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(L)-2,5-DIHYDROPHENYLALANINE FROM THE FIREBLIGHT PATHOGEN *ERWINIA AMYLOVORA**

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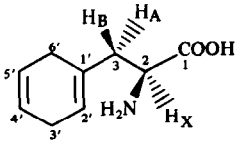
Key Word Index—*Pyrus communis*; Rosaceae; pear, *Erwinia amylovora*, (L)-(−)-2, 5-dihydrophenylalanine; (*S*)-(−)-α-amino-1, 4-cyclohexadiene-1-propanoic acid, 6-thioguanine, fireblight, reactive oxygen species; hypersensitive reaction

Abstract—(L)-(−)-2, 5-Dihydrophenylalanine isolated from axenically grown *Erwinia amylovora* induces necrosis of pear cells in culture

INTRODUCTION

Fireblight of rosaceous species [2] is an economically important plant disease, particularly in the apple and pear industry. In European countries, which prohibit the use of antibiotics such as streptomycin in agriculture, there is no cure for it other than burning Previous attempts to clarify the virulence mechanisms of fireblight have been controversial [3–7], and it appears that the disease is caused by more than one virulence factor Of particular interest is the cause of cell leakage and necrosis [8–11] which, supposedly, through liberation of phenolic compounds from the vacuoles and their subsequent oxidation, gives rise to the typical firetorched appearance of the diseased plant One suggestion has been that a bacterial metabolite, a 'putative necrotoxin' [5], is involved, but attempts to isolate it from infected pear tissue culture have been unsuccessful. Like with other bacterial pathogens [12], isolation of the putative necrotoxin from an axenic culture of the causal bacterium, in this case *Erwinia amylovora* (Burril) Winslow *et al*, may be easier. Since we were able to simulate some of the symptoms of the disease (growth inhibition, discoloration, and necrosis) with partially purified fractions of the culture filtrate of virulent *E. amylovora* EA213 in a Bartlett pear suspension culture (BASC) [10, 13], the author followed this approach to purify and characterize a metabolite of *E amylovora*, which, as shown below, is necrotoxic to pear cells and was identified as (*S*)-(−)-α-amino-1,4-cyclohexadiene-1-propanoic acid (**1**) [(L)-(−)-2,5-dihydro-phenylalanine, DHP].



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* Part 6 in the series 'Secondary Metabolites of *Erwinia*' [1]

RESULTS AND DISCUSSION

Details of the BASC bioassay have been reported elsewhere [10, 13]. In short, it is based on growth curves for the cultured pear cells, monitored by sedimentation measurements in side arm flasks The test for necrotoxicity was regarded as positive when the fraction under consideration produced both growth inhibition and a grey discoloration of the cells, thereby indicating massive necrosis (typically overnight). Figure 1 gives an example and also extends our previous studies in that it shows that a necrotoxin is produced not only in galactose minimal medium but also in sucrose minimal medium provided the medium is vigorously aerated. In a similar experiment [10] where the sucrose medium was only moderately aerated, no necrotoxicity was observed. This is an important finding, as it makes us aware of the fact that necrotoxin production is dependent on environmental factors. Ultimately, any model for a virulence mechanism needs to accommodate this fact While such a regulatory control opens a new frontier in fireblight toxin research, an intriguing hypothesis which considers the hypersensitive

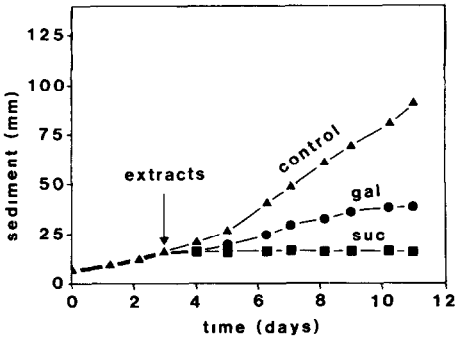


Fig. 1. Response of BASC to extracts from culture filtrates of *E amylovora* grown under vigorous aeration on sucrose (suc) or galactose (gal), compared to BASC which received no extracts (control)

reaction (HR) as a possible source of 'oxygen stress' and as a trigger for DHP production, is presented below

The necrotoxin in the galactose medium has been unambiguously identified as DHP (*vide infra*); evidence that the necrotoxin in the sucrose medium also is DHP is as yet indirect and derives from preliminary studies investigating the production of DHP in sucrose minimal medium by two avirulent strains of *E amylovora*, namely EA212, which produces necrosis of BASC [10], and P66, which does not The spent media were analysed by HPLC and a peak with the correct R, for DHP was found for EA212, but not for P66 This result is consistent with the assumption that DHP is produced in sucrose medium, but only by strains of *E amylovora* able to induce necrosis of pear tissue More strains need to be investigated before a firm conclusion can be reached Nevertheless, taken altogether, the data and arguments presented in this paper make DHP a strong candidate for playing a primary role in fireblight disease

To characterize the toxin, it was mass-produced in galactose minimal medium Our previous attempts to isolate a necrotoxin from *E amylovora* had led to the identification of 6-thioguanine (6-TG) [10] At that time, we found that both 6-TG and the putative necrotoxin could be extracted from the culture filtrate by means of a cation exchange column, whereas on an anion exchange column 6-TG was retained but the necrotoxin eluted with the void volume This procedure was followed again in the present study The resulting crude toxin preparation was extracted with methanol and the extract purified in two stages by silica gel chromatography to give three ninhydrin-positive components which were labelled ASA, ASB, and ASC [13] in the order of increasing polarity

Compound ASC has not yet been identified. By mass spectrometric and NMR spectroscopic investigations and comparison with published data [14–20], ASB was identified as valine and ASA as a mixture of phenylalanine and DHP Identification of DHP was hampered by the fact that the EIMS of ASA did not show a distinctive molecular ion, neither for DHP nor for phenylalanine A strong ion at *m/z* 166 nominally corresponded to an $[M+H]^+$ ion for phenylalanine which may be observed under EIMS conditions which favour auto-chemical ionization [21] However, as a control measurement with authentic phenylalanine revealed, such conditions did not prevail Later it became apparent that the ion at *m/z* 166 was the $[M-H]^+$ ion of DHP The $[M-H]^+$ ions are usually not observed with such abundance under EIMS conditions [21, 22], but have been noted for DHP before [16] When the mass spectrum of ASA was acquired using chemical ionization (CI), $[M+H]^+$ ions for phenylalanine and DHP could be seen. This allowed confirmation of the elemental composition of DHP by accurate mass measurement (Complete mass spectra of DHP may be found in ref [23].) The structure identification was corroborated by comparison with authentic DHP synthesized according to ref [19]

The necrotoxicity resided with fraction ASA, which, as judged by NMR, was a mixture of DHP. phenylalanine of *ca* 2.1 Purification of DHP could only be achieved after its chemical nature had been elucidated and the appropriate precautions could be implemented to prevent its decomposition Decomposition of DHP to phenylalanine is known to occur with ease, particularly, when DHP is dried *in vacuo* [16, 19, 24]. In the present study, a sample of DHP shown to be pure by ^{13}C NMR spectroscopy was found to be 50% decomposed in the subsequent ^1H NMR experiment, in between the sample had been dried by rotary evaporation This instability of DHP may explain why previous attempts to isolate the putative necrotoxin have been unsuccessful. In alkaline medium, oxidation of DHP not only to phenylalanine but also to a black polymer occurs within days and DHP may thus contribute to the black discoloration in fireblight disease However, DHP seems to be quite stable in neutral (or slightly acidic) aqueous solution, since its ^1H NMR spectrum showed little change during a three week observation period The author's method of choice to establish a stock solution of pure DHP for biological studies is RP-HPLC using water as eluent and concentration of the aqueous DHP fractions to *ca* 10 mg DHP/ml by rotary evaporation without ever drying the sample.

This was done with synthetic DHP before it was tested on a pear cell suspension culture to confirm that the observed necrosis was indeed due to DHP and not to a minor, but very potent, toxic contaminant in the DHP preparation derived from *E amylovora* Since at this point the BASC bioassay was no longer at hand, the author used a Passe Crassane pear cell suspension culture (PASC) at the Department of Pomology, University of California, Davis (courtesy of R Romani and B Hess) Discoloration of the pear cells (light grey compared to the light yellow of healthy tissue) was seen in the experiments which used 67 or 133 ppm DHP Growth was measured by determining the packed cell volume at the beginning and the end of the experiment (Fig 2) The lowest concentration of DHP which led to growth inhibition and necrosis (the latter judged by viability staining) was 8 3 mg/1, equivalent to 8 3 ppm or 5×10^{-5} M However, up to a concentration of 33 ppm some of the pear cells survived and eventually resumed normal growth In those experiments which tested 67 and 133 ppm DHP and

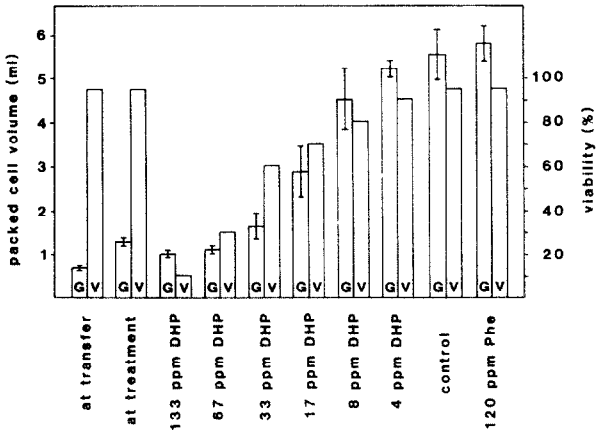


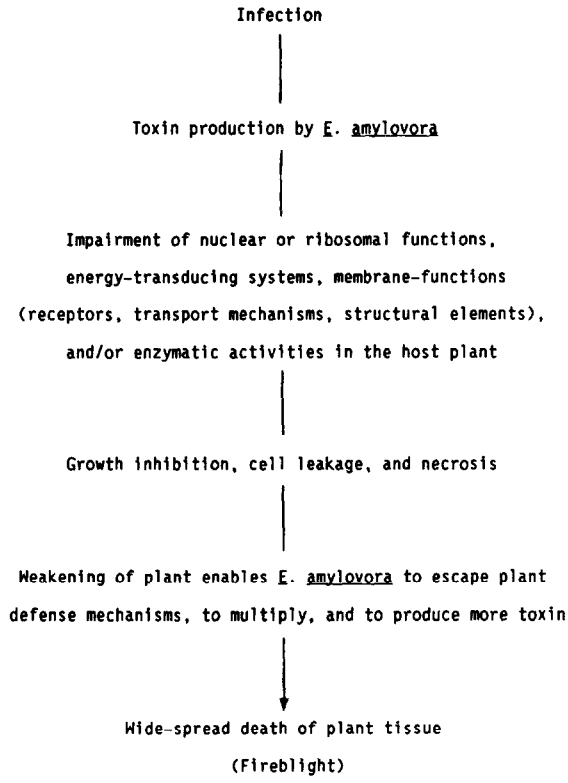
Fig 2 Effect of various concentrations of synthetic DHP on growth (G), derived from measurements of packed cell volumes, and viability (V) of PASC From left to right fresh subculture of PASC on day O (at transfer). PASC showing growth on day 3, when DHP or phenylalanine (Phe) were added (at treatment), PASC on day 8, at the end of the experiment, for various concentrations of DHP (XX ppm DHP), PASC on day 8 that had not received any additives (control), and PASC on day 8 that had received 120 ppm Phe

where growth inhibition was complete, DHP could still be detected in the culture filtrate at the end of the bioassay. No DHP was detected in the experiment with 17 ppm DHP and only very little in the experiment with 33 ppm DHP On the other hand, two new compounds were observed in the culture media which, based on HPLC retention times and UV absorbance spectra have been tentatively identified as phenylalanine and tetrahydrophenylalanine (an authentic sample of the latter was obtained as a minor product during the synthesis of DHP) Whether this reflects a detoxification mechanism by the pear cells, is not yet known, but if so, it will undoubtedly handicap any attempt to isolate DHP from fireblighted tissue.

Assuming, for the sake of the argument, that the chemical identity of the putative fireblight necrotoxin is now known, one can make an educated guess about how this 'toxin' may act This will provide a basis for future hypothesis-driven investigations regarding the virulence mechanisms of fireblight. Interestingly, the characteristics of DHP allow for either an active role as in Scheme 1, or a passive role as in Scheme 2 (first proposed at the Fallen Leaf Lake Conference on Erwinia, September 1987).

Scheme 1 follows the common concepts of phytotoxin action [25, 26] according to which a toxin interferes with one or more of the vital functions in the host plant. Vital functions which might be affected by DHP are protein synthesis and enzymatic activities which involve NAD(P)H as a co-factor. The latter seems possible because the 1,4-cyclohexadiene moiety of DHP and the reduced nicotinamide of NAD(P)H are structurally related and also share the capability to serve as a reductant. Interference with protein synthesis and (because of normal protein turn-over) protein maintenance, is perhaps even more likely, since false feed back inhibition and repression of enzymes in the metabolic pathway of phenylalanine have already been demonstrated for DHP in the 1970's in connection with observations that DHP inhibits growth of microoganisms and the rat [27, 28]. Whether the same mechanisms occur in plants, needs to be established. In plants, lack of phenylalanine may also result in a compromised defence capability via blockage of the synthesis of lignin and phytoalexins, but the regulation of these pathways is complex [29] and such an effect is difficult to predict.

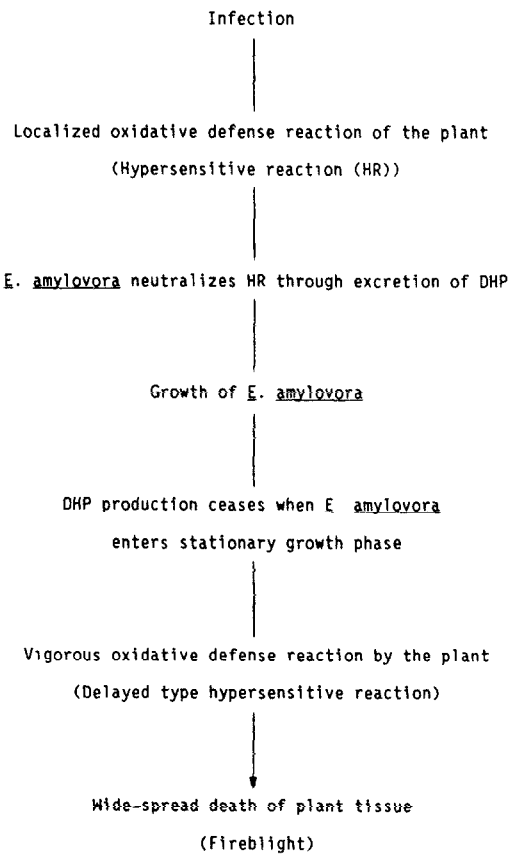
Scheme 2 considers the possibility that fireblight necrosis may be due to the HR and thus be self-afflicted by the plant The HR supposedly is a response of plant tissue towards stress such as wounding or invasion of a pathogen and seems to serve the purpose of sealing off the affected area [30] The exact molecular events during the HR are not known, but deleterious radical chain reactions initiated by reactive oxygen species, for example, superoxide, are attracting more and more attention [31–34] On the other hand, DHP is a reducing agent and seems to be produced by *E. amylovora* only in response to oxidative stimulus/stress. The crux of Scheme 2 is that production of DHP (and possibly 6-TG [35, 36]) by *E. amylovora* may lead to a steady state neutralization of the oxidative killing mechanisms of the plant and thus may enable the pathogen to multiply in its host. The HR may be prevented by DHP either through scavenging of oxygen radicals or through inhibition of enzymes (perhaps alternative terminal oxidases) which are involved in the production of these oxygen radicals; because in either mode each molecule of DHP would eliminate a chain



Scheme 1 Hypothetical 'toxic' involvement of DHP in fireblight disease.

reaction, DHP would be effective at low concentrations (sub-stoichiometric with regard to the damaging reactions prevented).

A priori, DHP may either prevent the HR, or do just the opposite, namely trigger the unspecific HR by grossly disturbing the redox state of the plant tissue; the latter scenario would probably require higher concentrations of DHP than the former. The effects of DHP on pear tissue culture may be explained either as a result of triggering the HR or as a result of an interruption of vital cell functions. In the fireblighted plant, however, where the HR may be induced by cell wall components of *E amylovora*, and where a DHP concentration high enough to be lethal or to induce the HR may never be reached, DHP may be functioning according to Scheme 2. Scheme 2 is supported by the following observations/arguments. (i) Massive necrosis is a late event in fireblight disease and occurs only after *E. amylovora* has reached a high cell number. For a toxin in the classical sense (Scheme 1), one would expect necrosis to *precede* colonization by *E. amylovora*, but this does not appear to be the case. (ii) Fireblight necrosis may be accompanied by symptoms of ripening [2], a process in which reactive oxygen species are supposedly involved. (iii) DHP has previously been identified as an inhibitor of several enzymes that catalyse the hydroxylation of aromatic rings [17, 37], a process which, at least in some cases, involves superoxide [37] (iv) The outbreak of fireblight in susceptible apple plants can be prevented by prior injection of galactose [11], which may be explained by stimulation of galactose oxidase and the production of superoxide during its normal activity [38] (although the



Scheme 2 Hypothetical HR suppression by DHP in fireblight disease

author of ref. [11] interprets his finding differently).

Clearly, it will be prudent to keep both schemes in mind when designing future experiments to unravel the virulence mechanisms of fireblight For example, Scheme 1 could be corroborated by showing (preferably in the intact plant) that induction of necrosis by DHP can be prevented by co-administration of phenylalanine. Scheme 2, on the other hand, would be supported if avirulent P66 could produce full-blown fireblight disease in a host which is concomitantly treated with DHP (at a concentration that does not produce necrosis by itself) Such experiments are in progress. To demonstrate the presence of DHP in a fireblighted plant as well as to study a possible detoxification reaction (*vide supra*), a sensitive and specific detector will be needed Preliminary studies (G Feistner and B L Beaman, unpublished results) have shown that thermospray mass spectrometry may be especially suitable for this purpose Proof that DHP is indeed of primary importance in fireblight disease, should also be secured by showing that single-site insertion mutagenesis [39] of *E. amylovora* EA213 (or other virulent strains), which results in deletion of DHP production, is always accompanied by loss of virulence (The reverse, i.e. avirulence always accompanied with lack of DHP production, is neither observed (see EA212) nor to be expected, since DHP is only one of several virulence factors) In contrast to degradative enzymes or toxic peptides/proteins, a secondary metabolite such as DHP is coded for by more than a single gene and may thus make the genetic analysis of fireblight disease difficult Indeed, multiple virulence genes have already been identified and some of them even indicate a possible involvement of HR inducing factors [40]

EXPERIMENTAL

Organisms E. amylovora EA213 (wildtype) and EA212 (avirulent, but induces necrosis of BASC [10]) were obtained from the collection of M P Starr, Department of Bacteriology, University of California at Davis (original cultures E9 and E8, respectively, from R N Goodman, Department of Plant Pathology, University of Missouri at Columbia) Strain P66 (avirulent and non-necrotic) was obtained from F Billing, East Malling Research Station, Maidstone Kent, U.K. All strains were maintained on YGC plates

Crude toxin extract used in the BASC assay EA213 was grown on a gyratory shaker (175 rpm) at 27° in 500 ml synthetic growth medium containing sucrose (galactose) as carbon source [10] Having reached the stationary growth phase after 3 days (4 days, when galactose-grown), the bacteria were removed by centrifugation The culture filtrate was evapd to dryness and extracted with 500 ml MeOH The extract was filtered and evapd and the procedure repeated with 100 ml MeOH The final extract gave 0.962 g (1.52 g) residue, which was taken up in H_2O/1M KOH to give 10 ml (20 ml) soln of pH 5.0 (pH 5.1) 1 ml of this soln, which corresponded to 50 ml (25 ml) bacterial culture filtrate, was applied to 60 ml BASC through a sterile 0.22 μm filter The toxin extract from sucrose-grown *E. amylovora* was prepared double strength as compared to the extract from galactose-grown *E. amylovora*, because it was intended to demonstrate that the toxin would be produced in the galactose medium only, the result proved the opposite

Analysis of culture filtrates for DHP The presence of DHP in the culture filtrate from EA212 (but not from P66) axenically grown on sucrose minimal medium was tentatively concluded from a C_{18}-RP-HPLC assay (ODS-10 25 cm × 4.6 mm, ODS-5 guard column) which used an injection vol. of 580 μl culture filtrate, a flow of 2.4 ml H_2O/min and monitored UV absorbance at 210 nm, in this system DHP eluted at 9.5 min, Phe at 5.5 min and 6-TG at 4.3 min

Isolation and identification of DHP For the isolation of DHP, EA213 was grown in 70 l galactose medium as previously described [10] DHP was extracted by passage over a column of DOWEX AG50WX8 (H^+-form, 3 lb, 75 × 5 cm), washed with H_2O (10 l), and eluted with 4 l 10% NH_3 Elution of DHP was followed by spot-testing for ninhydrin-positive compounds The positive fractions were pooled, evapd to dryness *in vacuo* at 40° dissolved in 200 ml 10% NH_3, and filtered To remove 6-TG the filtrate was passed over DEAE Sephadex A25 (19 × 2 cm) Evaporation yielded a wet, yellow-green residue (7 g), which was treated with 4 × 100 ml MeOH to extract most of the ninhydrin-positive components After passage over a short silica gel column (15 × 5 cm) and evapn, *ca* 3 g 'ASI' were obtained A methanolic soln of ASI, to which some silica gel had been added, was evapd to dryness *in vacuo* and the resulting free running powder was applied as a 2 cm upper layer on top of a silica gel column (45 × 5 cm) The column was eluted with toluene–MeOH, the polarity of which was gradually increased, starting with toluene–MeOH (2:1), and the eluate collected in five fractions Fraction 1 contained 616 mg of a yellow, ninhydrin-negative oil As revealed by TLC using toluene–MeOH–HOAc–H_2O (50:50:1:1) (solvent 1), fractions 2–4 (123 mg, 231 mg, and 590 mg, respectively, of a slightly yellow solid) contained varying amounts of two ninhydrin-positive compounds, labelled ASA (brick-orange colour reaction R_f 0.38) and ASB (pink colour

reaction, R_f 0 29) Fraction 5 (700 mg of a yellow solid), eluted with MeOH, contained ASB and a third ninhydrin-positive component, labelled ASC (pink colour reaction, R_f 0 15) Fraction 5 was dissolved as much as possible in 25 ml solvent 1 and re-chromatographed over silica gel (30 × 5 5 cm) to give 102 mg ASB and 202 mg ASC. ASB was identified as valine on grounds of its $[M+H]^+$ ion (*m/z* 118) in fast atom bombardment mass spectrometry and its NMR spectra ^{1}H NMR (90 MHz, D_2O, MeOH 3 39) δ1.0 (6H, *dd*), 3 0 (1H, *d*), 2 0 (1H, *m*), ^{13}C NMR (90 MHz, H_2O, CD_3CN 119 728) δ17.349 (Q, 126 Hz), 18.727 (Q, 126 Hz), 29 812 (D, 132 Hz), 61 192 (D, 122 Hz), 174 515 (S) Fraction 3 was re-chromatographed over silica gel (32 × 5.5 cm) using $CHCl_3$–MeOH (1·1) + 1% aq. NH_3 (58%) to give 67 mg ASA CIMS (R 10,000, 10% valley, 2×10^{-5} mbar methane) found for DHP *m/z* 168 1015, calc. for $C_9H_{14}NO_2$ ($[M+H]^+$). 168.1025, found for phenylalanine *m/z* 166 0870, calc for $C_9H_{12}NO_2$ 166 0868, ^{13}C NMR (90 MHz, H_2O, CD_3CN 119 728) phenylalanine δ 37 51 (T), 57 04 (D), 130 02 (M); DHP. δ 26 97 (T, 127 Hz), 28.27 (T, 127 Hz), 39.68 (T, 130 Hz), 53.35 (D, 145 Hz), 124.80 (D (*br*), 160 Hz), 125 05 (D, 152 Hz), 129 88 (m), 175 08 (S). Additional ASA, which was contaminated with ASB, was collected as a fraction labelled ASA/B (32 mg). Pure DHP (13 mg) was obtained from fraction 2 of ASI using the RP-HPLC system described above for the analysis of culture filtrates of EA212/P66 and characterized in the following order ^{13}C NMR (90 MHz, H_2O, CDCN 119.728) δ 26 85, 28 36 (C-3′, C-6′), 39.44 (C-3); 53.28 (C-2), 124 71, 124 87 (C-4′, C-5′), 125 16 (C-2′), 129 77 (C-1′), 175 08 (C-1), diluted with H_2O to 6 3 ml, $[\alpha]_D^{20} - 54°$ (H_2O–CD_3CN, *c* 0.2), dried (for ^{1}H NMR) and weighed (for $[\alpha]$), thereafter the ^{1}H NMR showed again signals for both DHP and phenylalanine, with synthetic DHP little decomposition occurred and the following ^{1}H NMR (400 MHz, D_2O, HOD 4 69) data were obtained δ 2 36 (1 H, *dd*, J_{BX} = 9 6 Hz, J_{AB} = 14.8 Hz, H_B), 2 5–2 65 (5 H, *m*, H_A, H_2–3′, H_2–6′), 3 73 (1 H, *dd*, J_{AX} = 4 4 Hz, J_{BX} = 9 6 Hz, H_X), 5 58 (1 H, *s* (*br*), H-2′), 5 67 (2 H, *s* (*br*), H-4′, H-5′)

Toxicity of synthetic DHP towards PASC PASC was grown for 8 days on a gyratory shaker at 110 rpm, 25°, in constant light, and in the same medium as used for BASC [10], except that the 2,4-D concentration was lowered to 0 5 mg/l Starting with 1 ml aq soln containing 13 3 mg DHP, a 1 2 dilution series with H_2O gave rise to 1 ml samples containing 6 65, 3 32, 1 66, 0 83, 0 42, and 0 21 mg, respectively, of DHP, which, on day 3, were applied through sterile 0 22 μm filters to 50 ml aliquots each of PASC in 125 ml Erlenmeyer flasks Control experiments with 6 mg phenylalanine and without any additives were run in parallel. To exclude that growth inhibition of PASC was caused by microbial contamination, at the end of the experiment a loop of each culture medium was streaked onto a yeast–glucose–agar plate; no bacteria or fungi were detected within the next two weeks The packed cell volumes of the pear cells were measured by centrifugation (2000 *g*) of 15 ml PASC aliquots in conical, graduated test tubes The mean values of duplicate experiments are shown in Fig. 2 Viability of the pear cells was evaluated by microscopic examination of cell staining by Evans Blue [41] Presence of DHP in 100 μl spent PASC culture filtrate was determined in a C_{18}-RP-HPLC assay (ODS-5 25 cm × 4 6 mm, ODS-5 guard column), which used, over 40 min, a linear gradient of 5–45% MeCN in H_2O (+0 1% TFA), a flow rate of 1 ml/min, and detection at 215 nm [detection limit *ca* 0 5 μg (absolute) or 5 ppm (concentration in PASC)]; in this system DHP eluted at 11 4 min and what appeared to be phenylalanine and tetrahydrophenylalanine at 9 3 min and 15 7 min, respectively

Chemical abstracts service reference numbers are as follows (L)-2,5-dihydrophenylalanine [16055–12–2], 6-thioguanine [154–42–7], superoxide [11062–77–4]

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Note added in proof Recently, E M Steinberger and S V Beer have published their work (only briefly described in ref [40]) on transposon mutagenesis of *E amylovora* and the genetic linkage between pathogenicity and hypersensitivity determinants in. *Molecular Plant–Microbe Interactions* (1988) **1**, 135