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Unsaturated fatty acids from zoospores of *Sclerospora graminicola* induce resistance in pearl millet

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Abstract

Downy mildew of pearl millet, caused by *Sclerospora graminicola*, is a devastating disease, resulting in high economic losses in the semi-arid regions of the world. Recently, induction of host plant resistance using biotic and abiotic inducers are included among disease management practices as an eco-friendly approach. Unsaturated fatty acids are considered as a new generation of plant disease resistance inducers. In the present study, six unsaturated fatty acids, viz. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), linolenic acid, linoleic acid and oleic acid, all originally detected in the zoospores of *S. graminicola,* were applied to seeds of susceptible cultivars of pearl millet to examine their ability to protect against downy mildew under greenhouse and field conditions. In greenhouse experiments, EPA and AA induced a maximum of 78.6% and 76.5% protection, whereas linoleic acid, DHA and linolenic acid provided up to 66.3%, 61.2% and 24.5% protection, respectively. Oleic acid was not effective in protecting pearl millet (only 5.1% protection). A time interval of four days between treatment of seeds and challenge inoculation was required to obtain optimum protection. Plants raised from treated seeds and challenge inoculated at the tillering and inflorescence stages showed enhanced resistance, resulting in higher grain yield compared to untreated plants of the same cultivar. Chitinase activity was found to be higher in susceptible seedlings of pearl millet after treatment with the fatty acids and pathogen inoculation than in seedlings only inoculated with the pathogen. This indicates that host defence responses are activated and thus that induced resistance is involved in the protection observed. The role of unsaturated fatty acids as activators of resistance against downy mildew in pearl millet is discussed.

Abbreviations: AA – arachidonic acid; DHA – docosahexaenoic acid; DMDI – downy mildew disease incidence; EPA – eicosapentaenoic acid; R – resistance; RI – resistant inoculated; RU – resistant uninoculated; S – susceptible; SI – susceptible inoculated, SU – susceptible uninoculated.

Introduction

There is a worldwide interest in developing agents and strategies to control plant disease with as few unwanted side effects as possible (Hammerschmidt, 2000; Oostendorp et al., 2001). A current trend in plant disease control is to reduce the use of conventional pesticides and, as an alternative, apply

compounds, which will stimulate the inherent defence mechanisms of the host plant against its pathogens. Several chemicals trigger such responses and this principle is known as 'induced resistance' (Kuc, 2001; Buonaurio et al., 2002; Decapdiville et al., 2003). Systemic induced resistance is phenotypically manifested as protection, which is often long lasting and active against a broad spectrum of pathogens (Kuc, 2001). A few inducer chemicals like benzothiadiazole (BTH) have already been commercialized as plant defence activators. However, development of new alternatives is necessary (Agostini et al., 2003; Decapdiville et al., 2003).

Downy mildew caused by *Sclerospora graminicola* (Sacc.) Schroeter is a major biotic constraint in pearl millet (*Pennisetum glaucum* (L.) R. Br.) production. *S. graminicola* belongs to Oomycota and may cause up to 80% yield loss in F1 hybrids of pearl millet (Thakur et al., 2003). The physiology of oomycetes is distinct from true fungal pathogens (Eumycota) and therefore conventional fungicides are often not effective (Tyler, 2001). On the other hand, seed treatment with resistance inducers like calcium chloride, hydrogen peroxide and BTH have been reported to protect pearl millet against *S. graminicola* (Geetha and Shetty, 2002).

Fatty acids have been recognised as signal molecules involved in activation of defence responses in plants. The signalling pathways include activation of other signal compounds such as jasmonic acid which in turn activates the octadecanoid pathway and oxylipin biosynthesis (Farmer, 1994; Itoh et al., 2002; Lee and Howe, 2003). Zoospores of *S. graminicola* contains an array of cellular fatty acids, which were detected by Gas Chromatography Mass Spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectroscopy (Geetha et al., 2002). Six unsaturated fatty acids, viz. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), linolenic acid, linoleic acid and oleic acid, were reported to induce systemic resistance in potato against infection by *Phytophthora infestans* (Cohen et al., 1991). The aim of the present study was to investigate the potential of these six unsaturated fatty acids as inducers of resistance after treatment of pearl millet seeds. Furthermore, the involvement of induced resistance against *S. graminicola* in the protection exerted by the fatty acids was studied by comparing selected defence responses of pearl millet seedlings with and without treatment.

Materials and methods

Plants and experimental design

Seeds of the pearl millet cultivars HB3, Kalucombu (KK), 852B, 7042S and 843B, all highly susceptible to downy mildew and IP18293, a highly resistant cultivar, were obtained from the 'All India Coordinated Pearl Millet Improvement Project' (AICPMIP), Jodhpur, Rajasthan, India, and the International Crop Research Institute in Semi-Arid Tropics (ICRISAT), Patencheru, India. Plants in the greenhouse (25 ± 2 °C, 85% RH) were grown in 18-li earthen pots with a mixture of soil, sand and manure (2:1:1). All experiments were conducted as completely randomized block experiments with four replications and each experiment were repeated three times.

Preparation of unsaturated fatty acids and seed treatment

The six unsaturated fatty acids were obtained from Sigma–Aldrich Chemicals, St. Louis, USA, viz. docosahexaenoic acid, DHA (D-2659), eicosapentaenoic acid, EPA (E-2011), arachidonic acid, AA (A-9298), linolenic acid (L-2376), linoleic acid (L-1376) and oleic acid (O-4754). The fatty acids were prepared as described by Vaughn and Lulai (1992). Five mg/ml (stock) of each was weighed in a sterile eppendorf tube, sonicated in 1000 μl of sterile distilled water and stored at 4 °C until use. Seeds were soaked separately in each of the six fatty acids (5 μg/ml) or distilled water (control) at 26 ± 2 °C and stirred for 9 h.

Seedling vigour analysis

After treatment with either of the six fatty acids or distilled water, seeds of cv. HB3 were washed 5–6 times with sterile distilled water and subjected to a germination test by the paper towel method (ISTA, 2003). After 7 days, the germination percentage, root length and shoot length were recorded and the seedling vigour calculated (Abdul-Baki and Anderson, 1973) from 100 seedlings per treatment in each replication.

Collection of zoospores and preparation of inoculum

Leaves from infected pearl millet plants showing symptoms of downy mildew were collected in the evening, washed in running tap water to remove remnants of previous sporulation, blotted dry and placed in a moist chamber for sporulation. The following morning, fresh sporangia were collected and the released zoospores used as inoculum (Safeeulla, 1976) at a concentration of 4×10^4 zoospores/ml.

Effect of fatty acids on S. graminicola *sporangial formation and zoospore release from sporangia*

For studies of the formation of sporangia and zoospore release from sporangia, infected leaves of cv. HB3 were collected, washed, blot dried, cut into pieces of 1 cm^2 size and treated with different concentrations of fatty acids (1, 2.5, 5, 7.5 and 10 μg/ml) for 1 h. Distilled water treated leaves served as control. The leaf pieces were subsequently blot dried and placed in a moist chamber for sporulation overnight. The next morning, the number of sporangia releasing zoospores was recorded by microscopy. Each treatment in each replication was represented by three leaf pieces. On each leaf piece, the number of sporangia was counted in five fields of vision at 400 $\times$ magnification (total area in field of vision approximately 0.79 mm^2).

The influence of the six unsaturated fatty acids on percent sporangia releasing zoospores was tested by mixing 100 μl sporangial suspension (4×10^4 sporangia/ml), prepared from untreated leaves, with 100 μl of either of the fatty acids (1, 2.5, 5, 7.5 or 10 μg/ml) in depression glass boxes. Treatment with distilled water served as control. The boxes were incubated at 25 $\pm$ 2 °C in darkness for 30 min. The percentage of sporangia releasing zoospores was recorded by microscopy. Each treatment in each replication was represented by three depression glass boxes. From each box, the number of sporangia releasing zoospores was counted in five fields of vision.

Demonstration of induced systemic resistance (ISR) against S. graminicola

Two-day-old seedlings raised from pearl millet seeds of cv. HB3, treated with either of the six fatty acids or distilled water (control), were inoculated with *S. graminicola* by the root-dip technique (Safeeulla, 1976). The seedlings were transplanted into earthen pots (10 seedlings per pot), maintained under greenhouse conditions and observed for disease expression. Each treatment in each replication was represented by 10 pots. At 30 days after inoculation, the disease incidence was recorded. Based on this value, percent protection from each treatment was calculated as: [number of diseased plants in the control – number of diseased plants in each treatment] $\times$ 100/number of diseased plants in the control.

Effect of time interval for building up of resistance

Due to the poor performance of oleic acid in reducing disease incidence, this fatty acid was omitted from the remaining experiments. Seeds of the susceptible pearl millet cv. HB3, soaked in each of the five remaining fatty acids for 9 h, were sown separately in earthen pots (10 plants per pot) under greenhouse conditions. The emerging seedlings were inoculated into the whorl region with a zoospore suspension of *S. graminicola* (Singh and Gopinath, 1985) at 1, 2, 3, 4 or 5 days after transplanting. Each treatment in each replication was represented by 10 pots. The number of diseased plants was recorded at 30 and 60 days after emergence and percentage protection induced by the fatty acids calculated as described above. Only data for 60 days are presented.

Determination of the durability of ISR

Plants of cultivar HB3, grown under greenhouse conditions in earthen pots (10 plants per pot) and initially treated with either of the five fatty acids or distilled water (control), were challenge inoculated twice with *S. graminicola*. The first inoculation was performed on 2-day-old seedlings and the second to the whorls of the nodal tillers and inflorescence primordia at the boot leaf stage 40 days after sowing. Each treatment in each replication was represented by 10 pots. Inflorescences and the young shoot whorls were challenge inoculated by depositing the inoculum between the leaf bases of the whorl, using a syringe. Number of plants with infected nodal tillers and inflorescences were recorded at the soft-dough stage 60 days after emergence and percent protection calculated as before.

Induction of ISR in different cultivars of pearl millet

The five fatty acids (EPA, AA, DHA, linolenic acid and linoleic acid) as well as distilled water (control) were applied to seeds of the highly susceptible cultivars KK, 852B, 7042S and 843B as before. The seeds were sown in earthen pots (10 plants per pot) under greenhouse conditions and 2-day-old seedlings were inoculated into the whorl region as before. Each treatment in each replication was represented by 100 seedlings. Disease incidence was recorded 60 days after sowing and percent protection calculated as before.

Induction of ISR under field conditions

Field trials were conducted to evaluate the downy mildew disease incidence and the protection offered by different unsaturated fatty acids in the Downy Mildew Sick Plot (Safeeulla, 1976). The plot has been naturally infested with zoospores of *S. graminicola* for three decades and provided the primary inoculum. The five fatty acids (EPA, AA, DHA, linolenic acid and linoleic acid) as well as distilled water (control) were applied to seeds of cv. HB3 as before and sown in the field. Disease screening was done using the infector row system as described by Williams (1984). Normal agronomic practices were followed to raise the crop (Niranjan Raj et al., 2003). Disease incidence was recorded 30 and 60 days after sowing and plants were rated as diseased when they showed any of the typical downy mildew symptoms (Safeeulla, 1976). The experiment was repeated three times during the same year. Only data from 60 days after sowing are presented from one experiment.

Influence of fatty acid seed treatment on growth parameters of pearl millet

The following growth parameters of the crop raised from fatty acid treated seeds in the Downy Mildew Sick Plot were studied 60 days after sowing: (1) the height of the plants, (2) number of days required for 50% flowering, (3) number of productive ears formed, (4) total number of productive tillers, (5) length and (6) girth of ears, (7) 1000 seed weight and (8) yield per hectare. All parameters were recorded on 25 plants per treatment in each replication of the experiment.

Chitinase activity

Pearl millet seeds of a resistant (IP18293) and a susceptible (HB3) cultivar were used for studying. Treatments were (1) HB3 treated with DHA, (2) HB3 treated with EPA, (3) HB3 treated with AA, (4) HB3 treated with linolenic acid, (5) HB3 treated with linoleic acid (nos. 1–5 were all inoculated with *S. graminicola*), (6) IP18293 (R) inoculated with *S. graminicola*, (7) IP18293 (R) treated with distilled water alone, (8) HB3 (S) inoculated with *S. graminicola*, (9) HB3 (S) treated with distilled water alone. All inoculations took place on 2-day-old seedlings by the root-dip technique (Safeeulla, 1976). Twenty-four hours after inoculation, 1 g of fresh seedling tissue of each sample were macerated using 0.05 M sodium acetate buffer, pH 5.2 (1 ml/g fresh weight) and acid washed glass beads at 4 °C. The samples were centrifuged at 10,000 rpm for 30 min. (Himac Centrifuge, HITACHI) and the supernatant used as crude extract. Protein content in extracts was estimated according to Bradford (1976), using bovine serum albumin (Sigma) as a standard. Chitinase was assayed following the method of Isaac and Gokhale (1982) with *N*-acetyl glucosamine (Sigma) as standard. Colloidal chitin in 0.05 M sodium acetate buffer (pH 5.2), purified from chitin (Sigma) following the method of Skujins et al. (1965), was used as a substrate. The concentration of *N*-acetyl glucosamine released after incubation were measured spectrophotometrically at 585 nm, using dimethyl amino benzaldehyde reagent (Reissig et al., 1955). The enzyme activity was expressed in terms of nmol min^{-1} mg^{-1} protein. Three independent experiments were performed.

Statistical analysis

Data from the studies of disease incidence represent discrete variables since it was recorded whether or not a plant was diseased. Consequently, these data were analysed by logistic regression, assuming a binomial distribution (corrected for overdispersion when present) (Collett, 1991). For comparison of the variables (percentages), odds ratios (Collett, 1991) were calculated using water treatment as a reference (odds ratio = 1.00). For example, the odds ratio for percent seeds germinating after AA treatment (Table 1) is 1.88. This means that odds

Table 1. Seed germination and seedling vigour (7 days after sowing) of pearl millet cv. HB3 after seed treatment with six unsaturated fatty acids or distilled water (control) under greenhouse conditions

Fatty acid[a]	Percent germination	Odds ratio[b]	Vigour index[c]
DHA	91.0	1.00^{NS}	1125.0
EPA	92.0	1.14^{NS}	1152.0
AA	95.0	1.88^{NS}	1205.0
Linolenic acid	92.0	1.14^{NS}	1028.0
Linoleic acid	93.0	1.31^{NS}	1101.0
Oleic	92.0	1.14^{NS}	850.0
Control	91.0	1.00	938.0
P-value	0.3307		< 0.0001
$LSD_{.95}$[d]	–		2.8

[a] DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; AA: arachidonic acid.
[b] Odds ratio for comparison of treatments (control used as a reference, odds ratio = 1.00). NS: non-significant difference.
[c] Vigour index was calculated from the germination percentage and mean root and shoot lengths of the seedlings.
[d] LSD-value for comparison of vigour index between treatments.

($P[1 - P]^{-1}$, in which P is the probability of a seed germinating) in the treated plants is about 1.9 times higher than odds for control plants. Other parameters measured are continuous variables, e.g. chitinase activity, 1000 seed weight, yield, etc. These parameters were analysed by analysis of variance and means were separated by LSD-values. Hypotheses were rejected at $P \leq 0.05$. All data were analysed by PC-SAS (release 8.2; SAS Institute, Cary, NC). All experiments were performed three times with similar results. Only representative data are presented.

Results

Seed germination and seedling vigour after treatment with fatty acids

Treatment with unsaturated fatty acids, viz. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), linolenic acid, linoleic acid or oleic acid did not significantly affect the seed germination percentage compared to the control (Table 1). On the other hand, vigour index was significantly increased by all fatty acids except oleic acid. The maximum seedling vigour was observed in seeds treated with AA (index 1205) and EPA (index 1152). Seedlings raised from oleic acid-treated seeds had significantly lower seedling vigour (index 850) compared to that of the control (index 938).

Effect of fatty acids on S. graminicola *sporangial formation and zoospore release from sporangia*

The formation of sporangia per cm^2 and percent sporangia releasing zoospores were tested after treatment of infected leaves with different concentrations of the six fatty acids (Figure 1a and b). For all fatty acids, application of any concentration significantly reduced the number of sporangia compared to the water treated leaves (Figure 1a). Increasing the fatty acid concentration from 1.0 to 5 μg/ml significantly increased sporangial number for all fatty acids, except linolenic acid, which only showed an increase from 1.0 to 2.5 μg/ml. Higher concentrations (7.5 and 10.0 μg/ml) significantly decreased sporangial number for all fatty acids. Application of increasingly higher concentrations of oleic acid resulted in gradually lower sporangial numbers.

Percent sporangia releasing zoospores was also significantly lower after treatment with any of the six fatty acids compared to water treatment (Figure 1b). However, increasing the concentration from 1.0 to 5.0 μg/ml resulted in significant increases in percent sporangia releasing zoospores for all fatty acids, except oleic acid. The highest concentrations of fatty acids (7.5 and 10.0 μg/ml) all resulted in subsequently larger reductions in percent sporangia releasing zoospores and oleic acid completely inhibited zoospore release at a concentration of 10.0 μg/ml.

Demonstration of induced systemic resistance (ISR) against S. graminicola

Disease incidence after treatment with each of the six fatty acids was studied in cv. HB3 at 30 days after inoculation in the greenhouse (Table 2). Maximum disease incidence (98%) was observed in seedlings raised from water treated seeds. Treatment with any of the fatty acids resulted in significantly lower disease incidence. Thus, the highest protection was observed in seedlings raised from seeds treated with EPA (78.6%) and AA (76.5%) and the lowest after treatment with oleic acid (5.1%).

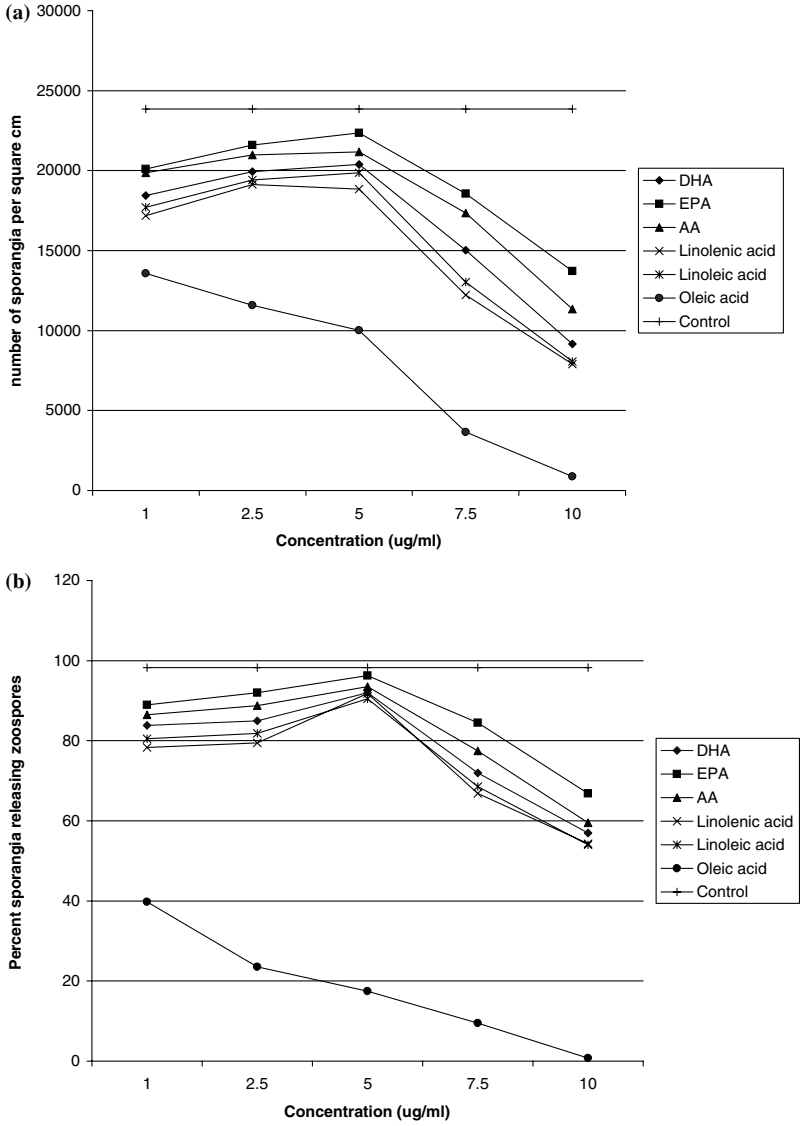

Figure 1. (a) Number of sporangia of *Sclerospora graminicola* per cm^2 of leaf and (b) percent sporangia releasing zoospores after treatment with six unsaturated fatty acids. For all fatty acids, sporangial numbers and percent sporangia releasing zoospores are significantly different ($P < 0.0001$) at all concentrations. Abbreviations as in Table 1.

Effect of time interval between fatty acid treatment and inoculation with S. graminicola

Due to the poor performance of oleic acid in reducing disease incidence, this fatty acid was not considered in the following experiments. Seedlings from seeds treated with either of the five unsaturated fatty acids were inoculated 1, 2, 3, 4 or 5 days after emergence (Figure 2). An interval of 4 days resulted in the lowest disease incidence for all fatty acids compared to 5 days, which was used as a reference (5.3–8.9% reduction). However, the difference was only significant for DHA and EPA. Disease incidence for 2 and 3 days were often significantly higher compared to 5 days (represented by negative values in Figure 2).

Determination of the durability of ISR

The durability of protection by fatty acid treatments was demonstrated by challenge inoculating with *S. graminicola* twice (Table 3). The first

Table 2. Downy mildew disease incidence (DMDI) and percent protection after seed treatment of pearl millet cv. HB3 with six unsaturated fatty acids or distilled water (control) under greenhouse conditions 30 days after inoculation with *S. graminicola*

Fatty acid[a]	DMDI	Odds ratio[b]	Percent protection[c]
DHA	38.0	0.01^{***}	61.2
EPA	21.0	0.01^{***}	78.6
AA	23.0	0.01^{***}	76.5
Linolenic acid	74.0	0.06^{***}	24.5
Linoleic acid	33.0	0.01^{***}	66.3
Oleic	93.0	0.27^{**}	5.1
Control	98.0	1.00	–
P-value	< 0.0001		

[a] Abbreviations as in Table 1.
[b] Odds ratio for comparison of treatments (control used as a reference, odds ratio = 1.00). The number of asterisks indicates the degree of significance. ***: significant at $P \leq 0.001$.
[c] Compared to the control.

inoculation took place on 2-day-old seedlings and the second to the nodal tillers and inflorescence primordia 40 days after sowing. The lowest disease incidence after the second challenge inoculation was recorded for EPA treatment (91.7% and 88.3% protection after inoculation of nodal tillers and inflorescences, respectively), whereas the highest disease incidence was observed after application of linolenic acid (59.8% and 54.5% protection, respectively).

Induction of ISR in different cultivars of pearl millet

Seeds of the highly susceptible cultivars of pearl millet KK, 852B, 7042S and 843B were treated with the five fatty acids (DHA, EPA, AA, linolenic acid and linoleic acid). 2-day-old seedlings of each cultivar were inoculated with the pathogen. The fatty acids reduced disease incidence to varying degrees in the different cultivars. All fatty acids significantly reduced disease incidence in the cultivars 852B (11.1–39.4% protection), 7042S (21.9–50.0% protection) and 843B (11.5–32.0% protection) (Table 4). For each cultivar, different fatty acids provided the highest protection, i.e. linolenic acid for 7042S, and DHA for 852B and 843B. In cv. KK, only DHA and linolenic acid gave significant reductions in disease (24.5% and 44.6%, respectively).

Induction of ISR under field conditions

Under field conditions (Table 5), the lowest disease incidence was recorded after treatment with

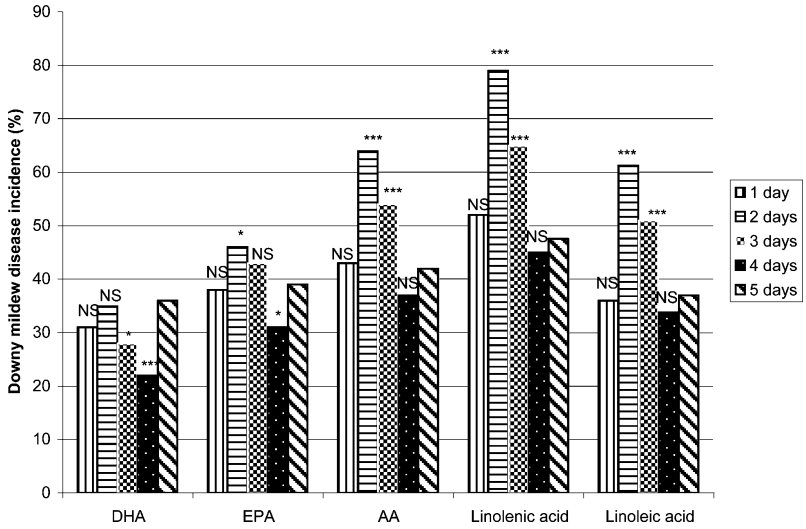

Figure 2. Downy mildew disease incidence (DMDI) of 60-day-old pearl millet plants of cv. HB3 raised from fatty acid treated seeds. The seeds were treated with either of five unsaturated fatty acids or water (control). The seedlings were challenge inoculated with *Sclerospora graminicola* at 1, 2, 3, 4 or 5 days after emergence. Comparisons are possible within each fatty acid (5 days used as a reference): The number of asterisks indicates the degree of significance. NS: non-significant difference, ***: significant at $P \leq 0.001$, *: significant at $P \leq 0.05$. Figures under the *x*-axis denote percent 'protection' (difference in disease incidence) of the individual time points compared to 5 days within each fatty acid. Abbreviations as in Table 1.

Table 3. Downy mildew disease incidence (DMDI) of pearl millet nodal tillers and inflorescences in plants raised from seeds treated with either of five unsaturated fatty acid or water (control) and challenge inoculated after 30 days under greenhouse conditions

Fatty acid[a]	DMDI nodal tillers	Odds ratio[b]	Percent protection[b]	DMDI inflorescences	Odds ratio[c]	Percent protection[c]
DHA	17.0	0.08***	76.9	16.0	0.06***	79.2
EPA	6.0	0.02***	91.7	9.0	0.03***	88.3
AA	10.0	0.04***	86.1	12.0	0.04***	84.4
Linolenic acid	29.0	0.16***	59.8	35.0	0.16***	54.5
Linoleic acid	20.0	0.10***	72.2	19.0	0.07***	75.4
Control	72.0	1.00	–	77.0	1.00	–
P-value	< 0.0001			< 0.0001		

[a] Abbreviations as in Table 1.
[b] Odds ratio for comparison of treatments (control used as a reference, odds ratio = 1.00). The number of asterisks indicates the degree of significance. ***: significant at $P \leq 0.001$.
[c] Compared to the control.

Table 4. Downy mildew disease incidence (DMDI) and percent protection after seed treatment of different pearl millet cultivars with five unsaturated fatty acids or distilled water (control) under greenhouse conditions

Fatty acid[a]	Cultivar											
	KK			852B			7042S			843B		
	DMDI	Odds ratio[b]	Percent protection[c]	DMDI	Odds ratio	Percent protection	DMDI	Odds ratio	Percent protection	DMDI	Odds ratio	Percent protection
DHA	49.0	0.52***	24.5	52.0	0.27***	39.4	58.0	0.06***	39.6	53.0	0.32***	32.0
EPA	65.0	1.00NS	0.0	60.0	0.38***	25.0	75.0	0.13***	21.9	58.0	0.39***	25.6
AA	60.3	0.82NS	7.3	53.8	0.29***	32.8	70.3	0.10***	26.8	63.0	0.48***	19.2
Linolenic acid	36.0	0.30***	44.6	54.0	0.29***	32.5	48.0	0.04***	50.0	57.0	0.37***	26.9
Linoleic acid	60.0	0.81NS	7.6	71.0	0.61**	11.1	68.0	0.09***	29.1	69.0	0.63**	11.5
Control	65.0	1.00	–	80.0	1.00	–	96.0	1.00	–	78.0	1.00	–
P-value		< 0.0001			< 0.0001			< 0.0001			< 0.0001	

[a] Abbreviations as in Table 1.
[b] Odds ratio for comparison of treatments (control used as a reference, odds ratio = 1.00). The number of asterisks indicates the degree of significance. ***: significant at $P \leq 0.001$, **: significant at $P \leq 0.01$.
[c] Compared to the control.

EPA and AA and the highest for linolenic acid. EPA conferred 69.4% protection, AA 65.9% protection and linolenic acid only 19.9% protection.

Influence of fatty acid seed treatment on growth parameters of pearl millet

The unsaturated fatty acids tested in this study not only resulted in higher protection against *S. graminicola*, but also significantly enhanced growth of the plants raised from treated seeds (Table 6). Plant height was significantly increased by up to 28 cm and flowering was 4–6 days earlier. The number of reproductive tillers increased from 2.0 in control plants to 3.2–3.9 after fatty acid treatment and basal tillers and nodal tillers increased from 1.0 and 2.0, respectively, in control plants to 2.9–5.0 and 3.0–4.0, respectively after fatty acid treatment. The length of ears increased from 9.5 in control plants to 10.7–14.3 cm in treated plants and likewise, the girth of ears increased from 3.6 cm (control plants) to 4.2–4.9 cm after treatment with fatty acids. Finally, 1000 seed weight and yield increased from 7.2 g and 1212 kg/ha, respectively, in control plants to 9.3–11.7 g and 1372–1499 kg/ha, respectively, after treatment.

Table 5. Field experiment showing downy mildew disease incidence (DMDI) and percent protection 30 days after inoculation with *S. graminicola* following seed treatment of pearl millet cv. HB3 with five unsaturated fatty acids or distilled water (control)

Fatty acid[a]	DMDI	Odds ratio[b]	Percent Protection[c]
DHA	43.5	0.01^{***}	56.1
EPA	30.3	0.01^{***}	69.4
AA	33.8	0.01^{***}	65.9
Linolenicacid	79.3	0.04^{***}	19.9
Linoleic acid	42.8	0.01^{***}	56.8
Control	99.0	1.00	–
P-value	< 0.0001		

[a] Abbreviations as in Table 1.
[b] Odds ratio for comparison of treatments (control used as a reference, odds ratio = 1.00). The number of asterisks indicates the degree of significance. ***: significant at $P \leq 0.001$.
[c] Compared to the control.

Chitinase activity

Chitinase activity (Figure 3) was selected as a host defence response to be studied in order to verify that induced resistance is involved in protection exerted by the fatty acids. A differential response of chitinase activity was observed when susceptible seeds were treated with fatty acids and 2-day-old seedlings challenge-inoculated with the pathogen. All fatty acids induced significantly higher chitinase activity in inoculated pearl millet seedlings than in the susceptible inoculated seedlings (SI) (4.6 nmol min^{-1} mg^{-1} protein). Likewise, there was a significantly higher chitinase activity in resistant inoculated (RI) and susceptible uninoculated (SU) seedlings compared to susceptible inoculated seedlings (SI), but not in resistant uninoculated seedlings (RU).

Discussion

In the present study, seeds of downy mildew susceptible pearl millet cultivars were treated with six unsaturated fatty acids, i.e. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), linoleic acid, linolenic acid and oleic acid. These acids have all been detected as constituents of the zoospores of *S. graminicola* (Geetha et al., 2002). Among the unsaturated fatty acids, application of EPA and AA resulted in the highest protection against *S. graminicola* (78.6% and 76.5%, respectively) whereas the lowest protection was offered by oleic acid, which was subsequently omitted from the experiments. It is interesting to note that oleic acid did not give any high protection in spite of the fact that it is found to be present in the zoospores of *S. graminicola* and it is the most efficient inhibitor of sporangial formation and zoospore release from sporangia. The unsaturated fatty acids tested here are also present in sporangia of *Phytophthora infestans* and play a pivotal role in inducing resistance to late blight disease in potato (Cohen et al., 1991; Coquoz et al., 1995; Merzlyak et al., 2000). In the present experiments, these unsaturated fatty acids were used as seed amendments for the first time.

A number of biotic and abiotic inducers of disease resistance have been reported, but very few

Table 6. Effect of seed treatment with five unsaturated fatty acids or distilled water (control) on growth parameters of pearl millet plants (cv. HB3) under field conditions

Fatty acid[a]	Height (cm)	Days required for 50% flowering	Total number of reproductive tillers per plant	Number of basal tillers	Number of nodal tillers	Length of ears (cm)	Girth of ears (cm)	1000 seed weight (g)	Yield (kg per ha)
DHA	74.7	38.0	3.2	4.0	3.0	11.3	4.6	10.5	1408.5
EPA	71.6	38.0	3.6	3.0	3.0	10.7	4.2	9.8	1394.8
AA	70.5	39.0	3.5	2.9	3.0	11.0	4.3	9.3	1372.3
Linolenic acid	84.0	40.0	3.4	4.0	3.0	14.3	4.8	11.6	1498.5
Linoleic acid	83.3	41.0	3.9	5.0	4.0	12.9	4.9	11.7	1385.3
Control	55.9	44.0	2.0	1.0	2.0	9.5	3.6	7.2	1211.5
P-value	< 0.0001	0.0254	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
$LSD_{.95}$	2.4	3.6	0.4	0.4	0.2	0.2	0.3	0.2	8.7

[a] Abbreviations as in Table 1.

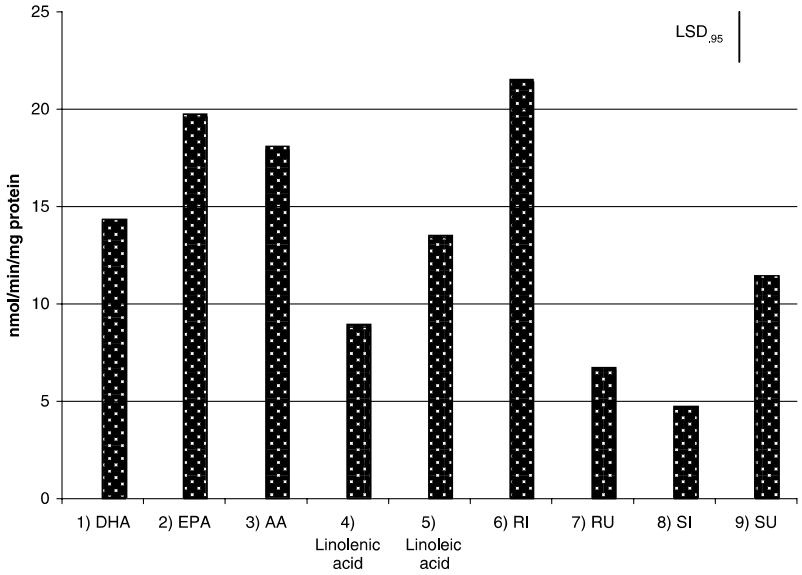

Figure 3. Chitinase activity (nmol min^{-1} mg^{-1} protein) in 2-day-old pearl millet seedlings raised from seeds treated with either of five unsaturated fatty acids and inoculated with *Sclerospora graminicola*. Treatments were (1) HB3 treated with DHA, (2) HB3 treated with EPA, (3) HB3 treated with AA, (4) HB3 treated with linolenic acid, (5) HB3 treated with linoleic acid (nos. 1–5 were all inoculated with *S. graminicola*), (6) IP18293 (R) inoculated with *S. graminicola*, (7) IP18293 (R) treated with distilled water alone, (8) HB3 (S) inoculated with *S. graminicola*, (9) HB3 (S) treated with distilled water alone. Abbreviations as in Table 1.

have been shown to reduce the disease incidence under field conditions (Oostendorp et al., 2001). Our study is the first report, testing the efficacy of unsaturated fatty acids in inducing systemic resistance in a monocot plant against a devastating oomycete pathogen under both greenhouse and field conditions. The study revealed that also under field conditions, the unsaturated fatty acids were effective in reducing the downy mildew incidence in pearl millet. We found a similar level of protection against disease under field conditions as under greenhouse conditions. Often higher protection is observed under controlled conditions than in the field due to the more stable environment under greenhouse conditions.

It was demonstrated that the protection induced by the different fatty acids varied among the different susceptible cultivars. This might indicate that the level of protection depends on host cultivar specific defence mechanisms. Kamoun et al. (1993) examined host cultivar-specificity of purified elicitin proteins from the culture filtrate of *Phytophthora parasitica* and *Ph. cryptogea* on plant species belonging to Solanaceae (*Nicotiana*) and Brassicaceae. The proteins were found to be genus-specific elicitors within the Solanaceae and cultivar-specific elicitors within the Brassicaceae. In potato, treatment with AA lead to systemic protection not only against *Ph. infestans*, an oomycete, but also against the causal agent of early blight, the ascomycete *Alternaria solani*. This indicates that the fatty acids may also be efficient against organisms other than oomycetes (Coquoz et al., 1995). The distribution and metabolism of the active fatty acids in different pathogens should be studied in order to evaluate their direct role in resistance and to demonstrate whether the effect should be designated as ISR as defined by Kuc (2001).

Induced systemic resistance normally requires a period of time between inducer treatment and challenge inoculation for gene activation of defence responses to take place (Ryals et al., 1996). In the present study, a one-day interval between inducer treatment and challenge inoculation did not give significant protection, but already a two-day interval gave significant protection for most fatty acids. Four to five days between inducer treatment and challenge inoculation was optimal for the resistance to build up, indicating that this is the time necessary for activation of a level of defence, sufficient to inhibit pathogen growth.

In order to verify that induced resistance is involved in the protection against *S. graminicola*, chitinase activity was measured after treatment with the fatty acids. Plants constitutively expressing higher levels of hydrolases such as β-1,3-glucanases, chitinases and peroxidases are shown to have enhanced resistance against fungal pathogens (Cachinero et al., 1996; Kasprzewska; 2003). In our study, the level of chitinase activity in the susceptible cultivar HB3 inoculated with *S. graminicola* was significantly higher after treatment with the fatty acids in all cases (Figure 3, nos. 1–5) compared to the control treatment (Figure 3, no. 8). These results are in concurrence with observations from other host-pathogen systems (Cachinero et al., 1996; Kasprzewska, 2003) where it was shown that increases in activity and accumulation of chitinases occurred after induction of resistance in maize against *Fusarium moniliforme* and in pepper against *Phytophthora capsici*. There was a rather high chitinase activity in cv. HB3 not inoculated with *S. graminicola* (Figure 3, no. 9). The reason for this is not known. However, a possible explanation could be that the chitinase activity is suppressed in the susceptible, but not in the resistant cultivar after pathogen inoculation. Nevertheless, since the inoculated plants showed a clear increase in chitinase activity after fatty acid treatment compared to the control, this indicate that host defence responses are activated and consequently that induced resistance is involved in the protection observed. However, the significantly lower sporangial formation and lower percent sporangia releasing zoospores also indicate that the fatty acids have a direct inhibitory effect on *S. graminicola*, 5.0 μg/ml generally having the lowest inhibitory effect. Further studies are needed in order to elucidate which other defence reactions are activated by the fatty acids and the extent to which direct toxic effects participate in the protection.

It is interesting to note that the fatty acids present in the surface of *S. graminicola* zoospores act as elicitors and trigger defence responses in the host since this is actually not beneficial for the survival of the pathogen. Ponchet et al. (1999) suggested that oomycetes having surface-borne elicitors in their life cycle enhance the leakage of nutrients (sterol carriers) from the host plant and thus survival of the pathogen. In this way, pathogen elicitins stabilise the evolutionary equilibrium between host and the pathogen (Kirchner and Roy, 2000).

There are several advantages of applying fatty acids to pearl millet seeds. Thus, the fatty acids tested in the present study were able to systemically protect plants against disease. Pearl millet is a short duration crop, ready for harvest in 80–90 days. However, the plants are continuously producing nodal tillers and these fresh nodal tillers are potential infection sites for downy mildew. The durability of ISR was tested by inoculating such nodal tillers and here, seed treatment with fatty acids was observed to offer a high level of protection, indicating that resistance is durable throughout the growth period. Furthermore, seed treatment with the fatty acids also increased the pearl millet growth parameters like plant height, number of tillers, number of ears, length of ears, ear girth, 1000 seed weight and yield when compared to those of the untreated control. Among the fatty acids tested, linolenic acid and DHA were the most efficient in enhancing growth and yield. A similar effect was demonstrated in earlier studies in pearl millet where it was shown that rhizobacteria are potential growth enhancers as well (Niranjan Raj et al., 2003).

A few fatty acids have been developed as commercial disease protection agents. Salicylic acid, a known inducer used in combination with EPA and poly unsaturated fatty acids (PUFA) are developed specifically against downy mildews (Agostini et al., 2003; Decapdiville et al., 2003). Furthermore, EPA in combination with harpins, is used against many fungal pathogens, insects and nematodes on cucumber, oranges, cotton and roses, sold under the product name Messenger™ by Eden Bioscience, Bothell, WA (Agostini et al., 2003; Decapdiville et al., 2003). EPA, tested in the present study, has been marketed under the name Adjust™ by the Stoller Company for control of downy mildews as well as other fungal pathogens and insect pests (Agostini et al., 2003; Decapdiville et al., 2003).

There are good prospects for a future commercial use of the fatty acids tested in the present study for downy mildew control, like it has already happened for EPA. The fatty acids confer a high and long lasting protection against *S. graminicola* and are easy to apply as a seed treatment. In addition to the disease control, the fatty acids also promote plant growth, thus helping in securing a higher yield.

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References

Abdul-Baki AAA and Anderson JD (1973) Vigor determination in soybean seed by multiple criteria. Crop Science 13: 630–633

Agostini JP, Bushong PM and Timmer LW (2003) Greenhouse evaluation products that induce resistance for control of scab, melanose and Alternaria brown spot of citrus. Plant Disease 87: 69–74

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254

Buonaurio R, Scarponi L, Ferrera M, Sidott P and Bertona A (2002) Induction of systemic acquired resistance in tomato by acibenzolar-*s*-methyl against bacterial spot disease. European Journal of Plant Pathology 108: 41–49

Cachinero JM, Cabello F, Jorrin J and Tena M (1996) Induction of different chitinase and β-1,3-glucanase isoenzymes in sunflower (*Helianthus annuus* L.) seedling in response to infection by *Plasmopara halstedii*. European Journal of Plant Pathology. 102: 401–405

Cohen Y, Gisi U and Mossinger E (1991) Systemic resistance of potato against *Phytophthora infestans* induced by unsaturated fatty acids. Physiological and Molecular Plant Pathology 38: 255–263

Collett D (1991) Modelling Binary Data. Chapman & Hall. London

Coquoz J-L, Buchala A, Meuwly Ph and Metraux J-P (1995) Arachidonic acid induces local but not systemic synthesis of salicylic acid and confers systemic resistance to *Phytophthora infestans* and *Alternaria solani*. Phytopathology 85: 1219–1224

Decapdiville G, Beer SV, Watkins CB, Charles L, Wilson CL, Luis O and Tedeshi LO (2003) Pre- and post-harvest harpin treatment of apples induce resistance to blue mold. Plant Disease 87: 39–44

Farmer EE (1994) Fatty acid signaling in plants and their associated microorganisms. Plant Molecular Biology 26: 1423–1437

Geetha HM and Shetty HS (2002) Induction of resistance in pearl millet against downy mildew disease caused by *Sclerospora graminicola* using benzothiadiazole, calcium chloride and hydrogen peroxide – a comparative evaluation. Crop Protection 21: 601–610

Geetha NP, Amruthesh KN and Shetty HS (2002) Determination of cellular fatty acid components in five pathotypes of *Sclerospora graminicola,* the downy mildew pathogen of pearl millet. Journal of Mycology and Plant Pathology 32: 345–353

Hammerschmidt R (2000) Changing susceptibility into resistance. Physiological and Molecular Plant Pathology 57: 1–3

Isaac S and Gokhale AV (1982) Autolysis: A tool for protoplast production from *Aspergillus nidulans*. Transactions of the British Mycological Society 78: 389–394

ISTA (2003) International Rules for Seed Testing (2003) International Seed Testing Association, Chapter V

Itoh A, Schilmiller AL, McCaig B and Howe GA (2002) Identification of a jasmonate-regulated allene oxide synthase that metabolizes 9-hydroperoxides of linoleic and linolenic acids. Journal of Biological Chemistry 277: 46051–46058

Kamoun S, Young M, Glascock CB and Tyler BM (1993) Extracellular protein elicitors from *Phytophthora*: Host-specificity and induction of resistance to bacterial and fungal phytopathogens. Molecular Plant–Microbe Interactions 6: 15–25

Kasprzewska A (2003) Plant chitinases – regulation and function. Cellular and Molecular Biology Letters 8: 809–824

Kirchner JW and Roy BA (2000). Evolutionary implications of host-pathogen specificity: The fitness consequences of host life history traits. Evolutionary Ecology 14: 665–692

Kuc J (2001) Concepts and direction of induced systemic resistance in plants and its application. European Journal of Plant Pathology 107: 7–12

Lee GI and Howe GA (2003) The tomato mutant spr1 is defective in systemin perception and the production of a systemic wound signal for defense gene expression. Plant Journal 33: 567–576

Merzlyak MN, Chivkunova OB and Maximova NI (2000) On the involvement of arachidonic and eicosapentaenoic acids in the elicitation of a defense process in potato plants. Journal of the Russian Phytopathological Society 1: 51–57

Niranjan Raj S, Chaluvaraju G, Amruthesh KN, Shetty HS, Reddy MS and Kloepper JW (2003) Induction of growth promotion and resistance against downy mildew on pearl millet (*Pennisetum glaucum*) by rhizobacteria. Plant Disease 87: 380–384

Oostendorp M, Kunz W, Dietrich B and Staum T (2001) Induced disease resistance in plants by chemicals. European Journal of Plant Pathology 107: 19–28

Ponchet M, Panabieres F, Milat ML, Mikes V, Montillet JL, Suty L, Triantaphylides C, Tirilly Y and Blein JP (1999). Are elicitins cryptograms in plant-oomycete communications? CMLS Cell and Molecular Life Sciences 56: 1020–1047

Reissig JL, Strominger JL and Leloir LF (1955) A modified colorimetric method for the estimation of *N*-acetylamino sugars. Journal of Biological Chemistry 217: 959–966

Ryals JA, Neuschwander UM, Willitis MG, Molina A, Steiner H and Hunt MO (1996) Systemic acquired resistance. Plant Cell 8: 1809–1819

Safeeulla KM (1976) Biology and Control of Downy Mildews of Pearl Millet, Sorghum and Finger Millet. Wesley Press, Mysore

Singh SD and Gopinath R (1985) A seedling inoculation technique for detecting downy mildew resistance in pearl millet. Plant Disease 72: 425–428

Skujjins JJ, Potgieter HJ and Alexander M (1965) Dissolution of fungal cell walls by a streptomycete chitinase and β-(1,3) glucanase. Archives in Biochemistry and Biophysics 111: 358–364

Thakur RP, Rao VP, Amruthesh KN, Shetty HS, Datar VV (2003) Field surveys of pearl millet downy mildew – effects of hybrids, fungicides and cropping sequence. Journal of Mycology and Plant Pathology 33: 387–394

Tyler (2001) Genetics and genomics of the oomycete–host interface. Trends in Genetics 17: 611–614

Vaughn SF and Lulai EC (1992) Further evidence that lipoxygenase activity is required for arachidonic acid elicited hypersensitivity in potato callus cultures. Plant Science 84: 91–98

Williams RJ (1984) Downy mildew of tropical cereals. In: DS Ingram and PH Williams (eds) Advances in Plant Pathology (pp 1–103) Academic Press, London, Vol. 2.