and adjusted to an absorbance of 0.3 at 280 nm) were used to coat the wells of microtitre polyvinyl plates: 100 µl of antigen was added to each well. The unreacted sites in the wells were blocked with 20% skimmed milk powder in distilled water for 2 h.

After washing the wells with PBS, 100 µl of antiserum (1 : 4000) was added to each test well and incubated at 37 °C for 2 h. The wells were washed thoroughly with PBS, and 100 µl biotin-conjugated anti-rabbit goat IgG (Genei, Bangalore) was added to each well followed by incubation at 37 °C for 2 h. Bound antibody in the wells was detected using avidin-conjugated peroxidase (Genei, Bangalore). For the enzyme reaction, 0.005% H_2O_2 in 0.04% *O*-phenylene diamine was used as the substrate. The reaction was stopped after 30 min with 2 M H_2SO_4 and read at 490 nm using a Dynatech ELISA reader. Antigen treated with pre-immune serum or wells containing all additions except the antibody were used as blanks. Concentrations of *S. graminicola* in the test samples were calculated by extrapolating the ELISA reading in the standard graph (Kumar et al., 1993). At least four replicates were maintained for the assay and it was repeated three times.

Results

RNase activity in pearl millet seedlings

Three-day-old seedlings that were not inoculated with the downy mildew pathogen were used. Seedlings showed RNase activity between 10 and 15 units (Table 1). Pearl millet seedlings of HB 3 and IP 18292 showed higher RNase activity of 14.79 and 14.06 units respectively, than 843 B and ICMP 451, 10.39 and 10.52 units respectively. These results indicate that constitutive RNase activity of different pearl millet cultivars is not related to their resistance to the downy mildew pathogen.

Table 1. RNase activity in seedlings of pearl millet cultivars that are not inoculated with the downy mildew pathogen

Cultivar	RNase activity (Units) ($\triangle$ Abs at 260 nm h^{-1} mg^{-1} of protein)*
HB 3 (Highly susceptible)	14.79 ± 0.24a
843 B (Susceptible)	10.39 ± 7.8c
ICMP 451(Resistant)	10.52 ± 0.27c
IP18292 (Highly resistant)	14.06 ± 0.12b

Data are means $\pm$ SE of triplicate samples of two independent experiments. The values in the column followed by same letter(s) are not significantly different from each other ($P = 0.05$).
*Average of two independent experiments each with three replicates.

Identification of major RNase of pearl millet cultivars

The profile of major RNase isozymes of pearl millet cultivars as characterised by a substrate gel assay identified 15–20 isozymes with an approximate molecular mass ranging from 6.5 to 121 kDa (Figure 1; Table 2). Twenty isozymes were resolved in HB 3 cultivar while the cultivars IP 18292, ICMP 451 and 843 B recorded 16, 16 and 15 isozymes, respectively. Three isozymes representing 106.9, 89.4 and 71.6 kDa were present only in HB 3 ICMP 451 and IP 18292 cultivars, while HB 3 and 843 B shared the isozymes of 65.6, 59.2 and 50.8 kDa. Similarly, the isozymes of 53.5 and 7.2 kDa were detected only in ICMP 451 and IP 18282. RNase isozymes of 110.2 and 93.2 kDa were specific to HB 3 and an isozyme of 121.0 kDa was present in HB 3 and ICMP 451 cultivars. On the other hand, the RNase isozyme of 95.6 kDa was present only in 843 B cultivar. The other RNase isozymes (117.4, 38.1, 34.7, 25.1, 22.4, 18.1, 14.1, 12.4, 10.8, 9.4 and 6.5 kDa) were common to all the pearl millet cultivars. Four of the isozymes with molecular masses of 12.4, 10.8, 9.4 and 7.2 kDa had the strongest activity in the cultivars IP 18292 and ICMP 451 and sometimes appeared very close to each other as two bands. In cultivars HB 3 and 843 B, while the isozyme of 7.2 kDa was not detected, the other three were expressed mildly.

Tissue-specific study

Tissue specific activity of RNase in the seedlings of HB 3, 843 B, ICMP 451 and IP 18292 was assayed spectrophotometrically. The enzyme activity was higher in the root tissue than in the shoot tissue in all the seedlings tested (Figure 2). In HB 3, shoot tissue showed RNase activity of 11.54 units whereas root tissue exhibited 38.84 units. RNase enzyme activity of 9.95 and 59.61, 9.46 and 39.90 and 9.49 and 49.23 units were observed in shoot and root tissues of 843 B, ICMP 451 and IP 18292 cultivars, respectively.

Since the profile of RNase recorded the maximum number of isozymes in the pearl millet cultivar HB 3 (Figure 1), the same cultivar was used to characterise the tissue-specific expression on the substrate gel. Electrophoretic analysis of tissue homogenate of HB 3 cultivar identified 20 isozymes in the extract of whole seedlings and 18 and 15 isozymes in the shoot and root tissues respectively. Though most of

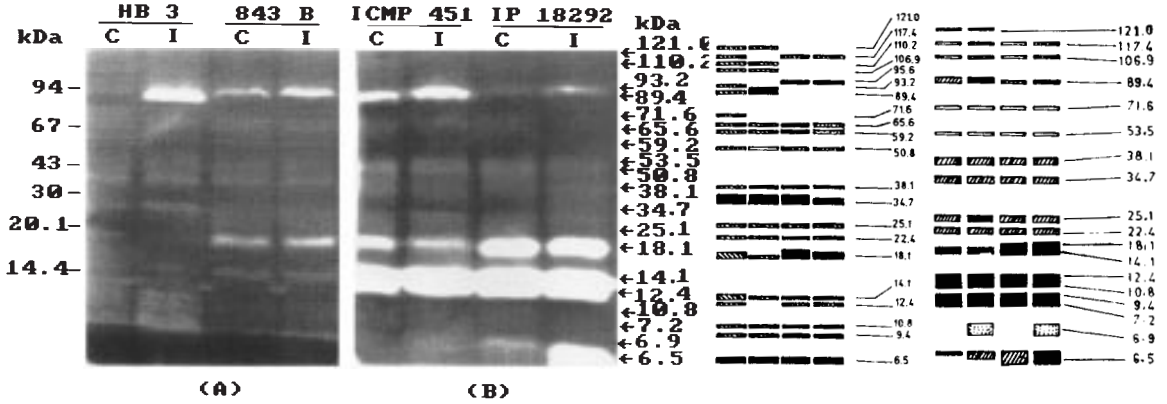

Figure 1. RNase pattern in seedlings of pearl millet cultivars uninoculated and inoculated with zoospores of *S. graminicola*. The RNase activities are represented schematically to the right of the lanes with their apparent molecular masses. Positions of the molecular mass markers are shown to the left of the gel. Gel showing RNase isozymes pattern of cultivars HB 3 (HS) and 843 B (S) (A) and, ICMP 451 (R) and IP 18292 (HR) (B).

Table 2. Properties of the major RNase of seedlings of pearl millet cultivars

Molecular mass (kDa)	Cultivars								pH optima	pH sensitivity	Response to						
	HB 3		843 B		ICMP 451		IP 18292				Zn^{++} (μM)			Ca^{++} (mM)		Mg^{++} (mM)	
	C	I	C	I	C	I	C	I			2	20	200	1	10	1	10
121.0	+	+	ND	ND	+	+	ND	ND	7–8	≤7–≤8	+	+	+	+	+	+	+
117.4	+	−	+	+	+	+	+	+	7–8	≤7–≤8	+	+	+	+	+	+	+
110.2	+	+	ND	ND	ND	ND	ND	ND	7–10	4	+	+	−	+	−	+	−
107.0	+	+	ND	ND	+	+	+	+	7–10	4	+	+	−	+	−	+	−
95.6	ND	ND	+	+	ND	ND	ND	ND	ND		ND	ND	ND	ND	ND	ND	ND
93.2	+	−	ND	ND	ND	ND	ND	ND	7	4	+	+	+	+	−	+	−
89.5	+	+	ND	ND	+	+	+	+	7	4	+	+	+	+	−	+	−
71.6	+	−	ND	ND	+	+	+	+	7		+	+	−	+	−	+	−
65.6	+	+	+	+	ND	ND	ND	ND	7–8		+	+	−	+	−	+	−
59.2	+	+	+	+	ND	ND	ND	ND	7–8		+	+	−	+	−	+	−
50.8	+	+	+	+	+	+	+	+	7		+	+	−	+	−	+	−
38.1	+	+	+	+	+	+	+	+	5–7	10	+	+	−	+	+	+	+
34.7	+	+	+	+	+	+	+	+	5–7	10	+	+	−	+	+	+	+
25.2	+	+	+	+	+	+	+	+	7		+	+	−	+	+	+	+
22.4	+	+	+	+	+	+	+	+	5–7		+	+	−	+	+	+	+
18.1	+	+	+	+	+	+	+	+	5–7		+	+	−	+	+	+	+
14.1	+	+	+	+	+	+	+	+	7		+	+	−	+	+	+	+
12.4	+	−	+	+	+	+	+	+	7		+	−	−	+	+	+	+
10.8	+	+	+	+	+	+	+	+	7		+	−	−	+	−	+	−
9.4	+	+	+	+	+	+	+	+	7–9	10	+	−	−	+	−	+	−
7.2	ND	ND	ND	ND	+	+	+	+	ND			ND		ND		ND	
6.9	ND	ND	ND	ND	−	+	−	+	ND			ND		ND		ND	
6.5	+	+	+	+	+	+	+	+	7–9		+	−	−	+	−	+	−

The difference in activity after inoculation with the downy mildew pathogen *S. graminicola* and sensitivity to pH, metal ions are indicated. −, sensitive, reduced activity; ND, no detected; +, sensitive, increased activity; C, uninoculated control; I, Inoculated with *S. graminicola*.

them were common to both the tissues, their intensity differed. Two of them, representing the isozymes 110.2 and 93.2 kDa, were specific to root tissues, while the shoot tissues resolved 38.1 and 14.1 kDa bands as characteristic markers (Figure 3).

Effect of pH and metal ions on RNase activity

pH affected RNase activity from host tissues differently (Table 2). The highest total RNase activity with 20 isozymes was found in gel slices treated with incubation buffer at pH 7.0, but considerable activity was evident at pH 6 and 8 (Figure 4) as well.

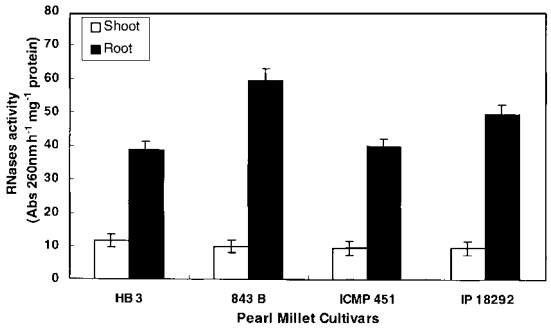

Figure 2. Tissue specific (shoot and root tissues) RNase activities of seedlings of pearl millet cultivars. One unit of enzyme was expressed as difference in OD at 260 nm h^{-1} mg^{-1} of protein. Data are triplicate samples of two independent experiments.

Isozymes of 14.1, 12.4, and 10.8 kDa, which had strongest activity at pH 7, showed decreased intensity with increased pH. On the other hand, isozymes of 34.7, 25.1 and 22.4 kDa showed stronger band intensity at alkaline pH. Similarly, the 117.4, 106.9 and 93.2 kDa bands showed more intense banding pattern at alkaline pH. The isozymes of 18.1 and 14.1 kDa were highly active throughout the acidic range but were sensitive to pH $\geq$ 8. The 38.1 and 34.7 kDa isozymes which were stronger at acidic pH were inhibited at pH $>$ 6. The 18.1 and 14.1 kDa isozymes had pH optima at 7 and were the most resistant to pH among the isozymes. The 25.1 kDa isozyme displayed a pH optimum of 6 but was sensitive to pH $\geq$ 8. The isozymes of 65.6 and 59.2 kDa were strongest at pH 8 but were sensitive to pH $\leq$ 6. The 50.8 kDa isozyme was highly active at basic pH with the maximum activity at pH 8 but was completely inhibited at pH $\leq$ 7. The isozymes of 110.2 and 107.4 kDa were highly active at pH 10 and showed decreasing activity with decreasing pH. The isozymes of 121.0 and 117.4 kDa were active at pH 7 and were completely inhibited at pH $\leq$ 6 and $\geq$8. The 6.5 kDa isozyme though displayed a pH optimum of 7 was active at pH 8 as well and is likely to have a pH optimum of 7.5. However, it was sensitive to pH 10 and $\leq$6.

With increasing concentrations of Zn^{++}, decreasing number of bands was observed and thus all the concentrations of Zn^{++} showed inhibition over RNase

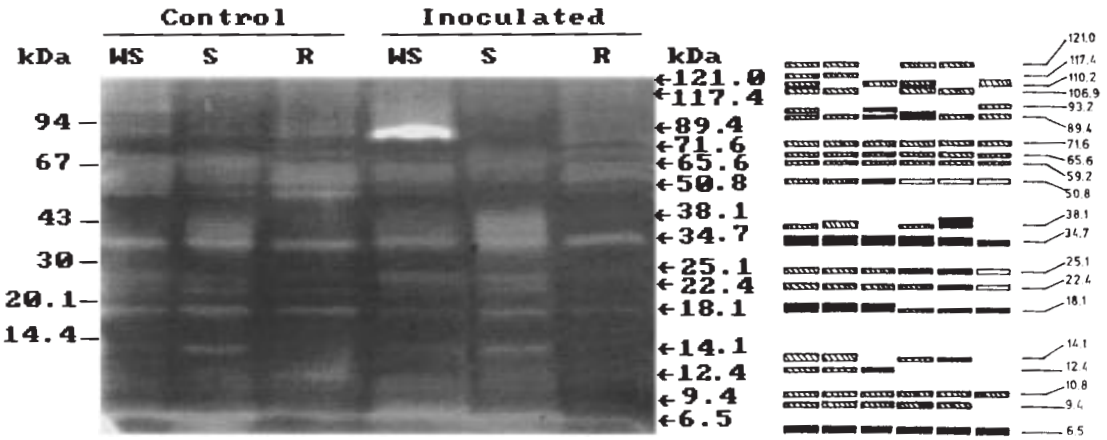

Figure 3. Tissue specific expression of RNase isozyme pattern highly susceptible (HB 3) seedlings of pearl millet cultivar uninoculated (control) and inoculated seedlings with zoospores of *S. graminicola*. The RNase activities are represented schematically to the right of the lanes with their apparent molecular masses. Positions of the molecular mass markers are shown to the left of the gel. WS: whole seedlings; S: Shoot; R: root. The RNase activities are represented schematically to the right of the lanes with their apparent molecular masses. Positions of the molecular mass markers are shown to the left of the gel.

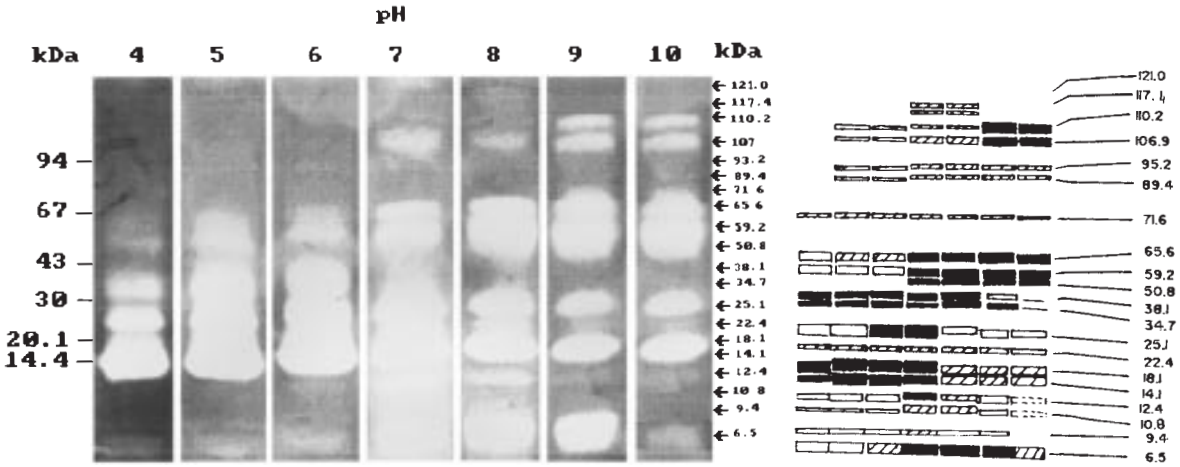

Figure 4. Effect of pH on RNase activities. After electrophoresis, slices of the gel were washed, incubated, stained and destined in the buffers at the pH values indicated above the lanes. The RNase activities are represented schematically to the right of the lanes with their apparent molecular masses. Positions of the molecular mass markers are shown to the left of the gel.

activity. In gel slices not treated with Zn^{++} (control) 20 isozymes were detected. Though a total of 19 bands were detected in 2 μM of Zn^{++} treatment, their intensity was very faint. Treatment with 20 and 200 μM detected 16 and 4 bands respectively. In 200 μM of Zn^{++}, only four isozymes (121.0, 117.4, 110.2 and 95.6 kDa) were detected. It was observed that increasing concentration of Zn^{++} affected the low molecular weight bands.

At a concentration of 1 mM, both Mg^{++} and Ca^{++} showed the same 20 isozymes as in control (Figure 5b). However, the isozymes of 34.7, 18.1, 14.1, and 12.4 kDa showed greater intensity. Similarly, the 121 kDa isozyme was enhanced on treatment with both salts but to lesser extent with Ca^{++}. At the higher concentration of 10 mM, only nine isozymes of 121.0, 117.4, 38.1, 34.7, 25.2, 22.4, 18.1, 14.1and 12.4 kDa were detected.

Effect of inoculation by S. graminicola *on RNase activity of seedlings of pearl millet cultivars*

Activity and isozyme pattern

On inoculation with the downy mildew pathogen, cultivars HB 3 and 843 B (in a compatible interaction with the downy mildew pathogen) showed a decrease of 12 and 4% respectively in enzyme activity over the respective uninoculated control. On the other hand, in incompatible interactions, inoculation with the pathogen enhanced the enzyme activity by 10 and 27% (ICMP 451 and IP 18292) (Figure 6). Gel electrophoretic results showed the disappearance of isozymes of 117.4, 93.2, 71.6 and 12.1 kDa of HB 3 cultivar, while the band intensity of 50.8, 18.1 and 14.1 kDa isozymes of HB 3 and 65.6, 59.2, 34.7 and 18.1 kDa of 843 B decreased. However, the band intensity of 89.4 and 38.1 kDa isozymes of HB 3 showed higher intensity on inoculation. Increase in total RNase activity of ICMP 451 and IP 18292 was correlated with the higher accumulation of isozymes of 121.0, 117.4, 106.9, 89.4, 25.1 and 6.5 kDa and 117.4, 106.9, 89.4 and 6.5 kDa respectively and appearance of a new isozyme of 6.9 kDa in both.

Tissue specific expression

In all the pearl millet cultivars, RNase activity was greater in root tissues than in shoot tissues. After inoculation, in highly susceptible and susceptible cultivars RNase activity decreased, whereas in highly resistant and resistant cultivar enzyme activity increased (Figure 6). In a highly susceptible pearl millet cultivar (HB 3), uninoculated shoot and root tissues showed RNase activity of 11.59 and 38.39 units respectively, whereas after the inoculation enzyme activity reduced to 11.00 and 26.00 units respectively. Similar trends were observed in the susceptible pearl millet cultivar 843 B. Uninoculated shoot and root tissues showed RNase activity of 10.07 and 59.59 units whilst in inoculated tissues shoot and root enzyme activity were 9.47

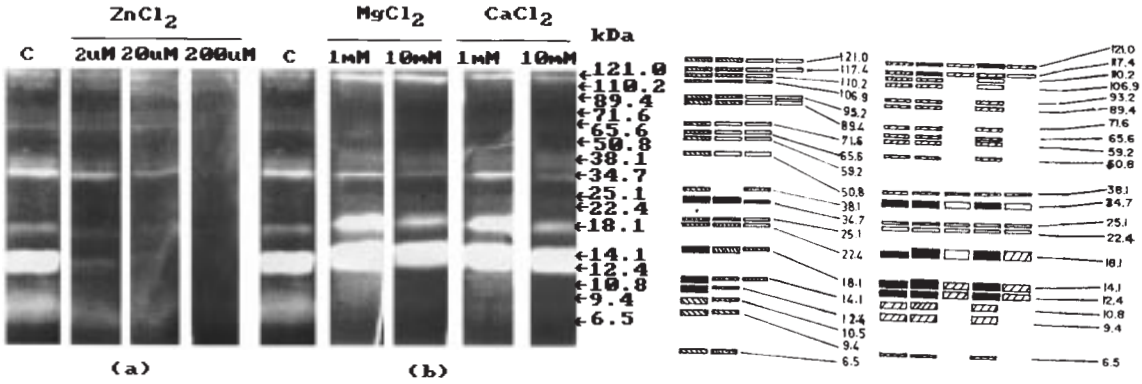

Figure 5. Effect of metal ions on RNase activities. After electrophoresis, slices of the gel were washed and incubated in 100 mM Tris buffer, pH 7.0 containing 2, 20, 200 μM of $ZnCl_2$ (a) and 1 and 10 mM of $MgCl_2$ and $CaCl_2$ (b). The RNase activities are represented schematically to the right of the lanes with their apparent molecular masses. Positions of the molecular mass markers are shown to the left of the gel.

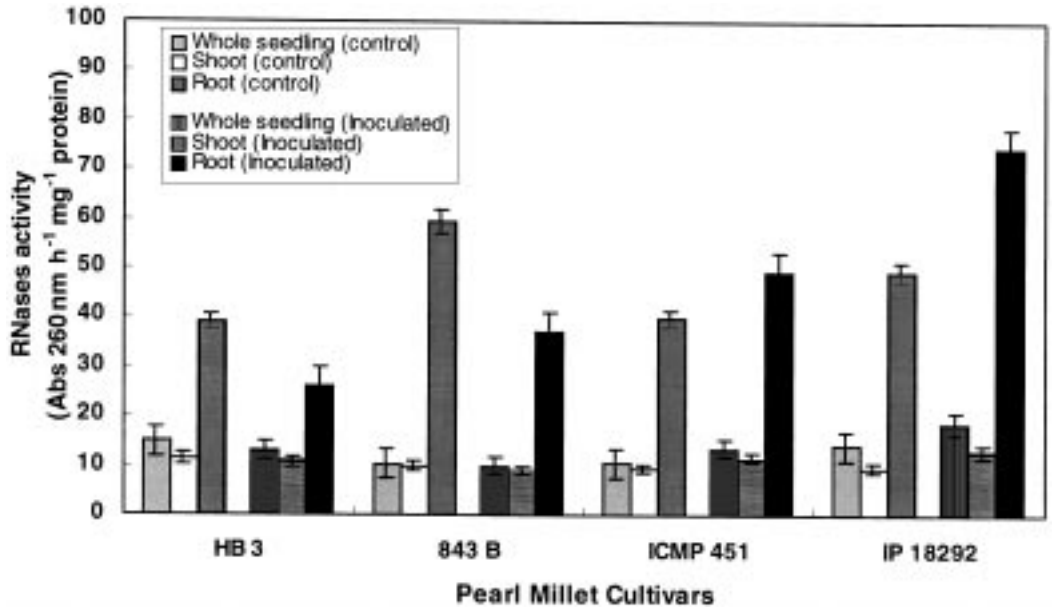

Figure 6. Tissue specific (whole seedlings, shoot and root tissues) RNase activities of seedlings of pearl millet cultivars uninoculated (control) and inoculated with zoospores of *S. graminicola*. One unit of enzyme activity was expressed as difference in OD at 260 nm h^{-1} mg^{-1} of protein. Data are means ± SE of triplicate samples of two independent experiments.

and 36.95 units. In highly resistant and resistant cultivars, both in shoot and root tissues, RNase activity was increased after inoculation. In the cultivar ICMP 451, 9.39 and 39.90 units of RNase activity were observed in uninoculated shoot and root tissues whereas in inoculated shoot and root tissues 11.60 and 48.80 units, respectively were observed. Similarly, in IP 18292, 9.35 and 49.97 units of RNase activity were observed in uninoculated shoot and root tissues and in 13.16 and 73.29 enzyme units inoculated shoot and root tissues respectively.

Induction of resistance in cultivar HB 3 of pearl millet by seed treatment with ASA

On RNase activity

Spectrophotometric analysis (Table 3) showed an increase in RNase activity from 11.0 to 13.0 units in

Table 3. RNase activity in highly susceptible, induced resistant and highly resistant cultivars and quantification of *S. graminicola* biomass in seedlings of pearl millet cultivars using ELISA

Cultivars	RNase activity (Units) (△ Abs at 260 nm h^{-1} mg^{-1} of protein)*	Fungal dry weight (μg g^{-1} fresh weight (whole plant) as quantified by ELISA 24 h after inoculation*
Highly susceptible (HB 3 + SDW)	6.73 ± 0.53d (−8.0%)	8.00 ± 0.24a
Induced resistant (HB 3 + 0.5 mM ASA in 20% PEG)	13.07 ± 0.31a (+21.6%)	1.12 ± 0.67b
Highly resistant (IP 18292 + SDW)	13.37 ± 0.45a (+27.6%)	0.91 ± 0.73b

Seedlings raised from sterile distilled water treated seeds of pearl millet cultivar HB 3 were used as highly susceptible; 0.5 mM ASA in 20% PEG treated HB 3 seeds were used as induced resistant and sterile distilled water treated IP 18292 seeds were used as highly resistant cultivars. Data are means ± SE of triplicate samples of two independent experiments. Figures in parenthesis indicate % increase (+)/decrease (−) in RNase activity of inoculated seedlings over uninoculated control. The values in the column followed by same letter(s) are not significantly different from each other ($P = 0.05$).

*Average of two independent experiments each with three replicate.

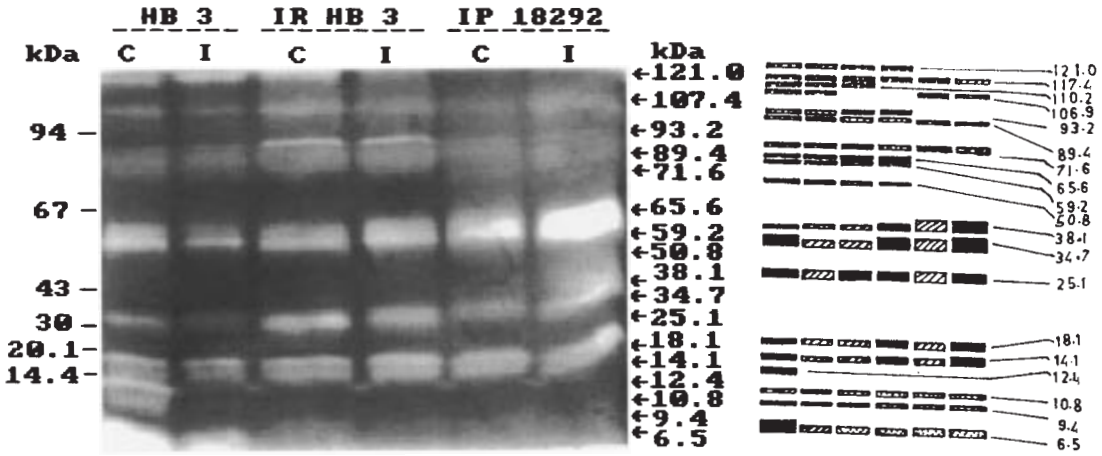

Figure 7. RNase isozyme profile of 3-day-old seedlings of HB 3, HB 3 (ASA treated), and IP 18292 pearl millet cultivars in uninoculated and inoculated seedlings inoculated with zoospores of *S. graminicola*. The RNase activities are represented schematically to the right of the lanes with their apparent molecular masses. Positions of the molecular mass markers are shown to the left of the gel.

ASA-treated HB 3 seedlings and from 10.5 to 13.5 units in highly resistant (IP 18292) seedlings 24 h after inoculation. On the other hand, highly susceptible (HB 3) seedlings recorded a decrease in enzyme activity from 7.6 to 6.4 units. These results were correlated with the results of gel electrophoretic analysis (Figure 7). Band intensities of the 71.6, 38.1, 34.7, 25.1, 18.1 and 14.1 kDa RNase isozymes in highly susceptible (HB 3) cultivar decreased after inoculation, whereas in highly resistant and ASA-treated cultivars the same proteins appeared as brighter bands compared to the control seedlings. In ASA-treated seedlings, a 93.2 kDa protein appeared as a distinct band. An RNase isozyme of 12.4 kDa presents only in the uninoculated highly susceptible (HB 3) cultivar and absent in all other samples. Band intensity of a 6.5 kDa isozyme, which was present only in highly susceptible (HB 3) cultivar, was decreased after inoculation.

On in vivo S. graminicola *biomass*

Effect of induction of resistance by seed treatment with 0.5 mM ASA on the infection and subsequent spread of the downy mildew pathogen was studied by estimating the *in vivo* fungal biomass in test seedlings

of pearl millet cultivars. ELISA assays were done at 24 h after inoculation. Fungal biomass in resistant and induced resistant seedlings was only 0.91 and 1.12 μg g^{-1} (fresh weight of whole seedlings), respectively. On the other hand, unprotected HB 3 recorded a much higher concentration of 8 μg (Table 3).

Discussion

In the present study, by assessment of molecular mass, pH optimum, and sensitivity to divalent cations, 15–20 RNase isozymes were identified. These differed in size from 6.5 to 121.0 kDa in seedlings of different pearl millet cultivars with varying resistance to downy mildew disease. This is the first report on RNase in pearl millet–downy mildew interactions. As for reproducibility, RNase pattern were always identical if the same extract was compared on the gel. Hence, care was taken to compare samples extracted on the same day and run on the same gel and all the experiments were repeated 3–4 times. RNase activity (spectrophotometric and pattern of isozymes on gel) in seedlings of pearl millet cultivars with varying resistance to downy mildew disease showed variation in the constitutive level of the cultivars tested. Similar results on the RNase pattern of host cultivars of different genetic backgrounds were shown in wheat (Barna et al., 1989). Tissue specificity of expression of RNase observed in the present study is consistent with the report available in wheat (Chang and Gallie, 1997). Similarly, studies carried out by Yen and Green (1991) identified more isozymes in stem than leaf of *Arabidopsis thaliana*. It has been suggested that tissue specificity may be due to the genes, encoding individual enzymes, responding to organ specific signal or may be that the enzyme activity of RNase may be regulated differentially in different tissues, perhaps via modification of enzyme (Yen and Green, 1991). In the present study, the pH optima for RNase of pearl millet were observed between 4 and 10 pH with the maximum activity at pH 7.0. In contrast, in *Arabiopsis thaliana*, optimum pH for RNase activity was between 6.5 and 7.5 and most of the isozymes were sensitive at $\leq$5.5 pH (Yen and Green, 1991), where as in maize, pH optimum was 6.4–8.0 (Fennoy et al., 1997). Our observation on the effect of metal ions on RNase activity recorded reduced numbers of isozymes with increasing concentration of Zn^{++}, indicating that Zn^{++} at all concentrations affects pearl millet RNase activity. This result is consistent with the general consideration that Zn^{++} has a negative effect on RNase activities from plants (Meza-Basso et al., 1986; Nguyen et al., 1988; Yen and Green, 1991). The inhibitory effect of cations on RNase activity observed in the present study also provides information about the ion requirements of specific isozymes. Zn^{++} reduced the activity of isozymes of RNase, whereas Mg^{++} and Ca^{++} were required for enzyme activity.

Pathogenic microorganisms are known to show reduced virulence on plants that have acquired systemic resistance. The induction of systemic resistance is also reported to be associated with rapid activation of multiple mechanisms including hydrolytic enzymes, antimicrobial compounds and RNase for defense that were either latent in susceptible plants or expressed too late to control disease (Lusso and Kuć, 1995). The increase in RNase activities reported in this paper may represent an additional resistance mechanism or they may function in programmed cell death. Increased RNase activity recorded in the ASA-treated HB 3 seedlings after challenge inoculation with the pathogen suggests the possible involvement of RNase in the biochemical mechanism of resistance induced in pearl millet seedlings. Accumulation of more than one RNase in the resistant and ASA-treated pearl millet seedlings suggests that the RNase activity, rather than one specific RNase, could be important in pearl millet defense. A 93.2 kDa isozyme detected only in extracts of ASA-treated seedlings indicates the possibility of specific activation of this isozyme expression in SAR. Such increases in RNase activity in host plants induced to SAR after challenge inoculation with pathogens have been reported in tobacco (Lusso and Kuć, 1995; Galiana et al., 1997). The sensitisation of the tissues of induced plants (Dean and Kuć, 1987; Pan et al., 1991) that result in a rapid response of the protected plants to inoculation is likely to be the result of a systemic signal(s) that moves systemically throughout the plant. Increased RNase activity observed in induced plants after challenge with *S. graminicola* is possibly a consequence of this sensitisation process as recorded in tobacco plants by Lusso and Kuć (1995). Challenge inoculated highly susceptible pearl millet seedlings showed 8% reduction in RNase activity. Whereas induced resistant and highly resistant pearl millet seedlings showed 21% and 27% increase in RNases activity respectively and suggested that seed treatment using 0.5 mM ASA, triggered the defense response in the otherwise susceptible pearl millet showed an increase similar to that of highly resistant

pearl millet seedlings. These results correlated with the decreased *S. graminicola* biomass in induced resistant seedlings and increased fungal biomass in susceptible pearl millet seedlings. There are also reports to support the hypothesis that RNase of plants are involved in control of pathogen development during expression of SAR (Galiana et al., 1997) against pathogen infection. Literature survey provides evidence for the existence of antifungal proteins that show similarity with RNase (Van Loon et al., 1994; Huynh et al., 1996). It has been suggested that SAR-RNase may induce pathogen growth resistance by using mechanisms as S-RNase of solanaceous plants with gametophytic self incompatibility which arrest the growth of pollen from the same genotype (Huang et al., 1994; Royo et al., 1994). From our studies, it appears that RNase are involved in the resistance of pearl millet to downy mildew system.

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