ELSEVIER SCIENTIFIC PUBLISHERS IRELAND

Plant Science 93 (1993) 55–61

PLANT SCIENCE

Scoparone eliciting activity released by phosphonic acid treatment of *Phytophthora citrophthora* mycelia mimics the incompatible response of phosphonic acid-treated *Citrus* leaves inoculated with this fungus

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(Received 3 February 1993; revision received 23 April 1993; accepted 16 June 1993)

Abstract

Citrus leaves floating on H_3PO_3 (10 μg/ml) were protected from infection by a strain of *Phytophthora citrophthora* sensitive in vitro to H_3PO_3 (S strain). Protection was associated with significant scoparone accumulation at the infection site. On the contrary, an H_3PO_3 relatively insensitive (RI) mutant of the fungus infected leaves floating on H_3PO_3 (10 μg/ml), and did not produce scoparone accumulation. S and RI strains were infectious in leaves floating on control buffer devoid of H_3PO_3. Both scoparone accumulation and inhibition of the S strain in *Citrus* leaves floating on H_3PO_3 (10 μg/ml) were reversed by pretreatment with AOA. In vitro incubation of *P. citrophthora* mycelia in H_3PO_3 (10 μg/ml) released a significant scoparone eliciting activity from the S strain, but not from the RI strain, as tested in *Citrus* leaves. Application of the scoparone eliciting preparation of S strain towards *Citrus* leaves protected them against infection by either S or RI strain; both scoparone accumulation and leaf protection were suppressed by pretreatment of the leaves with AOA. Thus, scoparone elicitors released in vitro by H_3PO_3 (10 μg/ml) treatment of mycelia of the S strain (but not of the RI strain) of *P. citrophthora*, mimic the incompatible response observed in H_3PO_3-treated *Citrus* leaves inoculated with the S strain (but not with the RI strain) of the fungus.

Key words: Phosphonic acid; *Phytophthora citrophthora*; *Citrus*; Elicitor; Scoparone

1. Introduction

Fosetyl-Al (aluminium tris-*O*-ethylphosphonate, trade name Aliette®), has little in vitro effect on mycelial growth of most oomycetes, while controlling a number of diseases they cause in plants.

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Abbreviations: AOA, α-aminoacetic acid; CEP, crude elicitor preparation; CMA, corn meal agar: EP, eliciting preparations; MES, *N*-morpholino ethane sulfonic acid.

SSDI 0168-9452(93)03679-P

In treated plants, fosetyl-Al is degraded to phosphonic acid (H_3PO_3), which appears to be the active component involved in disease control [1].

A number of observations supported the hypothesis that host reactions are involved in the protection of plants by phosphonates [2–4], but it was also suggested that these chemicals might act through a direct toxicity towards plant pathogens [5,6].

We have shown previously that floating leaves on fosetyl-Al or H_3PO_3 prevented infection by a fosetyl-Al and H_3PO_3 sensitive S strain of *Phytophthora citrophthora*, but not infection by a mutant of the fungus relatively insensitive (RI) to fosetyl-Al and H_3PO_3 [7]. We also found that preinoculation of leaves floating on H_3PO_3 with the S strain prevented infection by the subsequently inoculated R strain. This suggested that a general resistance mechanism was induced by inoculating the S strain (but not the RI strain) of *P. citrophthora* to fosetyl-Al (or H_3PO_3) treated *Citrus* leaves.

Resistance of *Citrus* stems towards *P. citrophthora* was found to be linked to the accumulation of scoparone, a phytoalexin associated with disease resistance in *Citrus* [8]. This led us to test whether elicitors of scoparone might be associated with the different behaviour of the S and RI strains of *P. citrophthora*, when inoculated to *Citrus* leaves in the presence of phosphonic acid. Our earlier observations have shown that in vitro incubation of the myceli of *P. citrophthora* in fosetyl-Al released scoparone eliciting activity from the S, but not from the RI strain [9].

In the present paper, we investigated the possible involvement of scoparone elicitors in the incompatible response observed when *Citrus* leaves, floating on H_3PO_3, were inoculated with the S strain of *P. citrophthora*, while a compatible reaction was obtained upon inoculation of the RI strain by itself.

In order to avoid the problems linked to possible direct effects of H_3PO_3 on the leaf or on the inoculum, we choose to use the in vitro release of scoparone elicitors by *P. citrophthora* mycelia as a differential marker of the in planta effects of phosphonic acid on leaf infection by the S or the RI strain.

2. Materials and methods

2.1. Fungal isolates, plants and inoculation procedure

Two strains of *P. citrophthora*, either sensitive (S strain; EC_{50} = 6.5 μg/ml), or relatively insensitive (RI) mutant (EC_{50} = 125.3 μg/ml) [7] to fosetyl-Al and H_3PO_3, were maintained on CMA at 25°C, and were inoculated onto wounded leaves of rough lemon (*Citrus jambhiri*) [10].

Surface-sterilized leaves were wounded along the main vein and were placed to float, adaxial side up, on a solution of 10 μg/ml H_3PO_3 buffered at pH 6.2 with 0.03 M MES, with/without 300 μM AOA. This concentration of phosphonic acid was chosen as in planta, it inhibited lesion size with the S strain, but not with the RI strain of *P. citrophthora*; such inhibition was entirely reversed by pretreatment with AOA. Inoculation was performed 2 h after wounding by placing an agar disc (0.4 cm in diameter) taken from 5-day-old colonies of *P. citrophthora*, at the site of the wound. After 5 days of incubation at 25°C in the dark, the diameter of lesions was recorded.

2.2. Release of scoparone elicitors by P. citrophthora mycelia

Mycelia of the S strain (or of the RI strain) of *P. citrophthora*, were incubated in vitro for 6 days in 0.03 M MES buffer, pH 6.2, with/without 10 μg/ml H_3PO_3. The incubation fluids were filtered, treated with ethanol, and the precipitates were resuspended in 0.03 M MES (pH 6.2) containing 10 μg/ml neomycin sulfate, to obtain CEP [9]. Carbohydrate concentration of the elicitor preparations were determined as D-glucose equivalents, using the phenol-sulfuric acid technique [11].

2.3. Fractionation of CEP on a Sephacryl S-300 column

CEP (500 μl containing 500 μg glucose equivalent) was applied to a 2.5 × 28 cm column of Sephacryl S-300 (Pharmacia) equilibrated and eluted with MES 0.03 M (pH 6.2) at 1 ml/min. Fractions (0.5-ml) were collected to make the EP. Carbohydrate concentration and scoparone-eliciting activity were determined as shown below.

2.4. Assay of elicitor preparations

Leaves taken from rough lemon plants grown in a greenhouse, were surface-sterilized for 5 min in 2% NaClO, followed by 2 washes with distilled water. They were then placed, adaxial side up, on moist filter papers. A longitudinal incision (5-mm-long) was made with a scalpel along the main vein of each leaf, followed by lifting up veinal tissue and some adjacent epidermis. Twenty μl of CEP or EP (containing 20 μg glucose equivalent in MES buffer) were deposited into each leaf incision; control leaves were treated similarly with MES buffer. To test the biological effects of CEP or EP on infection, leaves were then placed to float on 0.03 M MES (pH 6.2) for 2 h, before inoculating them as described above. The diameter of lesions was recorded after 5 days in the dark.

In order to evaluate scoparone induction by CEP or EP, non-inoculated control leaves were incubated for 4 days in the dark at 25°C. A leaf disk, 2 cm in diameter, containing the wounded site and the elicitor droplet, was then excised from each site. Scoparone concentrations were determined by the method of Afek and Sztejnberg [12] and Sulistyowati et al. [13], using 1 g fresh weight of tissue collected from several treated leaves.

3. Results

Results showed scoparone accumulation (71 μg/g fresh weight) and protection against infection (lesions of 9 mm), in *Citrus* leaves floating on 10 μg/ml H_3PO_3 and inoculated with the S strain, but not in leaves inoculated with the RI mutant (Table 1).

Pretreatment with AOA had no effect on lesion size and scoparone accumulation in leaves floating on MES. However, such pretreatment sharply decreased scoparone concentration (12.6 μg/g vs. 71 μg/g) and increased lesion size (25 mm vs. 9 mm), in leaves floating on H_3PO_3 (10 μg/ml) and inoculated with the S strain, while showing no effect on scoparone concentration or lesion size in leaves inoculated with the RI strain.

Eliciting activity of CEP (adjusted at 20 μg glucose equivalent in 20 μl) was estimated by quantifying scoparone accumulation in 1-g aliquots of leaf tissue. The protective effect of CEP treatment towards subsequent infection by *P. citrophthora*, was evaluated by measuring the diameter of lesions (Table 2).

Scoparone accumulation was observed in leaves treated with CEP released upon mycelia incuba-

Table 1
Diameter of lesions and concentration of scoparone in *Citrus* leaves floating on different solutions and inoculated with *P. citrophthora* strains

Floating solution	Inoculated strain[a]			
	S		RI	
	Lesion size (mm)	Scoparone[b] concentration	Lesion size (mm)	Scoparone[b] concentration
MES buffer (0.03M, pH 6.2)	28 ± 3.1[a]	10.6 ± 0.3[a]	35 ± 3.4	9.8 ± 0.3
AOA (300 μM) in MES	27 ± 4.0	9.8 ± 1.6	33 ± 3.5	11.3 ± 0.8
H_3PO_3 (10μg/ml) in MES	9 ± 2.3	71.5 ± 14.0	29 ± 3.2	15.3 ± 7.0
H_3PO_3 + AOA in MES	25 ± 3.7	12.6 ± 2.0	28 ± 3.7	11.8 ± 1.1

[a]S, H_3PO_3 sensitive strain; RI, H_3PO_3 relatively insensitive strain. Data are the mean result of 4 independent experiments with 10 replicates ± S.D.

[b]Scoparone concentrations are expressed as μg/g fresh weight of leaf tissue. Application of MES buffer onto the wound (non-inoculated control) produced 0.1 μg/g of scoparone.

Table 2
Scoparone eliciting activity and protective effect of crude eliciting preparation (CEP) obtained by in vitro incubation of mycelia of the S strain (or RI mutant) of *P. citrophthora* with phosphonic acid

CEP[b] origin	Inoculated strain of *P. citrophthora*							
	S[a]				RI[a]			
	Without AOA		With AOA		Without AOA		With AOA	
	Lesion size (mm)	Scoparone concentration (c)	Lesion size (mm)	Scoparone concentration (c)	Lesion size (mm)	Scoparone concentration (c)	Lesion size (mm)	Scoparone concentration (c)
S (H_3PO_3)	6 ± 1.2	71 ± 11.1	25 ± 4.1	21 ± 6.4	6 ± 3.1	75 ± 10.3	30 ± 5.1	19 ± 5.4
S (MES)	30 ± 4.0	14 ± 2.1	32 ± 5.2	11 ± 3.1	33 ± 3.5	12 ± 2.4	34 ± 5.3	15 ± 3.4
RI (H_3PO_3)	28 ± 3.4	10 ± 2.3	30 ± 2.4	12 ± 2.4	33 ± 4.1	17 ± 3.4	34 ± 3.7	13 ± 2.2
RI (MES)	29 ± 2.5	10 ± 3.1	28 ± 4.1	13 ± 3.1	33 ± 3.8	15 ± 4.1	33 ± 4.2	13 ± 3.1

[a]Leaves floating on MES buffer with/without 300 μM AOA, were inoculated with the S or the RI strain of *P. citrophthora* 2 h after CEP treatment. The diameter of lesion was measured after 5 days of incubation. S, H_3PO_3 sensitive strain; RI, relatively insensitive mutant.

[b]Mycelia were incubated in MES buffer containing 10 μg/ml H_3PO_3, to yield the crude elicitor preparation (CEP). Twenty μl of CEP (containing 20 μg/ml glucose equivalent) were applied on each wound. Application of MES buffer to wounds (control) produced 0.14 μg scoparone/g fresh weight of leaf tissue (mean of 3 replicates ± S.D.).

[c]Scoparone concentration expressed as μg/g fresh weight of leaf tissue. Data are the mean of 4 independent experiments with 10 replicates ± S.D.

Table 3
Scoparone eliciting activity of different preparations made from CEP of mycelia from the S strain of *Phytophthora citrophthora* incubated or non-incubated in phosphonic acid

Eliciting preparation[a]	Mycelia incubated in MES buffer		Mycelia incubated in 10 μg/ml H_3PO_3	
	% glucose	Scoparone concentration	% glucose	Scoparone concentration
CEP	100	10 ± 0.4[b]	100	74 ± 11.3
A	12	7 ± 2.4	26	46 ± 4.2
B	29	12 ± 3.2	21	14 ± 3.8
C	14	7 ± 3.0	32	12 ± 4.6
D	16	9 ± 2.5	18	8 ± 3.4

[a]CEP was obtained by incubating the mycelia of S strain in H_3PO_3 and was then submitted to chromatography on Sephacryl S-300 column. Eighty fractions were collected and pooled to make 4 eliciting preparations (A,B,C,D) which were applied at the rate of 20 μg glucose equivalent on each wounded site of *Citrus* leaves. Scoparone concentration are expressed as μg/g fresh weight of leaf tissue.
[b]Mean of 3 replicates ± S.D.

tion of the S strain of *P. citrophthora* in H_3PO_3 (but not with a similar preparation with the mycelia of RI mutant). Correlatively, the size of lesions induced by inoculating either S or RI strain was sharply reduced by treatment of leaves with CEP of the S strain (but not with a similar preparation of the RI mutant).

Pretreatment with AOA of leaves submitted to application of CEP of the S strain, reduced scoparone accumulation to the levels found in leaves without CEP, and increased correlatively the size of the lesions formed after inoculation of either S or RI strains.

CEP was fractionated into 80 fractions by column chromatography on Sephacryl S-300. A first peak (fractions 30–40) was present only in the preparation from S strain incubated in H_3PO_3. A second peak (fractions 40–60) was observed in all 4 combinations (S or RI strain incubated in either H_3PO_3 or MES buffer). Fractions 30–40 were pooled to make preparation A, while fractions 40–60 were used to make preparations B, C and D, which were tested for their scoparone eliciting activity and their protective effect (Table 3). Scoparone accumulation was found only in leaves treated with CEP of S strain incubated in H_3PO_3 (but not with CEP of S strain incubated in MES). After chromatography on Sephacryl column, scoparone eliciting activity was mainly located in preparation A, which contained 26% of CEP glucose equivalent (Table 3). Application of fraction A (20 μg glucose equivalent) to *Citrus* leaves induced the accumulation of 46.5 μg scoparone/g fresh weight of leaf tissue (as compared to 8.3–14.2 μg scoparone/g with fractions B, C or D).

Table 4
Effect of treatment of *Citrus* leaves with different scoparone eliciting preparations, on subsequent infection by *P. citrophthora*

Inoculated strain[a]	Eliciting preparations[b]	Size of lesions (mm)	
		MES-CEP[c]	H_3PO_3-CEP[c]
S	CEP	29 ± 3.1	6 ± 1.1
	A	30 ± 4.0	7 ± 1.4
	B	29 ± 2.3	27 ± 2.5
	C	29 ± 2.1	26 ± 3.7
	D	28 ± 3.2	28 ± 3.2
RI	CEP	33 ± 2.4	5 ± 1.5
	A	32 ± 2.7	6 ± 1.7
	B	34 ± 3.4	34 ± 2.2
	C	33 ± 3.5	33 ± 3.4
	D	34 ± 2.8	30 ± 4.2

[a]Inoculation with the H_3PO_3-sensitive S strain, or with the RI relatively insensitive mutant, were performed 2 h after treatment of *Citrus* leaves with the total crude eliciting preparation (CEP), or with the different eliciting preparations (30 μg glucose equivalent).
[b]A,B,C,D,: eliciting preparations obtained after chromatography of CEP on Sephacryl S-300 column.
[c]CEP obtained by incubating mycelia of S strain of *P. citrophthora* in MES pH 6.2 amended with/without 10 μg/ml H_3PO_3.

Correlatively, treatment of wounds with CEP released by S mycelia incubated in H_3PO_3 (or with preparation A obtained after CEP chromatography on Sephacryl), greatly reduced the diameter of lesions formed by inoculating the S strain or the RI mutant of *P. citrophthora*, while preparations B, C or D had no effect on lesion size for both strains (Table 4).

4. Discussion

Afek and Sztejnberg [8] reported that the phytoalexin scoparone accumulated in *Citrus* stems inoculated with *P. citrophthora* in the presence of fosetyl-Al. Scoparone accumulation was also observed in inoculated genetically resistant varieties [4]. Besides, Saindrenan et al. [14] showed that in vitro treatment of the mycelia of *Phytophthora cryptogea* with H_3PO_3 released compounds eliciting a resistance response towards this fungus in *Vigna unguiculata*.

We found that inoculation with *P. citrophthora* of detached *Citrus* leaves floating on 10 μg/ml H_3PO_3 provides a suitable experimental model to test the relationship between scoparone accumulation and induction of resistance to this fungus. In all our experiments, incompatible reactions in planta were associated with scoparone accumulation, as shown in H_3PO_3-treated leaves inoculated with the S strain, and in CEP-treated leaves inoculated with either the S or the RI strain. Correlatively, the compatible reaction observed in planta by inoculating H_3PO_3-treated leaves with the RI strain was linked to low scoparone content. Periodate oxidation of CEP gradually decreased its scoparone eliciting capacity, together with the protection effect it induced, thus suggesting that scoparone accumulation and leaf protection are related to the release of carbohydrate elicitors [15].

The cross protection phenomenon, by virtue of which *Citrus* leaves preinoculated with the S strain of *P. citrophthora* in the presence of H_3PO_3 were protected against superinfection by the RI strain [7], was also linked to scoparone accumulation (unpublished results). All incompatible reactions became compatible when H_3PO_3-treated leaves were pretreated with AOA thus suggesting a role of the phenylpropanoid pathway in inducing the defence mechanism correlated to scoparone accumulation. Our overall results indicate that application to *Citrus* leaves of scoparone elicitors released by in vitro incubation of the mycelia of the S strain of *P. citrophthora* in 10 μg/ml H_3PO_3, mimic all in planta results obtained upon inoculation by the S or the RI strain, respectively, both in terms of compatible (or incompatible) reactions and in terms of scoparone accumulation.

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