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Lipopolysaccharides of plant-growth promoting Pseudomonas sp. strain WCS417r induce resistance in carnation to Fusarium wilt

R. VAN PEER[1] and B. SCHIPPERS*

Department of Plant Ecology and Evolutionary Biology, Section Plant Pathology, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, the Netherlands

[1] Present address: Cyanamid, International Agricultural Division, Rue du Bosquet 15, B-1348 Louvain - la Neuve, Belgium

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Abstract

The numbers of diseased plants could significantly be reduced when microconidia of *Fusarium oxysporum* f.sp. *dianthi* were inoculated into the stem and viable-, heat-killed cells or purified LPS of the bacterium *Pseudomonas* sp. strain WCS417r were applied to the roots. Because the competition between *F. o. dianthi* and the bacterium could be excluded, the disease suppression seems to be due to an induced resistance. Accumulation of phytoalexins was found in the stem segments. No accumulation of these compounds was found when the plants were bacterized but noninfected. It is concluded that cell surface components present in the lipopolysaccharides of the bacterium are the inducing factors.

Increased peroxidase activity could be measured in root washes and root extracts after only bacterial preparations were added. No significant differences in peroxidase activity were found in stem extracts. The possible role of increased peroxidase activity in suppression of Fusarium wilt in carnation is discussed.

Additional keywords: carnation, induced resistance, lipopolysaccharides, peroxidase, *Pseudomonas*.

Introduction

Control of Fusarium wilt by pseudomonads has often been attributed to competition for certain nutrients such as iron (Van Peer et al., 1990; Scher and Baker, 1982) or carbohydrates (Elad and Baker, 1985; Lemanceau, 1989). Recently it was suggested that *Pseudomonas* sp. strain WCS417r, in addition to a siderophore-mediated competition with the fungus, also protected carnation cultivars against Fusarium wilt by means of enhancement of their resistance levels (Van Peer et al., 1991). In those experiments the competition between the fungus and *Pseudomonas* sp. WCS417r could be excluded because both microorganisms were spatially separated.

* Corresponding author: B. Schippers

Carnations respond to fungal infection by accumulation of a large number of new compounds, benzoic or cinnamic acid amides of (hydroxy) anthranalic acid or derivatives thereof, known as dianthramides (Niemann and Baayen, 1988; Ponchet et al., 1988). Several of these compounds appeared to be fungitoxic (Bouillant et al., 1983, Ponchet et al., 1988; Van Peer et al., 1991) and therefore are phytoalexins. Production of these phenolic amides is correlated to the resistance expression (Baayen and Niemann, 1989; Niemann and Baayen, 1988) and may be manipulated within a cultivar by pretreatment with salicylic acid or phenylserine (Niemann and Baayen, 1989; Ponchet et al., 1983) or by treatment with a saprophytic *Pseudomonas* strain WCS417r (Van Peer et al., 1991).

This study was initiated to investigate whether or not cell surface components of *Pseudomonas* sp. strain WCS417r are the inducing factor(s) leading to a plant protection along with enhanced phytoalexin accumulation. It has been demonstrated that cell surface components of the pathogenic *Pseudomonas syringae* pv.aptata (Minardi et al., 1989) or *Pseudomonas solanacearum* (Graham et al., 1977) were able to induce resistance in tobacco. To our knowledge, such information has not been reported before with regard to saprophytic rhizosphere inhabiting pseudomonads.

In addition, we investigated the possible role of peroxidases as 'alarm signals' being systemically transported through the plant roots to the stem after bacterization and inducing sensitization of defense mechanisms such as production of phytoalexins. Although the role of peroxidases in defense mechanisms of the plant is by no means clear (Van Loon, 1985), it has been shown that they are systemically transported rapidly through the plant (Hammerschmidt et al., 1982). In some cases, it has been suggested that peroxidases are involved in the formation of phytoalexins (Rathmel and Bendall, 1972; Veech et al., 1976) or in the integration of simple phenolics into cell walls (Negrel and L'Herminier, 1987).

Materials and methods

Plant cultivation. Carnation (*Dianthus caryophyllus* L.) cuttings of the moderately resistant cultivar Pallas, which had been rooted on rockwool granulate, were obtained from van Staaveren B.V., Aalsmeer, the Netherlands. The plants were placed on rockwool cubes (365 cm^3) (Grodan B.V., the Netherlands) and were grown in the glasshouse at 22 °C under a photoperiod of 16 h artificial natural daylight conditions for at least one week prior to the treatments. A nutrient solution (De Voogt, 1983), modified for iron (15 μM Fe-EDDHA) was given manually. Depending on the season, evaporation, and growth stage, plants received 25 to 50 ml of the nutrient solution three times a week.

Bacterization and inoculation of plants. *Pseudomonas* sp. strain WCS417 was isolated from wheat rhizosphere in a 'take all' suppressive field soil and demonstrated to be effective in suppression of take all disease caused by the soilborne fungus *Gaeumannomyces graminis* var. *tritici* in wheat (Lamers et al., 1988) and of *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen f. sp. *dianthi* in carnation (Van Peer et al., 1989, 1990, 1991, 1991a,). This strain also promoted growth of several crops (Van Peer et al., 1990). A rifampicin-resistant mutant of WCS417 (WCS417r) was used for bacterization of plant roots. A bacterial culture of *Pseudomonas* sp. strain WCS417r

was grown at 27 °C for 24 h on King's medium B (KB) agar (King et al., 1954). Cells that were collected from the agar plates were diluted in sterile tap water (final concentration approximated 107 cells ml^{-1}. A part of the bacterial suspension was killed by heat treatment (80 °C, 30 min). Viability of these heat-treated cells was checked by dilution plating of the suspension on KB. The plants were bacterized by pouring 25 ml of either dead or viable cells to the rockwool cube of each plant, seven days prior to inoculation with *F. o. dianthi*. Three days prior to inoculation with the fungus, the plants did not receive nutrient solution, but were fertilized an hour after inoculation.

A microconidial suspension of race 2 (isolate WCS816) of *Fusarium oxysporum* f. sp. *dianthi* was obtained from cultures grown for seven days on potato dextrose agar plates at 23 °C. Sterile distilled water (SDW) was poured on the plates and was recollected after carefully abrading the agar. The resulting microconidial suspension was filtered through sterile glasswool and adjusted to a concentration of 106 conidia ml^{-1}. One week after bacterization, the plants were stem-inoculated between the first and the second pair of leaves and about 3-5 cm above the stem base as was described previously (Van Peer et al., 1991). Thirty μl of the microconidial suspension were deposited in the axil of one artificial (Parafilm) pair of leaves tied to the stem. Thereafter the stems were incised horizontally at both sites through the droplets just into the xylem. The control treatment was mock-inoculated with SDW instead of the microconidial suspension. The various treatments are shown in Table 1.

To exclude competition between *F. o. dianthi* and *Pseudomonas* sp. strain WCS417r, the presence of this strain in the stems was checked both at the time of inoculation with *F. o. dianthi* and at the end of the experiments. Stem segments, 2 cm in length were taken just above the stem base, they were surface-sterilized by flaming after dipping in 70 % aethanol, sliced in pieces of 2 mm and incubated on KB agar plates supplemented with rifampicin (KBrif) (150 mg l^{-1}) for 2-4 days. Thereafter, growth of strain WCS417r on the medium was determined. Stem segments of non-bacterized plants were plated also.

Root colonization by *Pseudomonas* sp. strain WCS417r was determined at 0, 1, 2, 6 and 12 weeks after inoculation with *F. o. dianthi*. Random samples of root pieces (0.3 g) were shaken (1 min) in glass test tubes containing 5 ml 0.1 M $MgSO_4$ and 2.5 g of glass beads (0.17 mm diam). Serial dilutions were plated on KBrif media and incubated at 27 °C for 48 h. Root pieces from non-bacterized plants were similarly plated. Number of colony forming units (cfu)/g of root tissue were determined.

Disease development. The number of diseased plants and severity of wilting were scored at least once a week on 32 plants per treatment. The following scale according to Baayen and Niemann (1989) was used: 0, no disease symptoms; 1, initial but still questionable disease symptoms; 2, limited local symptoms; 3, well-developed symptoms on otherwise still healthy looking plants; 4, severe wilt, and 5, complete death. A plant was considered to be diseased when local symptoms due to Fusarium wilt (index 2) had developed.

Experimental design. Two separate experiments were performened. In each experiment in total 900 plants were used. Plants were placed in randomized block design and each treatment was replicated six times. A total of 32 plants per treatment were kept for evaluation of disease development.

Extraction and purification of bacterial lipopolysaccharides. Lipopolysaccharides (LPS) of *Pseudomonas* sp. strain WCS417r were extracted with hot phenol according to Westphal and Jann (1965). After extraction, the pooled aqueous extracts were dialyzed against distilled water for 4 days and ultra centrifuged at 29 500 × *g* for 6h followed by two times at 32 000 × *g* for 3 h. The extracted LPS was suspended in distilled water and stored at −20 °C. The amount of LPS extracted in this way was calculated from the original amount of cells in the culture, assuming a 10% loss due to the extraction procedure. The plants were treated with 25 ml of suspended LPS, corresponding with 107 viable cells ml^{-1}. Purity of LPS obtained in this way did not significantly differ from LPS obtained by the method described by de Weger et al. (1989) as compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This indicates that indeed mainly LPS (and no proteins) were extracted. This was also confirmed by treating the obtained LPS according to the method by Westphal and Jann (1965) with proteinase-K (0.1 mg ml^{-1}) followed by SDS-PAGE (R. van Peer, unpublished results).

Extraction of phytoalexins and high performance liquid chromatography. For extraction of phytoalexins, 5 cm long segments were taken just above the inoculation site at various times after inoculation with *F. o. dianthi* or after mock-inoculation. At each time, four samples per treatment were taken. Each sample consisted of stem segments from four plants. Thereafter, phytoalexins were extracted with acetone and analyzed by high-performance liquid chromatography as described before (Van Peer et al., 1991) using an LKB double-pump liquid chromatography equipped with a 2 × 10 cm Chromsep Lichrosorp RP18 (ID 3 mm) column, a Uvicord detector with 275 nm filter and a Baseline 810 chromatography work station. Peaks in the chromatograms were identified and numbered as described before (Van Peer et al., 1991). A number of the dianthramides (and dianthalexin) had previously been localized in the HPLC peaks, identification of the major constituents was among others based on HPLC-MS (Dr G.N. Niemann, personal communication) or after collection of HPLC peaks, rechromatographed and analyzed by PyMS and were necessary by high resolution mass spectrometry (Niemann et al., 1991; Dr G.N. Niemann, personal communication).

Differences in phytoalexin accumulation between the treatments with bacterial preparations or the non-treated control and inoculation with the pathogen alone were analyzed by polynomial regression analysis and student t-test (Snedecor and Cochran, 1980).

Preparation of root washes and root- and stem extracts. Root washes of the plants were prepared from cuttings rooted on perlite. Roots of three plants were washed in water for 15 min, by gentle agitation on a Griffin wrist shaker, thereby leaving the roots attached to the plants. The root wash was filtrated (Sleicher and Schull no. 604) and centrifuged at 10 000 × *g* for 10 min, the supernatant was then lyophilized. The resulting powder was solubilized in SDW (5 ml g^{-1} root fresh weight) before measuring peroxidase activity. Root- and stem extracts were also prepared from the rooted cuttings. Roots and stems were weighed and macerated with a homogenizer (ultra turrax T25) at 4 °C in 10 ml SDW g^{-1} tissue. The homogenate was filtered and centrifuged at 10 000 × *g* for 5 min. The supernatant then was immediately analyzed

for peroxidase activity.

Cells of *Pseudomonas* sp. strain WCS417r were assayed for production of peroxidase. Cells from stock cultures were inoculated on KB medium either with or without addition of root wash material (1 mg dry weight per ml culture) and grown for 24 h. Cells were collected by centrifugation (10 000 × *g*) for 20 min and resuspended in sterile tapwater (108 cells ml^{-1}). A part of the bacterial suspension was subjected to ultrasonic treatment. Thereafter, the sonicates and viable cells were examined for peroxidase activity.

Measurement of peroxidase activity. Peroxidase activity in 100 μl of the sample was measured at 20 °C in 1 ml reaction mixture containing 0.01 M phosphate buffer (pH 5.0), 0.25% (v/v) guaiacol and 1 μM H_2O_2 which was added last to initiate the reaction. Peroxidase activity was determined spectrophotometrically, reading the absorbance at 470 nm. Controls which lacked the acceptor or hydrogen peroxide were examined simultaneously. The activity was expressed as the change in absorbance when the absorbance increase was linear (starting approximately 30 s after addition of H_2O_2).

Statistical analysis. All experiments were repeated at least twice. Results were analyzed by analysis of variance, followed by Student's t-test to calculate minimum significant difference (Snedecor and Cochran, 1980).

Results

Disease development and root colonization by Pseudomonas sp. strain WCS417r. Disease development in carnations of the cultivar Pallas that were stem inoculated with *F. o. dianthi* was significantly reduced when roots of these plants were treated one week prior to inoculation with *F. o. dianthi* with either viable-or heat killed cells of *Pseudomonas* sp. strain WCS417r compared to the treatment with *F. o. dianthi* alone (Table 1). Addition of the purified LPS of this strain to the roots, also suppressed the disease development. Disease suppression of plants treated with LPS did not significantly differ from treatments with dead- or viable cells of *Pseudomonas* sp. strain WCS417r (Table 1). These results indicate that surface components of *Pseudomonas* sp. strain WCS417r are, at least partly, responsible for the observed disease suppression.

Pseudomonas sp. strain WCS417r extensively colonized the roots. After 12 weeks it could be recovered up to 6 × 104 cfu g^{-1} root. This strain was never detected on or in the surface sterilized or non-surface sterilized stem tissues of the plants, indicating that suppression of *Fusarium* wilt disease by viable cells of *Pseudomonas* sp. strain WCS417r can not be due to competition between the pathogen and the *Pseudomonas* cells.

Phytoalexin accumulation. Several dianthramides (including the phytoalexins) significantly accumulated in the stem segments of *F. o. dianthi* treated plants (Fig. 1). Treatment of roots with viable cells of *Pseudomonas* sp. strain WCS417r and stem inoculation with microconidia of *F. o. dianthi*, resulted in a significantly higher increase of phytoalexins in stem segments compared to plants that were treated with

Table 1. Mean percentages of diseased plants and the disease indices for the moderately resistant carnation cv. Pallas, 10 weeks after treatment of roots with either viable cells (Ps), heat-treated cells (Ps@) or purified LPS of *Pseudomonas* sp. strain WCS417r and/or stem-inoculation with *Fusarium oxysporum* f.sp. *dianthi* (Fod).

Treatment	1	2	3	4	5	6	7	8
day 0	– [x]	Ps	Ps@ (heat)	LPS	–	Ps	Ps@ (heat)	LPS
day 7	water	–	–	–	Fod	Fod	Fod	Fod
Exp. I	0	0	0	0	63a [y] (1.8) [z]	22b (0.6)	31b (0.9)	31b (1.0)
Exp. II	0	0	0	0	71a (1.9)	21b (0.5)	29b (0.7)	25b (0.6)

[x] Plants were not treated at all at the mentioned time.
[y] Corresponding letters indicate absence of significant differences between Fod inoculated plants ($P \leq 0.05$) within one experiment.
[z] Mean disease index (on a scale from 0 to 5 as described in materials and methods) calculated from 32 plants per treatment.

F. o. dianthi alone (Fig. 1). Such an increase was particularly found in the first seven days after inoculation with *F. o. dianthi*. Similar tendencies were found when roots of *F. o. dianthi* inoculated plants had been treated with either heat-killed cells or with purified LPS of *Pseudomonas* sp. strain WCS417r (Fig. 1). The higher accumulation of phytoalexins in LPS treated plants is noted. Only very low levels of these dianthramides could be detected in the water treated controls. There was no effect of bacteriza-

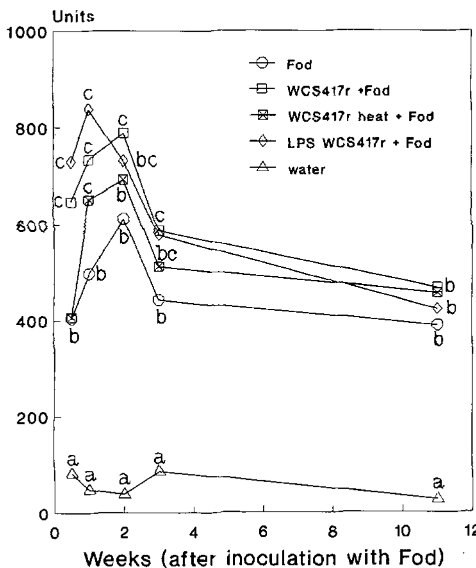

Fig. 1. Accumulation of phenolic compounds (phytoalexins) in the acetone extracts of stem segments of the carnation cultivar Pallas. The roots were treated with different preparations of *Pseudomonas* sp. strain WCS417r one week prior to stem inoculation with *Fusarium oxysporum* f. sp. *dianthi* (Fod). As a control, the stem was inoculated with water instead of Fod. Different letters at each sampling time indicate significant differences ($P < 0.05$).

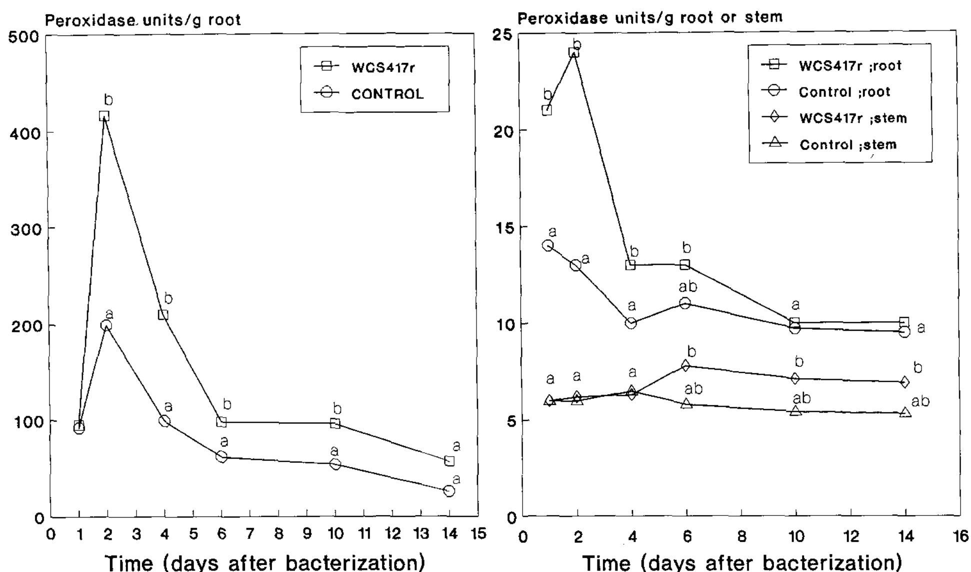

Fig. 2. (Left). Peroxidase activity in root washings from *Pseudomonas* sp. strain WCS417r bacterized (□) and non-bacterized (o) roots at different times after bacterization (Peroxidase activity in the root washes was assayed as described in 'Materials and Methods'). Data are based upon average values of four replicates each consisting of three plants. Peroxidase activities are expressed as units activity per g of root tissue. 1.0 unit of peroxidase activity produced an A change at 470 nm of 0.001 per second at 20 °C when the increase in absorbance was exponential. Corresponding letters indicate no significant difference at each time ($P < 0.05$).

Fig. 3. (Right). Peroxidase activity in root- and stem extracts from *Pseudomonas* sp. WCS417r bacterized (□) and non-bacterized (o) roots at different times after bacterization. For more details see Fig. 2.

tion on accumulation of these compounds in the absence of *F. o. dianthi* (data not shown).

Peroxidase activity. Root washes of carnation cuttings treated with cells of *Pseudomonas* sp. strain WCS417r consistently exhibited significantly higher peroxidase activities than the water treated controls, except after 14 days (Fig. 2). The maximum activity in both control- and *Pseudomonas* treated plants was found after two days and decreased thereafter.

Peroxidase activity in root- and stem extracts were lower than in root washes (Figs 2 and 3). Treatment of roots with viable cells of *Pseudomonas* sp. strain WCS417r resulted in already high peroxidase activity in the roots after two days which decreased thereafter. In contrast, in stem extracts maximum peroxidase activity was measured after six days, however not significantly different from the control treatment. Peroxidase activity was not measurable in the sonicates or viable cells of *Pseudomonas* sp. strain WCS417r that were grown on KB medium with or without root wash material.

Discussion

Bacterization with *Pseudomonas* sp. strain WCS417r of roots of the moderately resistant carnation cultivar Pallas, one week prior to stem inoculation with *F. o. dianthi*, significantly reduced disease development after 11 weeks. As *Pseudomonas* sp. strain WCS417r and *F. o. dianthi* were spatially separated, competition between these two micro-organisms has to be excluded. Therefore the disease suppression seems to be a result of an induced resistance. This is in agreement with previous reported results (Van Peer et al., 1991, 1991a). Both, treatments of roots with heat-treated cells of *Pseudomonas* sp. strain WCS417r or with purified LPS of this strain also significantly reduced Fusarium wilt in the carnations. It therefore appears that the inducing factor is located on cell surface components of the bacterium. LPS consists of a lipid A region, a core region and a O-polysaccharide chain. Particularly the latter structure has a specific nature (Hammond et al., 1985) which indicates that LPS may be suited as an agent for specificity of *Pseudomonas*-plant interactions. It has been demonstrated before that two strains of *Pseudomonas solanacearum* (K60 and B1) differing in their LPS composition also caused different responses in tobacco leaves (Whatley et al., 1980). Degradation of LPS at the cell wall could not be proven (Sequeira, 1983). Whether or not the cell surface components of *Pseudomonas* sp. strain WCS417r are strain specific remains to be elucidated. Preliminary results have shown that of two strains of *Pseudomonas* spp. (WCS417r and WCS358), both able to suppress Fusarium wilt in carnation (Van Peer et al., 1989), only WCS417r could reduce the disease suppression when spatially separated from the fungus (R. van Peer, unpublished results).

Inoculation of the stem with *F. o. dianthi* always coincided with a prominent accumulation of phytoalexins in the stem segments (Fig. 1). Such an increased accumulation was not found in water inoculated plants or in stem segments of plants that had been treated with purified LPS or with viable- or heat-treated cells of *Pseudomonas* sp. strain WCS417r and nót inoculated with *F. o. dianthi* (data not shown). This is in agreement with previously reported results (Van Peer et al., 1991). Phytoalexin accumulation in stem segments of *F. o. dianthi* inoculated plants receiving purified LPS, heat-treated or viable cells of *Pseudomonas* sp. strain WCS417r was always higher, except for 80 days after inoculation with the pathogen, than those that were inoculated with the pathogen alone (Fig. 1). These differences, however, were statistically significant during the first week for treatments with purified LPS, heat-treated or viable cells, after two weeks for viable cells and three weeks after inoculation with the pathogen for treatments with viable cells or LPS (Fig. 1). Similar results were obtained with repeated experiments. These results sustain our earlier results that induced resistance by WCS417r coincides with increased accumulation of phytoalexins (Van Peer et al., 1991). Although no causal relationship between phytoalexin accumulation and plant protection could be demonstrated, rather high amounts of the phytoalexins dianthalexin and methoxy-dianthramide S accumulated locally in *F. o. dianthi* inoculated plants (Niemann et al., 1990). Such amounts can be toxic to the pathogens (Ponchet et al., 1988; Van Peer et al., 1991) and may indeed directly affect growth of the fungus.

Natural infection of carnation by *F. o. dianthi*, however, proceeds either by direct penetration of the root epidermis or by entering the roots through wounds. It therefore

needs to be justified that the observed responses of the plant after inoculation of the pathogen into the stem, also reflect responses of the plant to root infection of the pathogen.

As both micro-organisms were spatially separated, it was supposed that some signals provided by *Pseudomonas* sp. strain WCS417r at the root surface induce sensitization of the stem. However, it cannot be excluded that the LPS or parts of it are aspirated through small wounds into the xylem where the fungus has been inoculated.

We demonstrated a significant increase of peroxidase activity in the root washes treated with *Pseudomonas* sp. strain WCS417r. The lack of peroxidase activity in preparations of *Pseudomonas* sp. strain WCS417r suggests that the increase in peroxidase activity is of plant origin. Peroxidase activity was more prominent in root washes than in root- or stem extracts (Figs. 2 and 3), which indicates that root cells in particular, may be involved in the response to bacterization. No significant increase of peroxidase could be demonstrated in the stems. Therefore, the hypotheses of Albert et al. (1987), that peroxidases are transported systemically through the plant, activating defense mechanisms at the infection site in the stem, is not supported by these experiments.

Increased peroxidase activity in root washes or -extracts of *Pseudomonas putida* inoculated beans was also reported by Albert and Anderson (1987). Treatment of roots with heat-treated cells, or with the purified LPS of *Pseudomonas* sp. strain WCS417r, also significantly increased peroxidase activity in roots but more irregularly (date not given). Association of enhanced peroxidase activity with induced resistance in cucumber by *Colletotrichum lagenarium* was also demonstrated by Hammerschmidt et al. (1982). Possibly, peroxidases are directly involved in the defense mechanisms of the plant. Peroxidases catalyze the final polymerization step of lignin synthesis, and may therefore be directly associated with the increased ability of systemically protected tissue to lignify (Gross et al., 1977; Hammerschmidt and Kuč, 1982; Vance et al., 1980). Sijmons et al (1985) suggested that a specific bean peroxidase is functioning in suberization. In addition, H_2O_2 and O_2^- which are generated by peroxidases at the root surface during oxidation of NAD(P)H (Halliwell, 1978) as well as the phenoxy radicals themselves (Rama Raje and Dunleavy, 1975), possess potent antimicrobial activity. When Fusarium enters the plant through penetration of root tissue, such as is the case in natural situations, the antimicrobial activity of superoxide anion and hydrogen peroxide at the root surface may contribute to the plant resistance mechanism. Recently, it was reported that a strain of *Pseudomonas putida* exhibited defense mechanisms to protect itself against superoxide anion and hydrogen peroxide (Katsuwon and Anderson, 1989).

Further studies will focus on the specific role of LPS of *Pseudomonas* sp. strain WCS417r using LPS-mutants and on the specificity of these bacterial cell surface components and their correlation with plant protection.

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