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==========1/25==========

1

Lipopolysaccharide-induced priming enhances NO-mediated activation of defense
1

responses in pearl millet challenged with Sclerospora graminicola
2

3

Abstract
4

Lipopolysaccharides (LPS) are resistance elicitors known to protect plants against various
5

pathogens. Lipopolysaccharides isolated from Pseudomonas fluorescens UOM SAR 14
6

effectively induced systemic and durable resistance against pearl millet downy mildew
7

disease caused by the oomycete Sclerospora graminicola both in the greenhouse and in the
8

field.[2] [3] [7] [21] ... Rapid and increased callose deposition and H2O2 accumulation was evidenced in
9

susceptible seeds pre-treated with LPS (SLPS) in comparison with the control seedlings,
10

which also correlated with expression of various other defense responses. Biochemical
11

analysis of enzymes and quantitative real-time polymerase chain reaction data suggest that
12

LPS protects pearl millet against downy mildew through the activation of basal defense
13

mechanisms of the plant, such as nitric oxide (NO) generation, enhanced activities of defense
14

enzymes and proteins. Induction and elevation of NO concentrations were shown to be

15

essential for LPS mediated defense manifestation in pearl millet and had an impact on the
16

other downstream defense responses like enhanced activation of enzymes and PR-proteins.
17

Temporal expression analysis of important defense enzymes and PR-proteins in SLPS
18

seedlings challenged with the downy mildew pathogen revealed that the enzymes peroxidase,
19

phenylalanine ammonia lyase, and the PR-proteins PR-1 and PR-5 were significantly
20

enhanced compared to the untreated control.[1] Higher gene expression and protein activities of
21

HRGPs were observed in SLPS seedlings which were similar to that of the resistant check.[1]
22

Collectively our results suggest that, in pearl millet-downy mildew interaction, LPS pre-
23

treatment affects defense signaling through the central regulator NO which triggers the
24

activities of POX, PAL, PR-1, PR-5, and HRGPs.
25

==========2/25==========
2

Key Words: Pearl millet downy mildew, lipopolysaccharides, induced resistance, nitric
26

oxide, defense enzymes, PR-proteins gene expression.
27

Introduction
28

Pearl millet [Pennisetum glaucum (L.) R. Br.] being more tolerant to drought stress, serves as
29

an important cereal crop, both in terms of food and fodder for millions of people living in the
30

semi-arid regions of Asia and Africa. As a staple food, nearly about 50 million Indians,
31

particularly in states of Rajasthan, Maharashtra, and Gujarat are dependent on pearl millet
32

which is grown in an area of seven million hectares with an annual production of 9.[1] [3] [2] 25 million
33

tonnes (Yadav, 2014). The downy mildew caused by the biotrophic oomycete
34

Sclerosporagraminicola (Sacc.) Schroet is the most destructive disease of pearl millet
35

severely affecting the production thereby incurring huge economic losses to the farmers.
36

Downy mildew caused crop losses up to 60% are reported and at times losses in individual
37

fields can reach nearly 100%, and are estimated to average 14% across India (Bidinger and
38

Hash, 2004;[3] Thakur et al., 2011). Popular and conventional methods like resistance breeding,
39

chemical control, and biological control are employed to manage the disease; however, they
40

have their own drawbacks. Chemical control of downy mildew is associated with safety risks
41

and also not economical for a crop like a pearl millet, and resistant hybrids succumb to the
42

breakdown of resistance due to the highly variable nature of the pathogen. In this context,
43

newer eco-friendly and safe options are being explored.
44

Resistance against pathogens can be triggered in plants through a phenomenon termed as
45

induced resistance which is projected as an eco-friendly and safe alternative strategy for plant
46

protection, and various studies across different plant-pathogen systems have shown its
47

efficiency in developing sustainable agriculture by minimizing the use of otherwise
48

hazardous chemicals. Induced resistance can be achieved by an array of biotic and abiotic
49

agents which act by eliciting an increased expression of innate immunity of plants against a
50

wide range of pathogens (Lyon, 2007; Walters et al., 2013). Among the several determinants
51

involved in the induction of systemic resistance by plant growth promoting rhizobacteria
52

(PGPR), the important are lipopolysaccharides (LPS) present in the outer membrane of gram-
53

negative bacterial cells (Van Loon et al.[26] [48] [13] , 1998; Ramamoorthy et al., 2001).
54

Lipopolysaccharides are ubiquitous, indispensable components of the cell surface of gram-
55

negative bacteria easily recognized by plants to directly trigger some plant defense-related
56

responses (Dow et al.[42], 2000; Newman et al., 2007; Erbs and Newman, 2012). LPS isolated
57

from a bacterial endophyte Burkholderia cepacia, pre-conditioned and protected tobacco
58

plants from Black-shank disease caused by Phytophthora nicotianae infection and the
59

treatment was shown enhance activities of several PR-proteins (Coventry and Dubery, 2001).
60

Enterobacter asburiae derived LPS induced systemic resistance in lettuce against soft rot
61

disease caused by Pectobacterium catovorum subsp. catovorum (Pcc), and this was
62

associated with increased activities of the defense enzymes superoxide dismutase and
63

peroxidase (Jetiyanon and Plianbangchang, 2013).
64

Induction of systemic and durable resistance against downy mildew of pearl millet has been
65

demonstrated in earlier studies from our laboratory using LPS from plant growth promoting
66

Pseudomonas fluorescens isolate UOM SAR 14 (Niranjan-Raj et al.[2] [7], 2011). However, the
67

mechanisms involved in the process of resistance were not studied.[12] The aim of the present
68

study was to study the role of different defense enzymes, PR proteins, resistant proteins at
69

biochemical and molecular levels during LPS induced resistance against pearl millet downy
70

mildew.[2] [12] [7]
71

Experimental procedures
72

Host
73

Two pearl millet cultivars were used as host plants for all the experiments.[0] The susceptible

pearl millet plants were raised from the seeds of the pearl millet cv.[0] 7042S which is recorded to be highly susceptible to downy mildew, and the resistant pearl millet plants were raised from the seeds of the pearl millet cv.[0] [15] 18292 which is highly resistant to downy mildew.[0] [4] [1] [3] ... Both 7042S and IP 18292 were sourced from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India and All India Coordinated Research Project on Pearl Millet (AICRP-PM), Mandor, Rajasthan, India.[0] [1] [3] [4] ...

Pathogen source and preparation of inoculum

The highly downy mildew susceptible 7042S pearl millet crop was raised in downy mildew sick plot which is heavily infested with Sclerospora graminicola oospores.[0] The downy mildew pathogen was maintained throughout the experimental period on this susceptible cultivar under field conditions.[0] [3] The downy mildew zoospores collected from these downy mildewed leaves was used as inoculum.[0] In the evening hours, the diseased leaves with high sporulation on the abaxial side were selected and collected, washed thoroughly in running tap water till the existing sporangia are removed.[0] [1] [3] [14] ... These leaves were cut into small pieces, blotted dry and were arranged in perspex plates (lined with moist blotters on both sides) and were kept in a dark, moist chamber for sporulation.[0] [1] [14] The following morning, the leaf pieces were observed for sporulation and the fresh crop of sporangia were scraped into sterile distilled water with a brush.[0] [4] The final concentration of the zoospores was adjusted to 40,000/ml using a haemocytometer, and used for inoculation.[0] [4]

LPS preparation and seed treatment

The bacterial isolate P.[0] fluorescens UOM SAR 14 was used as the source for the extraction of
95

Lipopolysaccharides.[0] The extraction, preparation and pearl millet seed treatment of LPS was
96

carried out according to the procedure earlier described by Niranjan Raj et al.[0] [53] , (2011).
97

Pseudomonas fluorescens UOM SAR 14 was inoculated on to KMB agar (270C) and
98

==========5/25==========
5

incubated for 24 h.[0] The bacterial cells were harvested, washed by centrifugation at 4000 g for
99

5 min, using phosphate buffered saline (10 mM, pH 7.[0] 2) followed by lyophilization.[0] The
100

cells were further suspended in 50 mM Tris HCl and 2 mM EDTA buffer (pH 8.[0] 5) and
101

subsequently sonicated at resonance amplitude six times for 30 s at 0–40C.[0] The intact cells
102

were separated by centrifugation, and the supernatant was further centrifuged at 8000 g for 60
103

min to obtain a pellet which was resuspended in 2 mM Tris HCl buffer of pH 7.[0] 8 and stored
104

at -200C. This pellet which contained the crude LPS, proteins and RNA was extensively
105

dialysed against water.[0] Further, the pellet was lyophilized and was extracted with
106

phenol/chloroform twice.[0] The resulting aqueous phase was pooled, again lyophilised and
107

suspended in Tris buffer.[0] The water phase was vigorously shaken and centrifuged at 16,000 g
108

for 5 min to separate and collect the LPS.[0] The water phase was shaken with equal volume of
109

chloroform to further remove the traces of phenol.[0] Protein, DNA and RNA contamination
110

were removed by treating the aqueous phase Proteinase K, DNase and RNase respectively.[0]
111

The resultant aqueous phase was vacuum dried, resuspended in Tris HCl buffer and stored at
112

-200C.[0] Purity of the LPS preparation was checked by mixing LPS sample with 1µl of
113

bromophenol blue, boiled and cooled for a minute and centrifuged at a speed of 12000xg.[0]
114

This sample was subjected to SDS-PAGE as described by Laemmli (1970) on 10 % gels run
115

at constant voltage (90V) and varying curent (<40 mA) at 4oC. The gels were stained using
116

the silver stain and scanned using a gel doc (Supplementary Fig.[0] 1).
117

Seeds of cv. 7042S were surface sterilized (0.[0] [6] 02% mercuric chloride for 5 min), thoroughly
118

rinsed in sterile distilled water (SDW) and were soaked in LPS solution (SLPS) at 50 µg/ml
119

concentration.[0] [3] [4] [1] ... One set of treatment comprising of SLPS followed by treatment with the NO
120

scavenger 25mM cPTIO (SLPS+cPTIO) 1 h prior to challenge inoculation was included as a
121

check to know the involvement of NO in defense responses induced by LPS treatment.[0] The
122

suspensions were incubated at 270C in a rotary shaker for 6 h time duration.[0] [1] [3] [4] ... Later, the seeds
123

==========6/25==========
6

were allowed to dry in an incubator at 300C.[0] [4] Resistant (R) and Susceptible (S) seeds soaked
124

in SDW for the same time duration served as positive control and negative control,
125

respectively.[0]
126

Pathogen inoculation and sampling of seedlings
127

The treated seeds were plated on moist blotters in Perspex plates and incubated for two days.[0] [1] [2] [3] ...
128

The emerging seedlings were inoculated with the S.[0] [1] [5] [2] ... graminicola zoospore suspension of 4 x
129

104 ml-1 concentration following the root-dip inoculation method and incubated at 25 ±10C in
130

dark.[0] [1] [3] One set of treated seedlings were not inoculated and were used as uninoculated
131

controls.[0] Seedlings were harvested at 0, 3, 6, 9, 12, 24, 48 and 72 h after inoculation (hai),
132

wrapped immediately in aluminium foil and stored at -800C until further use for biochemical
133

and molecular studies.[0] [1] [2] [13] ...

134

Estimation of nitric oxide
135

One gram of the harvested pearl millet seedlings was ground and homogenized in 1ml buffer
136

(0.[0] [1] [2] 1 M sodium acetate, 1 M NaCl and 1% (w/v) ascorbic acid, pH 7.[0] 0). The homogenate
137

obtained was incubated immediately with 10 mM diaminofluorescein-FM (DAF-FM) for 1 h,
138

followed by centrifugation at 10,000 rpm at 40C for 10 min.[0] The resultant supernatant was
139

collected and used for measurement of NO.[0] Fluorescence from diaminofluorescein-2T (DAF-
140

2T), the reaction product of DAF with NO, was measured by spectrofluorimeter with
141

excitation and emission wavelengths of 495 and 515 nm respectively (Kojima et al.[0] , 2001).
142

Histological studies
143

Time-course analysis of callose deposition
144

Callose deposition in pearl millet seedlings was analysed according to the method described
145

by Jensen (1962).[0] [1] The epidermal peelings were soaked in water-soluble aniline blue (0.[0] [1] 005%)
146

in 0.15 M di-potassium phosphate for 1 h.[0] [1] [4] The stained peelings were mounted in glycerol and
147

observed under fluorescence microscope where k = 365–405 nm.[0] [1] [4] Fluorescence was observed
148

along the walls of the cells with callose deposition.[0] Microscopic evaluation: in each case, 20
149

microscopic fields were counted for percentage calculation.[0] [1] [4] The experiment was done in four
150

replicates, each with 25 seedlings each and repeated 3 times.[0] [4] The peelings were examined
151

under x 500 and x1250 magnification for counting and photography, respectively.[0] [1] [9] [4] ...
152

Time-course analysis of localization of H2O2
153

Hydrogen peroxide localization in pearl millet seedlings was analysed according to the
154

method described by Thordal-Christensen et al.[0] [4] [1] (1997). The epidermal peelings were soaked
155

in 1 mg/ml 3,3'-diaminobenzidine (DAB), pH 3.[0] 8. The stained peelings were mounted in
156

30% glycerol, 30% lactic acid and observed under a microscope.[0] Regions with reddish-brown
157

staining of the cells indicated H2O2 accumulation.[0] Microscopic evaluation: in each case, 20
158

microscopic fields were counted for percentage calculation. The experiment was done in four
159

replicates, each with 25 seedlings each and repeated 3 times. The peelings were examined
160

under x 500 and x1250 magnification for counting and photography, respectively.
161

Biochemical studies
162

Enzyme assays
163

Enzyme extraction
164

Harvested pearl millet seedlings (1 g fresh weight) were finely ground to a paste in 1 ml of
165

extraction buffer.[0] [1] The obtained extract was centrifuged at 12,000g for 20 min at 40C and the
166

supernatant transferred to a new tube and used as the enzyme extract.[0] [1] [11] [10] ...
167

Protein estimation
168

To calculate the specific activities of the enzymes, protein content in the crude extract were
169

estimated by Lowry's method (Lowry et al.[0] [1] , 1951) using BSA (Sigma) as a standard.[0] [1] [6]
170

Phenylalanine ammonia-lyase assay
171

One gram of the harvested pearl millet seedlings was ground to a fine paste in 25 mM Tris
172

HCl buffer (pH 8.[0] [1] [10] 8). Activity of the PAL enzyme was assayed following the method
173

described by Beaudoin-Eagan and Thorpe (1985).[0] 100 µl of enzyme extract was mixed with

900 ml of 50mM L-Phenylalanine and 100 mM Tris HCl buffer solution, and kept in a water

bath at 400C for 120 min.[0] [1] The reaction was stopped by adding 60 ml of 5 N HCl.[0] [1] PAL

activity was determined as the amount of t-cinnamic acid formed from L-Phenylalanine per

mg of protein per min measured spectrophotometrically at a wavelength of 290 nm.[0] [1]

Peroxidase assay

One gram of the harvested pearl millet seedlings was ground to a fine paste in 1 ml of 10 mM

potassium phosphate buffer (pH 6.[0] [1] [10] 9) and the extract was centrifuged at 12,000g for 20 min at

40C and the supernatant was transferred to a new tube and used as the enzyme extract.[0] [1] [11] [43] ...

Peroxidase activity was assayed following the method described by Hammerschmidt et al.[0] [1] [11] ,

(1982). The reaction mixture (3 ml) consisted of 0.[0] [1] [5] 25% v/v guaiacol in 10 mM potassium

phosphate buffer (pH 6.[0] [1] [5] 0) containing 100 mM hydrogen peroxidase.[0] [1] [15] The crude enzyme (10

ml) was added to initiate the reaction, which was measured spectrophotometrically at 470

nm.[0] [15] [1] One unit of peroxidase enzyme activity is defined as the increase in absorbance recorded

at 470 nm.[0] [1] [15]

Hydroxyproline-rich glycoprotein assay

Preparation of cell wall and extraction of hydroxyproline (Hyp)

Extraction of pearl millet coleoptiles cell walls was carried out by slightly modifying the

procedure of York et al.[0] [20] [1] [28] ..., (1986). The roots and the coleoptile regions pearl millet seedlings

were separated and homogenised using pestle and mortar at 4oC in 0.[0] [1] [28] 5M potassium phosphate

buffer, pH 7.0. Complete disruption of the cells was ascertained by observing the homogenate

under microscope. The broken cells suspension was centrifuged at 2000×g for 10 min. Cell walls were washed repeatedly with the above buffer followed by SDW. Washed cell walls were vigorously stirred and suspended in 5 volumes of 1:1 chloroform–methanol. Subsequently, the organic solvent was carefully removed. Cell walls were repeatedly washed with 5 volumes of acetone and then air dried. The Hyp content in the cell wall hydrolysate was analysed to determine the amount of HRGPs. The hydrolysis of the cell walls was carried out in sealed tubes using 6N HCl for 18 h at 110 oC. The hydrolysates were evaporated to dryness to remove traces of HCl. Hyp was then extracted in minimum amount of SDW from the dried hydrolysed samples and estimated following the spectrophotometric procedure of Prockop and Udenfriend, (1960). Hyp content was expressed as _g Hyp mg−1 cell wall (dry weight).

Molecular studies

Quantitative real-time PCR analysis (qPCR)

The relative quantitation of PAL (NM_001174615.1), POX (EU492461), PR1 (HQ699781.1), PR5 (EU725133.1), and HRGP (GQ223398) mRNAs in harvested pearl millet seedling samples was done by using gene-specific primers designed with Primer Express version 3.0 software (Applied Biosystems) with PP2A (protein phosphatase 2A) as endogenous reference gene (Nayak et al., 2017) (Table 1). The primer specifications was confirmed by running an agarose gel electrophoresis. Every qPCR reaction (20 µL) had 1×

213

SYBR Green PCR master mix (SYBR Green mix, Applied Biosystems), 3 pmol primer and
214

20 ng cDNA and used StepOnePlus™ Real-Time PCR Systems (Applied Biosystems).[0] [2] [1] [3] ... The
215

qPCR steps included: denaturation for 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60
216

°C.[0] [1] [2] [3] ... A melting curve was created using a single cycle consisting of 15 s at 95 °C and 60 s at
217

60 °C at the end of each reaction.[1] [0] [2] [3] ... This was followed by a slow temperature increase to 95 °C
218

==========10/25==========
10

at the rate of 0.[0] [1] [2] [3] ...3 °C s−1. The quantification of target mRNAs used a comparative Ct method
219

(Livak and Schmittgen, 2001).[0] [2] [3] [1] ...
220

221

Effect of LPS pre-treatment on downy mildew disease incidence
222

Under greenhouse conditions
223

Pearl millet seeds treated with the inducers as described above were sown in earthen pots
224

containing autoclaved soil, sand and manure in the ratio 2:[0] [1] [3] 1:1 which were maintained at 25–
225

300C with 95% relative humidity.[0] The experiment consisted of four replicates for each
226

treatment and the pots were arranged in a complete randomized block design.[0] [4] Seedlings were
227

watered and fertilized when required.[0] Seeds treated with SDW served as control and seeds
228

treated with systemic fungicide Metalaxyl formulation Apron 35SD @ 6 g/kg served as a
229

chemical control.[0] [35] The emerging two-day-old seedlings were inoculated with downy mildew
230

pathogen by whorl inoculation method (Singh and Gopinath, 1985) with suspension of S.[0] [1] [3]
231

graminicola zoospores at a concentration of 4 x 104 zoospores/ml prepared as described
232

earlier.[0] [1] [18] [3] ... The inoculated plants were observed for downy mildew disease expression and rated
233

diseased when they expressed typical downy mildew symptoms like abaxial leaf sporulation,
234

chlorosis, stunting and earhead malformation.[0] [1] The downy mildew screening data were
235

consolidated at 60 days after sowing (DAS).[0]
236

Under field conditions
237

The field experiment was conducted to determine the effect of LPS on pearl millet downy
238

mildew disease incidence.[0] [8] [12] [49] ... Filed trials were conducted in Downy Mildew Nursery at the
239

University of Mysore, during 2015–2016.[0] The downy mildew nursery was a sick plot which
240

was naturally infested with oospores of S.[0] [1] graminicola, which served as the source of primary
241

inoculum.[0] [1] Infector rows raised 21 days prior to the raising of the test rows provided the
242

additional inoculum (Williams, 1984).[0] [1] Inducer treatments and controls were the same as
243

described above.[0] [1] Treated seeds were hand-sown with a minimum of four replications per
244

treatment.[0] Each replication was a single row of 5 m length, hand seeded with 100–150 seeds
245

per row.[0] The read loamy soil of the field was watered adequately when required and the
246

thinning of the plants was done after 21 days. The downy mildew disease was rated as
247

described for greenhouse studies.
248

Statistical analysis
249

All experiments were carried out in four replicates.[0] Data were analysed separately for each
250

experiment and was subjected to arcsine transformation and analysis of variance was carried
251

out with transformation values (JMP Software;[0] [1] [2] [3] ... SAS Institute Inc., Cary, NC). The
252

significance of effect of treatments was determined by the magnitude of the F value ($p \leq$
253

0.[0] [1] [2] [3] ...05). Tukey's HSD test was applied for separation of treatment means.
254

255

Results
256

LPS treatment enhances Nitric Oxide generation
257

NO is suggested to play an important role during induction of resistance in plants and it is
258

also demonstrated that NO is involved in induced resistance in pearl millet against downy
259

mildew.[5] [1] [4] [8] ... Therefore, to analyze the role of NO in LPS induced resistance in pearl millet, a time
260

course study was conducted to assess the generation of NO in treated seedlings was measured
261

compared to the control.[1] [5] [13] At constitutive level, NO generation was very low in all categories
262

of seedlings with or without pathogen inoculation.[1] [2] [13] [37] ... However, as time progressed, in all
263

categories of seedlings NO generation was significantly higher in inoculated seedlings
264

compared to the uninoculated seedlings (Fig.[1] [47] [2] 1). Up to 6 h, NO concentration generated was
265

high in resistant seedlings compared to SLPS seedlings.[2] [4] [9] However, after that and up to 24 h
266

NO levels were significantly higher in SLPS seedlings compared to the resistant seedlings,
267

beyond which again there was a fall in the levels of NO in SLPS seedlings.[2] [4] [9] However, without
268

inoculation, NO level in SLPS seedlings was considerably higher than that of resistant,
269

SLPS+cPTIO treated and susceptible seedlings.[4] [1] [2] In all categories of seedlings, NO
270

concentration peaked at 24 h with or without inoculation.[1] [2] Highest NO of 16.3 was recorded
271

in SLPS seedlings at 24 hai which was 1.21, 4.27, and 5.62 folds higher than that of resistant,

272

SLPS+cPTIO treated and susceptible seedlings.[2] [1] At all-time points, the concentration of NO
273

was significantly lower insusceptible seedlings compared to the resistant and SLPS,
274

SLPS+cPTIO treated seedlings with or without pathogen inoculation.[1] [2] [4] These results
275

demonstrated that NO generation is significantly enhanced due to LPS treatment in pearl
276

millet.
277

Histological studies
278

LPS treatment enhances Callose deposition
279

One of the early mechanisms of LPS elicited defense responses in plants is the alteration of
280

the host cell wall so as to inhibit the pathogen ingress at the site of infection, and callose
281

deposition is often reported to be an important cell wall reinforcement process initiated by
282

LPS. Therefore, to elucidate the role of LPS in pearl millet cell wall strengthening, callose
283

deposition was studied in the treated and untreated seedlings. Callose deposition at basal level
284

was observed for both inoculated and uninoculated seedlings of all categories. Among the
285

uninoculated seedlings, maximum callose deposition was observed in SLPS seedlings
286

compared to the uninoculated resistant, SLPS+cPTIO and susceptible seedlings (Fig.[1] 2A).
287

After inoculation, callose deposition gradually increased and by the end of 24 hai a total of
288

93.7% and 83.5% cells with callose deposition was observed in resistant and SLPS treated
289

seedlings.[1] At 24 hai, callose deposition in SLPS seedlings was 10.[1] 3 and 10.43 folds higher
290

than that of SLPS+cPTIO and susceptible control seedlings respectively (Fig. 2B). Callose
291

deposition results indicated that LPS treatment to pearl millet seeds will trigger earlier and
292

higher deposition of callose, particularly after downy mildew pathogen inoculation, in
293

comparison to the untreated control; and NO plays an important role in callose deposition.[12] [8] [10] [19] ...
294

LPS treatment increases H2O2 accumulation
295

It has been demonstrated that H2O2 accumulation is vital for activation of various defense
296

responses as it is an important early signaling molecule, and therefore to test whether H2O2 is
297

involved in LPS elicited systemic resistance in pearl millet against downy mildew, we studied
298

the time course pattern of H2O2 accumulation in the test seedlings.[4] [1] [5] [9] ... With or without pathogen
299

inoculation basal level of H2O2 accumulation was observed in all categories of seedlings (Fig.[1] [2] [6]
300

3A). However, following pathogen inoculation H2O2 accumulation gradually increased up to
301

24 h and plateaued thereafter. Maximum H2O2 accumulation was observed in resistant
302

seedlings followed by SLPS seedlings. After 24 hai H2O2 accumulation in SLPS seedlings
303

was 2.91 and 3.4 folds higher than that of SLPS+cPTIO and susceptible seedlings
304

respectively (Fig. 3B). In SLPS+cPTIO treated seedlings, maximum accumulation was
305

observed at 72 hai. The results showed that LPS treatment to pearl millet seeds triggers the
306

accumulation of H2O2 and scavenging of NO generation in LPS treated seedlings will
307

significantly reduce the H2O2 accumulation.
308

Biochemical studies
309

LPS treatment triggers increased activities of defense enzymes and HRGPs
310

PAL activity
311

It has been demonstrated previously that during induced systemic resistance, the enzyme
312

PAL plays a vital role and more so during LPS mediated immunity elicitation, therefore, to
313

determine the role of LPS induced resistance in pearl millet, PAL assay was conducted in the
314

test seedlings. Constitutive PAL activity was recorded in resistant, SLPS and susceptible
315

seedlings with or without pathogen inoculation which gradually and significantly increased
316

with time.[1] [2] PAL activity at constitutive level was significantly lesser in SLPS+cPTIO and
317

susceptible seedlings compared to resistant and SLPS seedlings with or without pathogen
318

inoculation.[1] [2] [37] [38] ... At all-time points tested PAL activity was higher in resistant seedlings
319

compared to SLPS, SLPS+cPTIO and susceptible seedlings with or without pathogen
320

inoculation.[1] [6] In both inoculated and uninoculated samples PAL activity peaked at 6 hai in
321

resistant and SLPS seedlings whereas in SLPS+cPTIO and susceptible seedlings maximum
322

PAL activity was observed at 9 hai.[1] In resistant seedlings, highest activity of 34.2 units was
323

recorded at 6 hai. In SLPS treated seedlings maximum PAL activity of 29.1 was recorded at 6
324

hai which was 3.54 and 4.20 folds higher than that of SLPS+cPTIO and susceptible at the
325

same time point. Among the uninoculated seedlings, at 6 hai PAL activity in SLPS was on
326

par with resistant seedlings and significantly higher than that of SLPS+cPTIO and susceptible
327

seedlings. Treatment with NO scavenger cPTIO before inoculation with pathogen resulted in
328

decreased PAL activities after 3 hai. At all-time points, the PAL activity was significantly
329

lower in susceptible seedlings compared to the resistant and LPS treated seedlings (Fig.[2] [4] [9] 4).
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Overall, PAL activity is enhanced significantly in LPS treated seedlings, particularly after
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pathogen inoculation. Further, NO was shown to be responsible for increased PAL activity

332

and scavenging of NO in the treated seedlings resulted in the poor activity of PAL.
333

Peroxidase activity
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Peroxidase is known to play a multifaceted role in plant defense which is involved in
335

lignification, cross-linking of phenolics and glycoproteins, suberization and phytoalexin
336

production and to determine the role of POX during LPS induced resistance in pearl millet
337

POX assay was carried out in the test seedlings.[17] [22] Constitutive peroxidase activity was
338

recorded in resistant, LPS treated and susceptible seedlings which gradually and significantly
339

increased with time. At all-time points tested peroxidase activity was higher in resistant
340

seedlings compared to SLPS and susceptible seedlings. In resistant and, SLPS treated
341

seedlings peak of POX activity was observed at 9 hai as against the SLPS+cPTIO and
342

susceptible seedlings where POX activity peaked at 12 hai with or without pathogen
343

inoculation. In resistant seedlings, highest activity of 74.68 units was recorded at 9 h after
344

inoculation. In SLPS treated seedlings maximum peroxidase activity of 71.63 was recorded at
345

9 hai which was 6.72 and 9.68 folds higher than that of SLPS+cPTIO and susceptible
346

seedlings respectively.[1] Treatment with NO scavenger cPTIO before inoculation with
347

pathogen resulted in decreased POX activities after 3 hai. At all-time points, the POX activity
348

was significantly lower in susceptible seedlings compared to the resistant and LPS treated
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seedlings (Fig.[2] 5). These results indicated that LPS treatment induces and enhances POX
350

activity through the generation of NO.
351

Hydroxyproline analysis

Increased cross-linking of cell wall proteins particularly hydroxyproline-rich glycoproteins is

known to be one of the important cell wall reinforcement mechanisms of resistance inducing

elicitors or inducers. In this study to elucidate the role of HRGPs in LPS induced resistance a

time course analysis of the accumulation of HRGPs wad done in the test seedlings.

Constitutive HRGPs activity was recorded in resistant, SLPS treated and susceptible

seedlings which gradually and significantly increased with time.[1] At all-time points tested

HRGPs activity was higher in resistant seedlings compared to SLPS, SLPS+cPTIO and

susceptible seedlings.[1] [6] Maximum HRGPs activity observed at 9 hai in resistant and SLPS

treated seedlings whereas maximum activity was delayed to 12 hai in SLPS+cPTIO and

susceptible seedlings. At 9 h after inoculation HRGPs activity of 0.976 in resistant seedlings

was significantly on par with 0.971 activity of SLPS treated seedlings. HRGPs activity in

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16

SLPS treated seedlings at 9 hai was 2.31 and 2.73 folds higher compared to SLPS+cPTIO

and susceptible seedlings. Treatment with NO scavenger cPTIO before inoculation with

pathogen resulted in decreased HRGPs activities after 3 hai. At all-time points, the HRGPs

activity was significantly lower in susceptible seedlings compared to the resistant and LPS

treated seedlings (Fig. 6). A significant increase in HRGPs activity in LPS treated seeds in

comparison to the untreated control showed that cell wall strengthening by HRGPs

crosslinking is induced by LPS treatment and this process is mediated by generation of NO.[2]

Molecular studies

LPS treatment upregulates defense genes expression
372

Induction of resistance is dependent on a coordinated expression of a set of complex defense
373

genes which majorly comprise of defense enzymes, defense proteins, and pathogenesis-
374

related (PR) proteins and to demonstrate the role of gene expression of different defense
375

enzymes and proteins we performed a Real-time analysis of the pattern of accumulation of
376

genes of some of the important enzymes and proteins in the test seedlings.[2] Quantification of
377

expression of the POX, PAL, PR-1, PR-5 and HRGPs gene in resistant, SLPS and susceptible
378

pearl millet samples indicated significant differences in the levels of gene expression.
379

Constitutive levels of PAL transcripts were detected in all categories of seedlings with or
380

without pathogen inoculation and the expression levels were higher in resistant and SLPS
381

seedlings compared to the susceptible controls.[2] [1] [4] [9] ... In all sets of seedlings, PAL gene expression
382

was higher in inoculated samples compared to the uninoculated samples at all time points.
383

The expression of PAL gene was highest at 6 hai in resistant and SLPS seedlings whereas in
384

susceptible seedlings maximum PAL gene expression was recorded at 9 hai.[2] The PAL gene
385

expression was highest in the resistant seedlings at all time points compared to the SLPS and
386

susceptible seedlings with or without pathogen inoculation.[2] In SLPS treated seedlings PAL
387

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17

expression at 6 hai was 3.[2] 33 and 3.82 folds higher than that of SLPS+cPTIO treated and
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susceptible seedlings at the same time point.[1] In uninoculated SLPS treated seedlings PAL
389

expression at 6 h was 2.03 and 2.58 folds higher than the SLPS+cPTIO treated and control
390

seedlings (Fig. 7A).

391

Strong signals of accumulation of peroxidase transcripts were detected at all time intervals in
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resistant, SLPS treated and susceptible seedlings with or without inoculation, however, POX
393

gene expression was higher in inoculated samples compared to the uninoculated samples. In
394

resistant and SLPS treated seedlings POX expression peaked at 9 h with or without
395

inoculation as against the susceptible seedlings where POX expression peaked at 12 h with or
396

without inoculation. Maximum POX expression was recorded at 9 hai and at this time point
397

POX gene expression in SLPS treated seedlings was on par with resistant seedlings;[1] and 6.49
398

and 13.11 folds higher than the SLPS+cPTIO treated and susceptible control seedlings
399

respectively (Fig. 7B).
400

Constitutive expression of HRGP transcripts was evident in all categories of seedlings with or
401

without inoculation and highest was in resistant seedlings.[2] [1] However, there was a marked
402

increase in the HRGP transcript accumulation as time progressed, and the expression peaked
403

at 9 h in resistant and SLPS seedlings whereas in susceptible seedlings expression peaked at
404

12 h.[2] At 9 h time point, HRGP expression in inoculated SLPS seedlings was 2.32, and 2.72
405

folds higher than that of SLPS+cPTIO treated and susceptible controls respectively. At 12 hai
406

HRGPs expression in SLPS treated seedlings was 1.21folds higher than the resistant
407

seedlings (Fig. 7C).
408

PR proteins induction and accumulation at higher levels during a pathogen infection is
409

important for eliciting defense responses hence, we have dissected the role of PR proteins
410

particularly PR-1 and PR-5 by a time course analysis of their expression pattern in the test
411

seedlings.[2] PR-1 transcript accumulation was noticed in all categories of seedling at the constitutive level, which increased with time. In all categories of seedlings, PR-1 gene expression was higher in inoculated samples compared to the uninoculated samples at all time points. It was noted that PR-1 expression peaked at 24 h time point in SLPS treated seedlings whereas in resistant, SLPS+cPTIO treated and susceptible controls maximum expression was at 48 h, with or without inoculation. At 24 hai PR-1 expression in SLPS treated seedlings was 1.69, 4.63 and 5.12 folds higher than resistant, SLPS+cPTIO treated and control seedlings respectively.[1] At 24 h time point, PR-1 expression in inoculated SLPS seedlings was 3.82, 5.11 and 16.29 folds higher than that of uninoculated SLPS, inoculated susceptible and uninoculated susceptible seedlings respectively (Fig.[2] 7D).

Similarly, PR-5 transcript accumulation was noticed in all categories of seedling at the constitutive level, which increased with time. In all seedlings, with or without pathogen inoculation, PR-5 gene expression peaked at 24 h time point.[1] From 3 hai to 24 hai PR-5 expression was higher in SLPS treated seedlings compared to both resistant and control seedlings.[1] Maximum PR-5 expression was recorded at 24 hai at which point PR-5 expression in SLPS treated seedlings was 1.[1] 13 folds higher than resistant seedlings.[3] [1] At 24 h time point, PR-5 expression in inoculated SLPS seedlings was 3.28 and 3.59 folds higher than that of SLPS+cPTIO treated and susceptible seedlings respectively.[2] However, from 48 hai onwards PR-5 expression decreased and was higher in resistant seedlings compare to SLPS treated and control seedlings (Fig. 7E).

These qPCR results demonstrated that LPS treatment is responsible for the earlier and higher
432

expression of genes of various defense enzymes like PAL, POX and defense proteins like
433

HRGPs, PR-1, and PR-5;[2] [1] [7] and all these defense gene expression enhancements in LPS treated
434

seeds is found to be mediated by the enhanced generation of NO.[3]
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LPS induces resistance against Downy mildew disease
436

A series of greenhouse and field experiments were conducted to demonstrate the ability of
437

LPS to protect pearl millet plants against downy mildew disease and also to examine the role
438

of NO in LPS mediated induced resistance.[3] [5] Downy mildew disease incidence was
439

significantly reduced due to SLPS treatment both under greenhouse and field conditions.[5] [3]
440

Further, it was noted that SLPS+cPTIO treatment drastically increased downy mildew
441

disease incidence suggesting a possible role for NO in LPS mediated resistance. Among all
442

the treatments Apron showed the lowest downy mildew incidence both under greenhouse and
443

field conditions (Fig. 8).
444

Under greenhouse conditions, maximum downy mildew reduction was in resistant seedlings
445

which showed 8.2% downy mildew. SLPS and SLPS+cPTIO treatments recorded 21.3 and
446

62.3% downy mildew respectively as against susceptible control which recorded 97% downy
447

mildew incidence (Fig. 9).
448

Under field conditions, maximum downy mildew reduction was in resistant seedlings which
449

showed 5.9 % downy mildew incidence. SLPS and SLPS+cPTIO treatments recorded 18.6
450

and 54.4% downy mildew as against susceptible control which showed 93% downy mildew

451

incidence (Fig. 9). The downy mildew reaction studies, both under greenhouse and field
452

conditions, clearly showed that LPS treatment is highly effective in managing downy mildew
453

disease by inducing systemic resistance in the host and such elicitation of resistance is
454

brought about by the enhanced generation of NO.
455

Discussion
456

Elicitor signaling primes the host defense responses and effectively contains pathogen
457

manifestation by mechanisms such as cell wall reinforcement, production of antimicrobial
458

metabolites, defense enzymes, PR proteins and hypersensitive response (Lorrain et al., 2003).
459

LPS act as elicitors of systemic resistance in many crop systems and their application is
460

known to have diverse effects in plants including synthesis of nitric oxide, phosphorylation of
461

MAPKs, and priming for more effective induction of various defense responses (Felix et al.,
462

1999; Dow et al., 2000; Gerber et al., 2004; Desaki et al., 2006; Newman et al., 2007; Erbs
463

and Newman, 2012). In this study, we examined the possible mechanisms underlying LPS
464

induced resistance in pearl millet against the oomycete Sclerospora graminicola by analyzing
465

the role of various defense enzymes and PR proteins during the process and furthermore we
466

attempted to elucidate the role of NO during LPS mediated resistance induction.[5] [25]
467

Nitric oxide is a vital signaling compound which acts as an early messenger which in turn
468

primes other signaling events (Garcia-Brugger et al., 2006). The time course analysis of LPS
469

treated pearl millet seedlings upon inoculation with Sclerospora graminicola, recorded earlier
470

generation and significant elevation in NO levels as compared to the untreated seedlings.[1] It

has been previously shown that NO production plays a vital role during innate-immune

responses triggered by LPS (Melotto et al., 2006). In Arabidopsis thaliana, LPS preparations

from different bacteria triggered innate immunity mediated by NO, indicating that NO was a

very early response to LPS treatment (Zeidler et al., 2004). In Arabidopsis, it was shown that

enhanced NO production is one of the immediate responses following LPS perception leading

to defense gene induction (Sun et al., 2012). Endogenous NO production was recorded in

plant cells challenged by avirulent pathogens and elicitors (Wendehenne et al., 2004;

Delledonne, 2005) emphasizing that NO is part of intracellular signaling cascades activated

in plant cells in response to pathogens or elicitors.

The histological studies in our experiments showed that LPS induced resistance mediated by

NO is associated with cell wall modifications like callose deposition and H2O2 accumulation.

Both callose deposition and H2O2 accumulation occurred quicker and in higher rate in SLPS

seedlings compared to the other checks with or without pathogen inoculation. Further, in the

presence of NO scavenger cPTIO, these cell wall modifications were slowed and weakened

confirming the role of NO in these defense responses. Our results corroborate earlier reports

stating that cell wall strengthening is an important aspect of induced resistance, and NO plays

an important in elicitor-induced wall modifications. LPS elicited immunity modulates

through NO production, induction of PR gene expression and cell wall alterations like

deposition of callose and phenolics (Erbs and Newman, 2012). Sun and Li et al., (2013)

demonstrated that, in Arabidopsis, LPS treatments induce an array of defense responses
491

which are modulated by NO generation such as enhanced callose deposition (Sun and Li,
492

2013). Pseudomonas fluorescens (CHA0) induced resistance against Rhizoctonia solani in
493

bean was found to be mediated by NO which increased H2O2 production;[3] while the use of NO
494

scavenger cPTIO resulted in decreased resistance and H2O2 production (Keshavarz-Tohid et
495

al., 2016). Increased cross-linking of cell wall proteins and callose deposition during NO-
496

mediated induced resistance in tomato against Colletotrichum coccodes and R. solani were
497

reported (Wang and Higgins, 2005; Noorbakhsh and Taheri, 2016).
498

Further, the present study showed that NO production was a very vital response of LPS
499

treatment to pearl millet and its production and concentration modulated various other
500

defense responses like the production of defense enzymes, PR proteins and HRGPs which
501

ultimately led to elevated resistance against downy mildew disease.[29] Further, scavenging of
502

NO in LPS treated seedlings resulted in significant decrease in all these defense responses
503

implicating that NO is central to LPS elicited resistance.
504

In plants, PAL plays a key role in the synthesis of lignins and isoflavonoid phytoalexins, both
505

of which are involved in plant defense reactions (Hahlbrock and Scheel, 1989).[19] In the present
506

investigations, PAL enzyme activity and also gene expression were significantly enhanced in
507

SLPS seedlings compared to untreated seedlings. Similar observations were reported in A.
508

thaliana treated with LPS derived from the bacterial pathogens Pectobacterium atrosepticum
509

and Pectobacterium carotovorum subsp. carotovorum significantly induced earlier and higher

510

expression of PAL transcripts (Mohamed et al., 2015). Furthermore, involvement of PAL
511

during pearl millet - S. graminicola interaction has been well documented conferring a major
512

role for this enzyme in resistance development (Nagarathna et al., 1993; Geetha et al., 2005).
513

Furthermore, NO-induced PAL enzymes in pearl millet seedlings during the induced
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resistance against downy mildew disease (Manjunatha et al., 2009a). Likewise, there are
515

several earlier reports which have confirmed the role of NO during enhanced activities of
516

PAL in various host-pathogen systems (Bowler et al., 1994). NO-mediated plant defense
517

activation significantly enhanced the expression of PAL in tobacco and wheat (Durner et al.,
518

1998; de Pinto et al., 2002; Guo et al., 2004).
519

Defense enzymes, especially peroxidase contain the pathogen spread through the formation of
520

polymerized phenolic barriers around the sites of infection (Smit and Dubery, 1997;[1] Li and
521

Steffens, 2002) and trigger the synthesis of anti-nutritive, antibiotic, and cytotoxic compounds
522

leading to enhanced resistance against pathogens (Hammerschmidt and Nicholson, 1999).[1] In
523

the current analysis, peroxidase enzyme activities and also an accumulation of peroxidase
524

transcripts were detected at all time intervals in resistant, SLPS and susceptible seedlings after
525

pathogen inoculation. However, the intensity was higher in resistant and SLPS seedlings
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compared to susceptible seedlings, further confirming the significance of this enzyme in pearl
527

millet downy mildew interaction. The role of peroxidase as an important enzyme and a
528

marker of systemic acquired resistance has been well established in numerous resistance
529

induction studies, however, its role in LPS induced resistance has been less reported.[3] [41] [44]
530

Nonetheless, the role of peroxidase as an important defense enzyme in imparting host

resistance by different inducers/elicitors against pearl millet downy mildew system is also

well demonstrated (Hindumathy et al., 2006; Deepak et al., 2007; Pushpalatha et al., 2007;

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Manjunatha et al., 2008; Devaiah and Shetty, 2009; Raj et al., 2012). In addition, peroxidase

seemed to be a vital enzyme whose levels were significantly enhanced during NO-mediated

resistance induction in pearl millet against downy mildew disease (Manjunatha et al.[3] [1] [4] [5] ..., 2009b).

NO-mediated induced resistance against Rhizoctonia solani in bean correlated with enhanced

peroxidase activities (Keshavarz-Tohid et al.[3] , 2016).

Hydroxyproline-rich glycoproteins (HRGPs) are important plant cell wall structural

components which are known to play a vital role in host defense responses to pathogen

invasion (Davies et al.[27] [36] [20] [8] ..., 1997). Pathogen infections or pathogen-derived elicitor treatments

have increased the level of HRGPs and subsequently induced resistance against various

pathogens (Bradley et al., 1992; Brownleader et al., 1995; Kang and Buchenauer, 2003).

Different elicitors induced downy mildew resistance in pearl millet which correlated with

increased HRGP content in the cell walls;[1] [4] [9] [3] ... particularly, maximum HRGP accumulation was

observed during Pseudomonas fluorescens UOM SAR 14 treatment (Sujeeth et al., 2010). It

is interesting to note here that the LPS used in the present study was obtained from

Pseudomonas fluorescens UOM SAR 14 which implied that LPS has a role in HRGP

accumulation.[12] The role of NO as a key signal component in accumulation of HRGPs was

also demonstrated by our earlier studies wherein priming of pearl millet seedlings with nitric

oxide (NO) donors effectively induced hypersensitive reactions (HR) and enhanced
551

accumulation of Proline/Hydroxyproline-rich glycoprotein (P/HRGP) during infection by
552

downy mildew pathogen Sclerospora graminicola; and endogenous NO concentration
553

regulated the degree of defense responses like HR development, H2O2 accumulation and
554

HRGPs cross-linking (Manjunatha et al., 2009a).
555

Pathogenesis-Related (PR) proteins are a group of diverse proteins whose accumulation is
556

triggered by pathogen attack or abiotic stress. PR-1 and PR-5 proteins, are widely studied and
557

are well established as markers of systemic acquired resistance in several host-pathogen
558

systems.[2] Moreover, PR-1 and PR-5 have been found to possess antifungal activity against
559

oomycetes. In earlier studies involving induced resistance against pearl millet downy mildew
560

disease, inducers like L-methionine, and Bacillus pumilus INR7 have shown accumulation of
561

PR-1 and PR-5 corresponding with the increased development of resistance (Sarosh et al.[2] [6] [7] [3] ...,
562

2005). Though there are several reports confirming the involvement of PR proteins during
563

NO-mediated resistance in plants, the role of LPS during such resistance development is less
564

reported. Our results corroborate earlier studies which have shown that LPS treatment primes
565

the induction and expression of various PR proteins in different host-pathogen systems (Dow
566

et al.[3] , 2000). Microarray studies in Arabidopsis plants showed that LPS treatment induced an
567

array of defense- or stress-associated genes, including glutathione S-transferases, cytochrome
568

P450, and many genes encoding PR proteins, both locally and systemically (Zeidler et al.[16] ,
569

2004). Further, Zeidler et al., showed that NOS mutant Arabidopsis plants, even when treated

570

with LPS completely, failed to express any defense-related genes, thus emphasizing that
571

perception of LPS and induction of NOS contribute toward the activation of plant defense
572

responses.[16] [30] [31] [32] ... Prior treatment of lipooligosaccharides (LOSs) from plant pathogen Xanthomonas
573

campestris pv. campestris induced the defense-related genes PR1 and PR2 in Arabidopsis
574

(Silipo et al., 2005). In, Arabidopsis, LPS treatment resulted in enhanced PR1 gene
575

expression and it was modulated by NO generation (Sun et al., 2012; Sun and Li, 2013).
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Conclusions
577

To our knowledge, this is the first report of the various defense enzymes/proteins and their
578

transcript accumulation pattern during LPS mediated induction of resistance in a monocot-
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oomycete system.[2] [34] [8] [29] ... For most of the enzyme activities and genes examined, the highest levels
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of activities and transcripts were observed in resistant seedlings followed by SLPS seedlings
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==========25/25==========
25

in comparison with the susceptible seedlings.[1] Measurement of mRNA accumulation
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demonstrates that genes encoding POX, PAL, HRGPs, PR-1, and PR-5, were induced very
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prominently by LPS treatment.[1] The enzyme products of the genes examined here are
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predicted to be involved in the biosynthesis of defense compounds, so it is not surprising that
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transcripts accumulated to high levels following pathogen inoculation.[40] [17] [22] The very rapid and
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large changes in the resistant and induced resistant seedlings, in contrast to the delayed,
587

smaller changes in the susceptible seedlings suggests that rate and magnitude of chemical
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defense responses are important for the effective expression of defense.[1]
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Enhanced accumulation of both defense enzymes and PR-proteins showed NO as the
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main gene-induction signal which is transported through the plant.[1] This result is
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indicative of NO as the main signal molecule triggered by LPS treatment, particularly
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during pearl millet-downy mildew host-pathogen interaction. Induced systemic
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resistance mediated by LPS has been demonstrated only in a few plant-pathogen systems
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and the biochemical and molecular mechanisms underlying this phenomenon is not
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investigated completely. Overall, the present study demonstrates the plausible
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involvement of the important defense enzymes like peroxidase, phenylalanine ammonia
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lyase, PR-proteins like PR-1 and PR-5, HRGPs leading to hypersensitive response
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during LPS mediated induced resistance against pearl millet downy mildew and that NO
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is a central signal to all these defense manifestations.[2] [3] [4] [7] ...
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