European Journal of Plant Pathology **104:** 125–132, 1997.

Biocontrol strain of *Bacillus subtilis* AF 1 rapidly induces lipoxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*

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Accepted 9 October 1997

Key words: groundnut, lipoxygenase, disease resistance, biological control, PGPR, *Bacillus subtilis, Aspergillus niger*

Abstract

Metabolic products of polyunsaturated fatty acids have been variously implicated in control of microbial pathogens. Induced resistance has been shown as one of the mechanisms of biological control by plant growth promoting rhizobacteria (PGPR). This paper reports a significant lipoxygenase (LOX) activity in groundnut seedlings with production of 13-hydroperoxyoctadecadienoic acid (13-HPODE) and 13-hydroperoxyoctadecatrienoic acid (13-HPOTrE) as major products with linoleic acid (LA) and α-linolenic acid (ALA), respectively. Both the hydroperoxides are inhibitory to the growth of *Aspergillus niger* as measured in micro titer plates. Ours is the first report on induction of LOX activities in groundnut on treatment with a PGPR strain *Bacillus subtilis* AF 1, and with crown-rot pathogen, *A. niger*. Treatment with *B. subtilis* AF 1 enhanced LOX levels in groundnut similarly but earlier to *A. niger* – treatment. This induction of LOX during activation of growth and pathogen infection was discussed in light of the reported involvement of LOX both in growth and development as well as in plant-pathogen interaction, particularly induced disease resistance.

Introduction

Rhizosphere bacteria that exhibit root colonization and exert beneficial effects on plants are termed PGPR (plant growth promoting rhizobacteria) (Kloepper and Schroth, 1978). Investigations into mechanisms for beneficial effects of most reported PGPR strains indicated that the PGPR increased growth indirectly by changing the microbial balance in the rhizosphere (Kloepper and Schroth, 1981). Iron chelating siderophores, antibiotics and hydrogen cyanide are produced by some PGPR and have been implicated in reduction of plant pathogens and deleterious rhizobacteria with a corresponding improvement in plant growth. Plants express resistance to microbes also by mechanisms involving metabolism of the polyunsaturated fatty acids (PUFAs) such as linoleic acid and linolenic acid via the lipoxygenase (LOX) pathway. Recent reports demonstrated LOX activation in plants following elicitor treatment or inoculation with pathogens (Croft et al., 1990; Koch et al., 1992; Ohta et al., 1991; Reddy et al., 1992; Rickauer, 1990; Slusarenko et al., 1993) and in incompatible plant-bacteria interactions (Croft et al., 1993). The products of LOX pathway contribute to defense reactions by inhibition of pathogen growth and development (Namai et al., 1990; Ohta et al., 1990), induction of phytoalexin accumulation (Li et al., 1991) and/or in signal transduction (Choi and Bostock, 1994). Preisig and Kuc (1987) provided evidence indicating that LOX acts as a mediator of arachidonic acid-induced elicitation of hypersensitive response (HR), using a range of LOX inhibitors with different mechanisms of action. The peroxidation of PUFAs by LOXs could be a major source of peroxides in biotically-stressed plant tissue. Peroxidized fatty acids are highly reactive and could be further metabolized to signal molecules such as jas-

monates, traumatin (Hildebrand, 1989) and hexenals (Croft et al., 1993).

Anderson and Guerra (1985) reported that bean roots colonized by a strain of *Pseudomonas putida*, which provided some protection from *Fusarium solani* f.sp. *phaseoli*, had higher lignin content than non-bacterized seedlings. Some PGPR are also able to induce systemic resistance in plants (Maurhofer et al., 1994; van Peer et al., 1991; Wei et al., 1991). *Bacillus subtilis* AF 1 causes extensive lysis of *Aspergillus niger* and reduces the incidence of crown-rot in groundnut (Podile and Prakash, 1996) and is a biocontrol PGPR. AF 1 stimulated phenylalanine ammonia lyase (PAL) and peroxidase in bacterized pigeonpea seedlings (Podile et al., 1995). Induction of host plant resistance in tobacco, carnation and cucumber suggests that some PGPR strains applied to seed(s) can influence the host plant susceptibility to the pathogens by inducing resistance. Also, there are evidences to implicate LOX in disease resistance. The purpose of this study was to determine whether a biocontrol PGPR strain like *B. subtilis* AF 1 mediates its action through LOX activity in groundnut. LOX activity in bacterized seedlings was therefore studied in comparison with groundnut infection by *Aspergillus niger*.

Materials and methods

Seeds and microbial cultures

Groundnut (*Arachis hypogaea* L.) seeds of ICG(FDRS)10 were procured from International Crop Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad, India. *Bacillus subtilis* AF 1 was isolated from soils that were non-conducive for pigeanpea wilt and had a broad spectrum antifungal and PGPR activity (Podile and Dube 1988). Pure culture of *A. niger* was isolated from crown rot infected groundnut plants from fields.

Seed bacterization with B. subtilis *AF 1*

Groundnut seeds were bacterized as described by Podile and Prakash (1996). Seeds surface sterilized with 0.02% mercuric chloride for 5 min and rinsed twice with sterile distilled water were bacterized with a PGPR strain of *B. subtilis* AF 1, grown as a lawn on PDA (Potato Dextrose Agar) (pH 7.0) for 48 h at 30 °C. The bacterial cells scrapped with a sterile glass rod, from two Petri plates (9 × 1.5 cm), were taken in 25 ml of 0.5% sterilized carboxy methyl cellulose (CMC). CMC was used as an adhesive. Surface sterilized seeds were bacterized by immersion in this bacterial suspension for about 15 min and then dried in flowing sterile air for 2 to 3 h. Surface sterilized groundnut seeds treated similarly with sterile distilled water served as non-bacterized controls. The dried bacterized seeds were transferred into sterile phosphate buffer (pH 6.8, 0.05M) and stirred magnetically for 15 min. Appropriate dilution of the suspensions were plated on PDA and the number of cfu /seed was determined. The population of introduced bacteria on eight randomly selected seeds varied between 6.5 and 6.6 log cfu /seed.

Soil infestation with A. niger

Native soil of the University of Hyderabad garden was mixed with farm yard manure in 1:1 ratio. *A. niger* was grown in potato dextrose broth for 48 h and the mycelium with spores was collected on coarse filter paper. The wet mycelial mat with spores was mixed thoroughly with the soil (2 g mycelium / kg soil) prepared as above.

Growth vigour and crown rot index of groundnut seedlings

In a total of 64 pots (15 cm × 10 cm), half were filled with *A. niger*-infested soil and the other half with unsterilized native soil with manure to 3/4th volume of the pot. Groundnut seeds, non-bacterized/bacterized with *B. subtilis* AF 1, twelve seeds in each pot, were sown separately in native soil and in the soil infested with *A. niger*. Seeds without any treatment served as controls. Vigour of the seedlings was recorded in terms of seedling emergence, fresh weight and dry weight in grams, and root length and shoot length in cm. Crown rot incidence in these treatments was also recorded. Seedlings with necrotic lesions, after thorough washing with sterile water were noted as crown-rot-infected seedlings.

Effect of microbial treatments on LOX activity

Preparation of LOX crude extract

The enzyme was prepared from groundnut seedlings after microbial seed treatments as described earlier by Kiran Kumar et al. (1992). Five g of groundnut seeds /seedlings were homogenized with 25 ml of 100 mM potassium phosphate buffer, pH 6.5 (20% homogenate

Table 1. Vigour and crown rot incidence in groundnut seedlings treated with *B. subtilis* AF 1, *A. niger*, *B. subtilis* + *A. niger* after seven days

Treatments		No. of seeds germinated (per pot)	Fresh weight (grams)	Dry weight (grams)	Root length (cm)	Shoot length (cm)	No. of seeds with crown-rot infection (per pot)
Native soil	Control	7.00 ± 0.10	2.45 ± 0.31	0.33 ± 0.05	2.86 ± 0.28	4.88 ± 0.27	1.04 ± 0.28
Native soil	AF 1	9.00 ± 0.10	3.48 ± 0.27	0.48 ± 0.02	3.81 ± 0.17	6.38 ± 0.32	0.90 ± 0.30
Infested soil	*A. niger*	5.00 ± 0.53	1.11 ± 0.32	0.11 ± 0.02	2.35 ± 0.30	2.64 ± 0.48	3.60 ± 0.36
Infested soil	Dual	8.00 ± 0.95	2.87 ± 0.11	0.42 ± 0.04	3.48 ± 0.26	5.93 ± 0.43	3.04 ± 0.28

In a total of 64 pots half were filled with *A. niger*-infested soil and the other half with unsterilized native soil with manure. Seeds non-bacterized/bacterized with *B. subtilis* AF 1, 12 seeds in each pot, were sown separately in native soil and in the soil infested with *A. niger*. Seeds without any treatment served as controls. The values for each treatment are significantly different ($P<0.05$) according to Student-Newman-Keul's method.

w/v) passed through two layers of cheese cloth and centrifuged at 10,000g for 30 min at 4 °C. The supernatant was filtered through two layers of cheese cloth to remove the floating lipid material and the filtrate was used as the crude enzyme source.

Measurement of LOX activity

The LOX activity was assayed polarographically in the seedlings at 24 h intervals for 7 days as described by Reddanna et al. (1990). The reaction mixture consisted of 2.9 ml of 0.1M phosphate buffer pH 6.5, 100 ml of enzyme solution and 266 μM linoleic acid (LA). The reaction was initiated with LA and oxygen consumption was recorded on a monitor. The activity was calculated from the slope and expressed as units/ ml. One unit of LOX activity corresponds to one μmol of oxygen consumed/min. The protein content was estimated by the method of Lowry et al. (1951) using BSA as standard. Specific activity was calculated as Units/mg protein.

HPLC analysis of groundnut LOX products

The hydroperoxides, 13-hydroperoxyoctadecadienoic acid (13-HPODE) and 13-hydroperoxyoctadecatrienoic acid (13-HPOTrE) were prepared with groundnut LOX by incubating with LA and ALA, respectively. The reaction mixture contained 100 ml of 100mM phosphate buffer, pH 6.5, enzyme solution (approximately 100 mg of protein) and 266 μM of LA/ALA at final concentration. The reaction initiated with LA/ALA was stopped by adding 6N HCl after 2 min. The products were extracted into an equal volume of hexane:ether (1:1) twice in a separating funnel. The upper phase was separated and evaporated in a rotary vacuum evaporator and redissolved in HPLC solvent (n-hexane:2-propanol:acetic acid 1000:15:1 v/v/v) and separated on Shimadzu HPLC using a silica column (CLC-SIL 4.5 mm × 250 mm) at a flow rate of 1 ml/min. The products were monitored at 235 nm. The major peak fractions were collected and identified based on UV-visible spectral characterization and co-chromatography with authentic standards.

Antifungal activity of LOX hydroperoxides

Antifungal activity of the hydroperoxides (13-HPODE and 13-HPOTrE) was studied in micro titer plates on the growth of *A. niger* as described by Podile and Prakash (1996) with minor modifications. LA/ALA hydroperoxides prepared as above were pooled and evaporated to dryness under nitrogen and redissolved in 10% ethanol. These hydroperoxides in 6.4μl were added to the micro titer plates to achieve a final concentration of 133 ng/μl. The volume is made up to 125 μl with PD broth. Each well was inoculated with 25 μl of *A. niger* spore suspension (A_{450} = 0.08) and incubated at 30 °C. Change in absorbance was monitored at 490 nm on an enzyme-linked immunosorbant assay (ELISA) reader every 7 h up to 28 h. PD broth and 6.4 μl of 10% alcohol (total volume 125 μl) inoculated with 25 μl *A. niger* spore suspension served as control. Every alternate well was kept empty to prevent interference and cross contamination. Triplicates were maintained for each concentration and average values of fungal growth in terms of the A_{490} was calculated. Given O.D values were calculated by subtracting the values of the first measurement from those at a specific time.

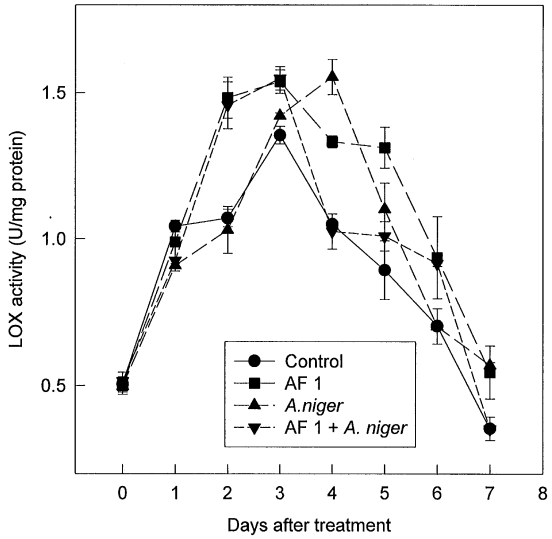

Figure 1. Lipoxygenase activity in germinating groundnut ICG(FDRS)10 seeds treated with distilled water (control), *B. subtilis* AF 1, *B. subtilis* + *A. niger* and *A. niger*. The surface sterilized seeds with respective treatments were germinated in pots (15 cm × 10 cm) filled with native soil and farm yard manure mixed in the ratio 1:1 and LOX activity was determined for one week. The experiments were run in triplicate and the mean values (± SD) expressed. Significance was calculated using Student's 't' test.

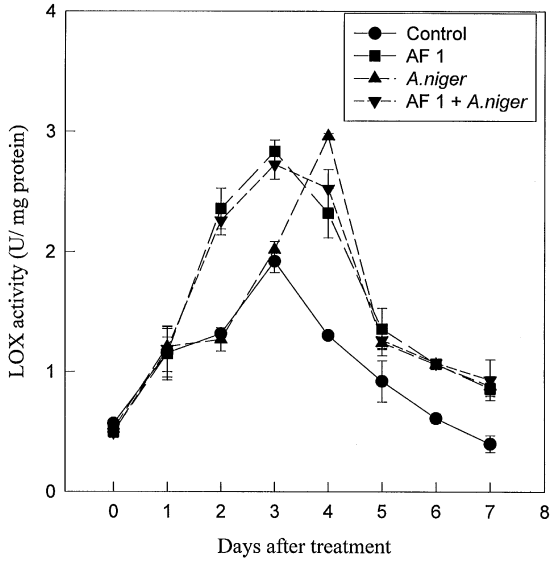

Figure 2. Lipoxygenase activity in germinating groundnut ICG(FDRS)10 seeds treated with distilled water (control), *B. subtilis* AF 1, *B. subtilis* + *A. niger* and *A. niger*. The surface sterilized seeds with respective treatments were germinated in pots (15 cm × 10 cm) filled with sterilized sand and LOX activity was determined for one week. The experiments were run in triplicate and the mean values (± SD) expressed. Significance was calculated using Student's 't' test.

Results

Effect of microbial seed treatments

The groundnut seeds treated with AF 1, *A. niger*, and AF 1 and *A. niger* (dual) were carefully dug out of the soil after seven days and the data on percentage emergence, fresh weight, dry weight, root length, shoot length and crown-rot infection were recorded in Table 1. The seeds treated with AF 1 alone or along with *A. niger* showed increased emergence of the seedlings (Table 1). The seeds treated with AF 1 were more vigorous, in terms of fresh weight, dry weight, root lenght and shoot length, compared to non-bacterized and/or *A. niger*-treated seeds (Table 1). The seeds treated with *A. niger* showed poor growth with higher disease incidence. In pathogen-infested soil, the seeds treated with AF 1 were vigorous compared to untreated controls, although they had necrotic lesions and the degree of infection was close to *A. niger* treatment.

Response of LOX to microbial seed treatment

LOX activity in germinating seeds of groundnut (ICG(FDRS)10) showed significant levels of LOX activity at neutral pH (4 U/ ml) with maximum at pH 6.5 (8 U/ml; 100mM phosphate buffer) with 266 μM LA. When groundnut seeds were treated separately with *B. subtilis* AF 1, *A. niger* and *B. subtilis* + *A. niger*, in native soil for seven days, the results indicated that AF 1 treatment induced a rapid accumulation of LOX (Figure 1). LOX activity reached a maximum by the 3rd day in both AF 1 treatments. Seeds treated with *A. niger* alone reached a maximum one day later, indicating that AF 1 induced LOX activity significantly faster than pathogen infection. When seeds treated with AF 1, *A. niger*, and AF 1 and *A. niger* were sown in sterilized sand, LOX activity showed a similar trend (Figure 2). The difference in LOX activity between bacterized seeds and non-bacterized seeds was more distinct on the 3rd day compared to non-sterile conditions. It is noted that the seeds treated with pathogen or AF 1 always had higher LOX activity compared to the controls in sterile sand.

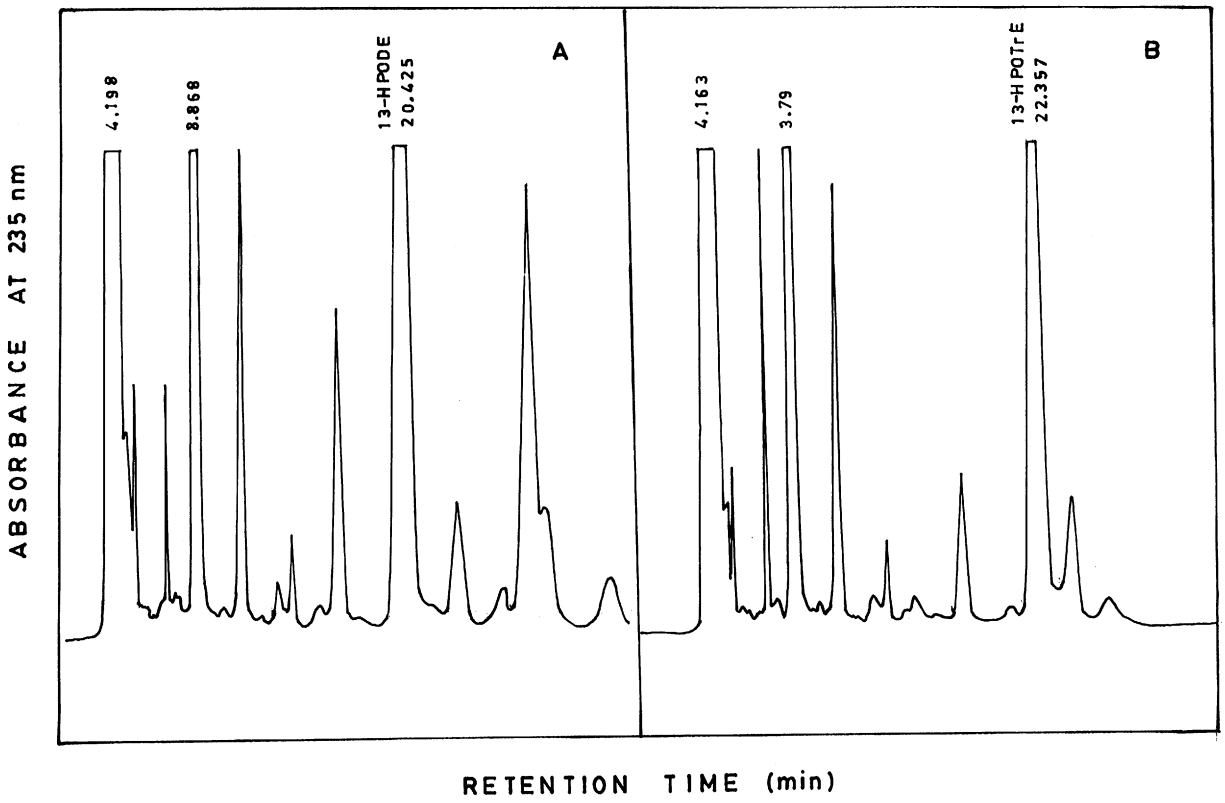

Figure 3. HPLC chromatograms showing the product profiles of groundnut LOX with A) linoleic acid and B) α-linoleic acid. HPLC was performed on a CLC-SIL (analytical) column, in hexane: 2-propanol: acetic acid (1000:15:1) as solvent at a flow rate of 1 mL/ min.

Groundnut LOX product(s)

The crude homogenate of 3-day old groundnut seedlings treated with AF 1, showing highest LOX activity, was incubated with LA and ALA and the products were extracted and separated using a semi preparative Shimadzu silica column (CLC-SIL) on HPLC with a solvent system of hexane: 2-propanol: acetic acid (1000:15:1) at a flow rate of 1 ml/ min.The HPLC analysis of the products with LA and ALA showed major peaks corresponding to standard 13-HPODE and 13-HPOTrE generated from soybean LOX (Figure 3.A,B), respectively, and eluted as a single peak with corresponding hydroperoxide on co-chromatography under identical conditions. The absorption spectra of the peak fractions had the characteristic spectrum of conjugated diene, with λ_{max} at 235 nm indicating that the products are fatty acid hydroperoxides.

Antimicrobial activity of LOX products

Hydroperoxides separated on HPLC (both 13-HPODE and 13-HPOTrE) were used to test their antifungal activity against *A. niger*. In PD broth the growth of *A. niger* showed a steady increase up to 28 h (Figure 4). Growth of *A. niger* was significantly inhibited by the hydroperoxides (13-HPODE and 13-HPOTrE) released from LA/ALA. The effect of 13-HPODE was marginally higher as compared to 13-HPOTrE (Figure 4).

Discussion

The seeds treated with AF 1 germinated vigorously when compared to non-bacterized and/or *A. niger*-treated seeds. Vegetative storage protein 94 of soybean was identified as LOX (Tranbarger et al., 1991). Liu et al. (1991) suggested that the auxins affect the LOX activity and LOXs are involved in germination com-

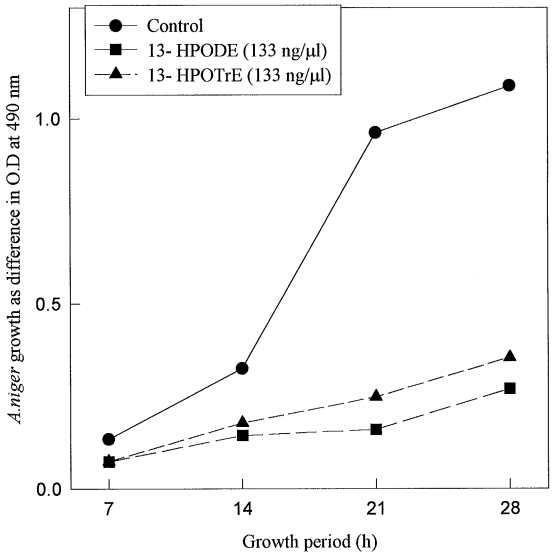

Figure 4. Antifungal activity of 13-HPODE and 13-HPOTrE on the growth of *A. niger* in micro titer plates as measured in an ELISA reader at 490 nm. Peak fractions of the hydroperoxides were pooled, evaporated, redissolved in 10% ethanol and added to the micro titer plates in 6.4 μl per well (final concentration of 133 ng/μl) in a total volume of 150 μl. Given O.D values are the average of triplicate observations calculated by subtracting the values of the first measurement from those at a specific time.

petency of soybean embryos. Some PGPR facilitate plant growth by altering the hormonal balance within the affected plant (Glick, 1995). *B. subtilis* and/or its interaction with groundnut may increase the hormone levels, which in turn induce LOX. Therefore, LOX activity could be associated with growth of the groundnut seedlings. Further studies, however, are required to analyse the specific LOX product(s) involved in growth and development.

Previous studies showed that LOX activity increased in resistant tissues in several host-pathogen combinations (Croft et al., 1990; Keppler and Novacky, 1986; Keppler and Novacky,1987; Yamamoto and Tani, 1986). Preisig and Kuc (1987) suggested that LOX plays a role in the hypersensitive reaction of potato to *Phytophthora infestans*. We show that LOX activity in groundnut seedlings was rapidly activated by seed treatment with a biocontrol PGPR strain of *B. subtilis*. This increase in activity with *B. subtilis* seed treatment was ahead by at least 24 h compared to the increase in *A. niger*-treated groundnut. Also, the LOX activity was significantly higher in bacterized seedlings early in the interaction.

In plant pathogen interactions LOX and its products were implicated in the inhibition of growth and development of pathogens (Croft et al., 1993). In the present study the products of groundnut LOX (13-HPODE and 13-HPOTrE) significantly inhibited the growth of *A. niger*. The increased LOX activity during germination and in response to AF 1 in groundnut may contribute to decrease the incidence of crown rot through the antimicrobial activity of its products against *A. niger*. Ohta et al.,(1990, 1991) reported that 13-HPODE and 13-HODE were potential inhibitors for the germination of the rice blast fungus *Magnaporthe grisea*. These LOX products, as precursors for jasmonic acid and methyl jasmonate, also could be involved in induced disease resistance (Xu et al., 1994).

LOX has been implicated in the generation of reactive oxygen species and mediation of lipid peroxidation, and plays an important role in induced resistance. Induction of SAR by plant growth promoting rhizobacteria/fungi is known (Alstrom, 1991; Leeman et al., 1995; Maurhofer et al., 1994; Meera et al., 1994; van Peer et al., 1991; Wei et al., 1991). The reported increase in LOX activity gives a preliminary evidence that *B. subtilis* AF 1 could induce disease resistance.

The induction of LOX in groundnut in response to a biocontrol PGPR strain of *B. subtilis* AF 1 was earlier than in response to a pathogen like *A. niger*. The products of LOX such as 13-HPODE and 13-HPOTrE show significant antifungal activity against *A. niger*. The timing of induction is a critical factor in induced host resistance. The early release of antifungal substances or molecules that induce resistance in bacterized seedlings could contribute to induced host resistance in biological control of *A. niger* in groundnut. This forms the first report on the induction of LOX in seedlings treated with biocontrol agents.

In light of the available evidences we suggest that the beneficial effects of seed treatment like increased growth, and disease control with *B. subtilis* could be partly due to changes in LOX activity contributing to induced resistance and antimicrobial activity.

Acknowledgements

The authors thank the Dean, School of life sciences, University of Hyderabad, for providing basic facilities and Dr. S. Nigam, Principal Scientist, Groundnut Breeding, ICRISAT for the supply of ICG(FDRS)10 seed material. ARP and PRS thank the Department of Science and Technology, and Council of Scientific and

Industrial Research, Government of India for sanctioning an R & D project and Research Fellowship, respectively. PR thanks Department of Biotechnology, Govt of India for financial assistance (Grant BT/18/06/96 – PID).

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