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Mechanisms of phosphate-induced disease resistance in cucumber

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Abstract

Certain phosphate salts are known inducers of systemic acquired resistance (SAR). In the present study, a local spray application of dipotassium hydrogenphosphate (K_2HPO_4) was effective in inducing a high level of systemic protection in cucumber plants against anthracnose caused by *Colletotrichum lagenarium*. Resistance induction by $K_2HPO_4$was associated with localized cell death in cucumber leaves treated with the phosphate salt. The cell death observed, subsequently resulted in the appearance of macroscopically visible, necrotic spots. Appearing lesions resembled those provoked by tobacco necrosis virus (TNV) during a hypersensitive response (HR) that leads to pathogen-induced activation of SAR. Phosphate-mediated cell death was preceeded by a rapid generation of superoxide and hydrogen peroxide. As a further consequence of phosphate application, a local and systemic increase in free and conjugated salicylic acid (SA) levels was detected. The phosphate-induced responses were also identified with a similar time range in cucumber leaves that had been pre-inoculated with TNV. In contrast, none of these responses was triggered by application of the commercial plant activator benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester (BTH), which nevertheless was highly effective in inducing SAR in cucumber against anthracnose. In conclusion, the chemical SAR inducer K_2HPO_4 and the biological inducer TNV share some common early steps in signal transduction leading to SAR in cucumber, which differ from those involved in BTH-mediated SAR.

Abbreviations: BTH – benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester; DAB – diaminobenzidine; HR – hypersensitive response; NBT – nitroblue tetrazolium; ROS – reactive oxygen species; SA – salicylic acid; SAR – systemic acquired resistance; TNV – tobacco necrosis virus.

Introduction

Over the past twenty years, induced disease resistance was demonstrated in a number of plant-pathogen systems by using biotic and abiotic inducing agents (Sticher et al., 1997). The major hallmark of this form of resistance is the ability of plants to defend themselves against a broad spectrum of pathogens by triggering plant species-specific defence responses (Métraux, 2001). The stimulus responsible for activating resistance in plants can either evolve from contact with necrosis-promoting microorganisms and viruses or from contact with non-pathogenic rhizobacteria causing no macroscopically visible symptoms (Van Loon, 1997). To distinguish between the two forms of induced resistance, rhizobacteria-mediated resistance was called induced systemic resistance (ISR) while pathogen-induced resistance has been termed systemic acquired resistance (SAR). In the case of SAR, salicylic acid (SA) was found to be an essential signal molecule envolved in triggering defense responses and/or in sensitizing plant cells for a faster and stronger response to further pathogen attack (for review, see Mauch-Mani and Métraux,

1998). In contrast, ISR is independent of SA-mediated processes (Pieterse and Van Loon, 1999). In addition to biological induction, elicitation of SAR by certain abiotic substances is of importance for integrating the induced resistance concept in agricultural application.

Various natural and synthetic agents that showed little or no structural relationship have been described as inducers of resistance in plants. Some of these may be useful for controling plant diseases under field conditions (for review, see Yamaguchi, 1998). Among these, benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester (BTH), the active ingredient of the commercial plant activator Acibenzolar-S-methyl, was investigated for resistance induction in a number of recently published studies (Ooostendorp et al., 2001 and citations therein). In addition to synthetic organic molecules such as BTH, inorganic substances also possess inducer capacity (Sticher et al., 1997). As one example, simple salt solutions have been shown to induce systemic resistance in several plant species against various pathogens (Reuveni and Reuveni, 1998). Dibasic and tribasic sodium or potassium phosphates with a pH value above 7 were highly effective in SAR induction (Gottstein and Kuć, 1989). Foliar application of such phosphate salts induced systemic resistance in cucumber (Gottstein and Kuć, 1989; Descalzo et al., 1990; Mucharromah and Kuć, 1991), broad bean (Walters and Murray, 1992), grapevine (Reuveni and Reuveni, 1995), maize (Reuveni et al., 1994), pepper (Reuveni et al., 1998) and rice (Manandhar et al., 1998) against pathogens belonging to different taxonomic groups. In cucumber, application of phosphate led to systemic protection against eight diseases caused by fungi, bacteria and viruses (Mucharromah and Kuć, 1991).

Despite the substantial number of studies dealing with phosphate-induced resistance in plants, little information exists about the mechanisms responsible for the initiation of phosphate-mediated resistance. However, activation of SAR by local application of phosphate strongly suggests systemic signalling that could result either from a translocation of the inducer itself or from signals that are generated in phosphate-treated tissues.

In the present study, a classical SAR test system consisting of cucumber and *C. lagenarium* was used to provide insight into primary processes triggered in plants by phosphates in comparison to mechanisms initiated by the biotic inducer tobacco necrosis virus (TNV) and the chemical inducer BTH.

Materials and methods

Materials

BTH was supplied by K.-L. Nau (Syngenta Ltd., Frankfurt, Germany). All other chemicals were from Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany).

Plants and pathogens

Cucumber (*Cucumis sativus* L.) cultivar Wisconsin SMR-58 was used for all experiments. Plants were grown in a 14 h/10 h day/night cycle at 24 °C and 18 °C, respectively. A liquid fertilizer (Wuxal Super®, 0.5% v/v, Aglukon, Düsseldorf, Germany) was applied twice a week. *C. lagenarium* was maintained on potato-dextrose agar at 20 °C in the dark and transferred to fresh agar plates every 10 days. Spore suspensions were prepared as previously described (Richmond et al., 1979). TNV inoculum was taken from heavily infected cucumber leaves as described by Anfoka and Buchenauer (1997).

Inducer treatments

Chemical treatment was performed by spraying an aqueous solution of K_2HPO_4 (1–100 mM) to the first true leaf (lower and upper surface) of 4–6-week old cucumber plants. BTH (0.05 mM) was applied in the form of the commerical plant activator Acibenzolar-S-methyl (WP 50). For biological induction of resistance, corresponding leaves of cucumber plants were inoculated with TNV using Celite as abrasive according to Jeun et al. (2000). One week after inducer application, plants were challenge inoculated with *C. lagenarium* on untreated upper leaves.

Challenge inoculation with C. lagenarium

Control plants and inducer-treated plants were challenge inoculated on leaf 2 by applying 15–30 droplets of 10 µl of a conidial suspension of *C. lagenarium* with 8×10^5 conidia per ml. After inoculation, plants were kept in a humid, dark chamber for 24 h and returned to the greenhouse after a short adaption to standard conditions. Assessment of disease symptoms was performed 8–10 days after challenge inoculation by counting the number of developed lesions.

Evaluation of cell death

Histochemical detection of cell death was done by infiltrating Evans blue into cucumber leaf discs at various times after inducer application according to Schraudner et al. (1998).

Detection of reactive oxygen species

Superoxide anion (O_2^-) generation was monitored by staining leaf discs with nitroblue tetrazolium (NBT) as described by Schraudner et al. (1998). Histochemical detection of hydrogen peroxide (H_2O_2) was performed by infiltrating 3,3′-diaminobenzidine 4 HCl (DAB) according to Thordal-Christensen et al. (1997). Infiltrated leaf discs were destained in ethanol : chloroform (4 : 1) containing 0.15% (w/v) TCA (Hückelhoven and Kogel, 1998).

Quantification of salicylic acid

Leaf material from cucumber plants was extracted for SA analysis (Siegrist et al., 2000). Contents of free and conjugated SA in cucumber leaves were determined according to Mölders et al. (1996), with modifications as described by Siegrist et al. (2000). SA content was quantified by HPLC (Pharmacia, Uppsala, Sweden) using a fluorescence detector (LC 304, Linear Instruments, Reno, USA). Recovery rates were determined by addition of SA standards to blank samples. SA levels of test samples were corrected respectively.

All experiments in the present study were repeated at least three times.

Results

Induction of systemic acquired resistance in cucumber by K_2HPO_4

A local spray application of 100 mM K_2HPO_4 was effective in inducing SAR in cucumber plants against anthracnose caused by *C. lagenarium* (Table 1) and powdery mildew caused by *Sphaerotheca fuliginea* (data not shown). Systemically induced resistance to anthracnose was evident at concentrations above 10 mM with maximum effects obtained between 50 and 100 mM (Figure 1). The level of protection that was achieved by treatment with K_2HPO_4 was similar to the one detected after biotic induction with TNV, whereas application of the chemical SAR inducer BTH resulted in complete resistance against *C. lagenarium* (Table 1).

Table 1. Effect of various inducers on the expression of systemic resistance in cucumber plants to *C. lagenarium*. Resistance was assessed by inoculating an upper leaf (leaf 2) with a conidial suspension (15 droplets) of *C. lagenarium*, 7 days after inducer application to leaf 1. (Necrotic lesions were counted 10 days after inoculation.)

Treatment	*Necrotic lesions*[a] (no.)
None (control)	14.9 ± 1.6
K_2HPO_4 (100 mM)	4.8 ± 2.3*
TNV	2.3 ± 1.6*
BTH (0.05 mM)	0[b]

*Means significantly different from control.
[a]Values are the means ± SD from a typical experiment with 5 plants per treatment.
[b]No disease symptoms.

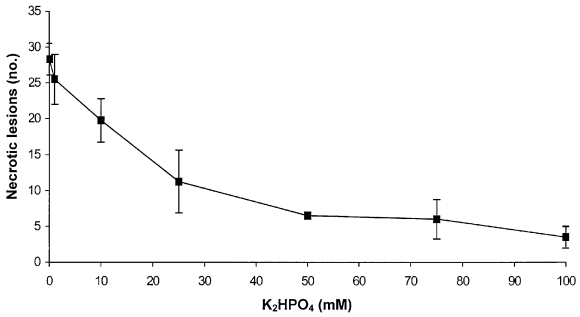

Figure 1. Dose response relationship of phosphate-mediated SAR in cucumber against anthracnose. Cucumber plants treated on leaf 1 with the indicated concentrations of K_2HPO_4 were challenge inoculated 7 days later on leaf 2 with 30 droplets of a conidial suspension from *C. lagenarium.* Necrotic lesions were counted 10 days after fungal inoculation. Values are means ± SD from a representative experiment with five plants per treatment.

Phosphate application causes localized cell death

Foliar application of K_2HPO_4 to cucumber plants led to the appearance of HR-like lesions on treated leaves. Necrotic spots, indicating cell death, were macroscopically visible within 48 h after phosphate treatment, especially on the adaxial leaf surface, and subsequently increased in size within the next days (not shown). The number of lesions appearing on cucumber leaves after phosphate treatment was strictly dependent on the concentration of the inducer (Figure 2) and seems to

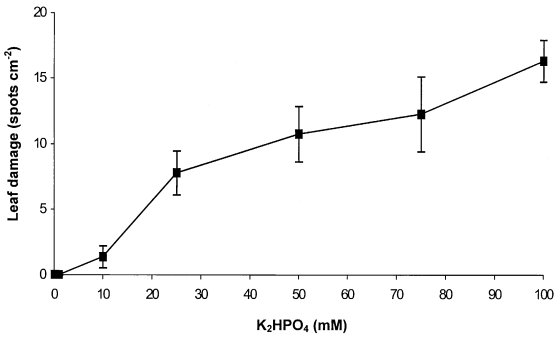

Figure 2. Determination of leaf damage caused by foliar applied K_2HPO_4. The number of chlorotic and necrotic spots on cucumber leaves was assessed 7 days after phosphate application. Values are the means ± SD from a representative experiment with five plants per treatment.

be correlated with the observed level of protection. It was found that phosphate concentrations of more than 10 mM were necessary for both triggering SAR and cell death (Figures 1 and 2). A nearly similar phenotype of necrotic spots also appeared after inoculation of cucumber leaves with the biotic inducer TNV (not shown). In contrast to K_2HPO_4 and TNV, cell death was not detected after application of 0.05 mM BTH (not shown).

Staining with Evans blue indicated a rapid initiation of cell death in cucumber leaves after phosphate application. Blue coloured spots appeared within 24 h and expanded up to 48 h after inducer treatment (Figure 3a). Histochemically assayed cell death was also evident in leaf tissues of cucumber plants that have been inoculated with the biotic inducer TNV, but not after application of BTH (Figure 3b).

Phosphate-induced generation of reactive oxygen species

Cell death in plant tissues is often correlated with the appearance of reactive oxygen species (ROS) (Jabs, 1999). Histochemical staining methods for the detection of O_2^- and H_2O_2, using NBT and DAB respectively, revealed that phosphate-mediated cell death was accompanied by a rapid generation of ROS. Accumulation of O_2^- was detected within 6 h after phosphate application and appeared more or less diffuse (Figure 4a). In contrast, H_2O_2 accumulation was demonstrated with a delay of about 3 h and proceeded for up to 72 h after phosphate treatment (Figure 5a). Accumulation of H_2O_2 was localized to regions where clusters of blue spots could be detected by staining with Evans blue (Figures 3a and 5a). Generation of ROS also occurred as a consequence of TNV inoculation, but was not seen after application of BTH (Figures 4b and 5b).

Phosphate-induced local and systemic accumulation of salicylic acid

SA is known to play a crucial role in SAR activation by cell death-promoting microorganisms and viruses (Mauch-Mani and Métraux, 1998). To estimate whether phosphate-mediated cell death also correlates with the accumulation of SA, the content of free and glucosylated SA in cucumber plants was determined in time course studies following phosphate application. Increased levels of free SA were found in phosphate-treated (local) and non-treated (systemic) cucumber leaves after spraying with K_2HPO_4 (Figure 6a,b). Forty-eight hours after phosphate application, the content of free SA was 145-fold higher in treated (local) and 16-fold higher in non-treated (systemic) leaves of induced plants compared to control plants. At the same time, the content of glucosylated SA was also markedly increased in treated (local) and non-treated (systemic) leaves of cucumber plants that had been sprayed with K_2HPO_4 (Figure 6c,d). However, at later time points no further increase of SA contents was detected in phosphate-treated plants (data not shown).

For comparison of the ability of different inducers to trigger accumulation of SA, the total SA contents (the sum of free and conjugated SA) were determined 72 h after application of BTH, K_2HPO_4 and after TNV inoculation. As shown in Figure 7, inoculation with TNV caused a massive accumulation of SA in inoculated and non-inoculated leaves of cucumber plants. Compared to the control, a 112-fold higher content of total SA was measured locally, whereas the systemic increase was about 6-fold. In contrast, SA contents in cucumber plants treated with the plant activator BTH remained at the level of the untreated control.

Discussion

Induced resistance is a principle that ideally fits in with environmental friendly plant protection strategies (Lyon and Newton, 1997). So far, only a limited number of inorganic, natural organic or synthetic compounds are known inducers of disease resistance in

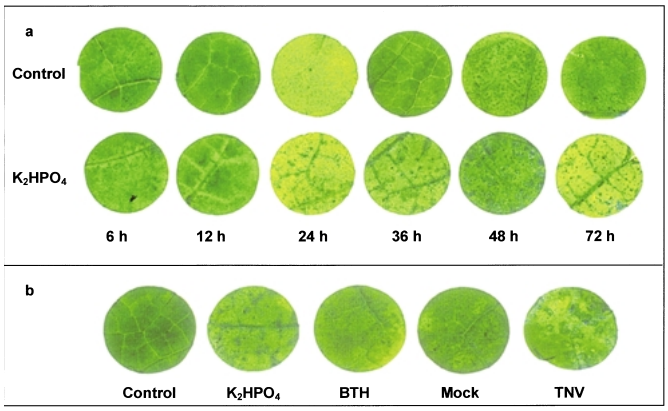

Figure 3. Histochemical detection of cell death by Evans Blue staining. (a) Time course of cell death appearance in cucumber tissue after application of 100 mM K_2HPO_4. (b) Cell death in cucumber tissue 48 h after application of 100 mM K_2HPO_4, 0.05 mM BTH, inoculation with TNV or treatment with abrasive (mock).

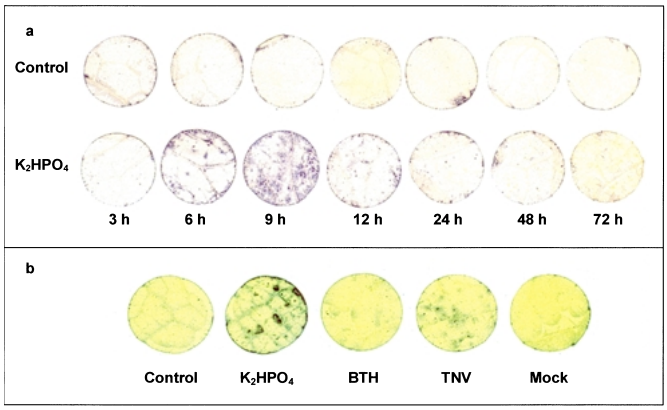

Figure 4. Histochemical detection of O_2^- by NBT staining. (a) Time course of O_2^- appearance in cucumber tissue after application of 100 mM K_2HPO_4. (b) Staining of O_2^- in cucumber tissue 9 h after application of 100 mM K_2HPO_4, 0.05 mM BTH, inoculation with TNV or treatment with abrasive (mock).

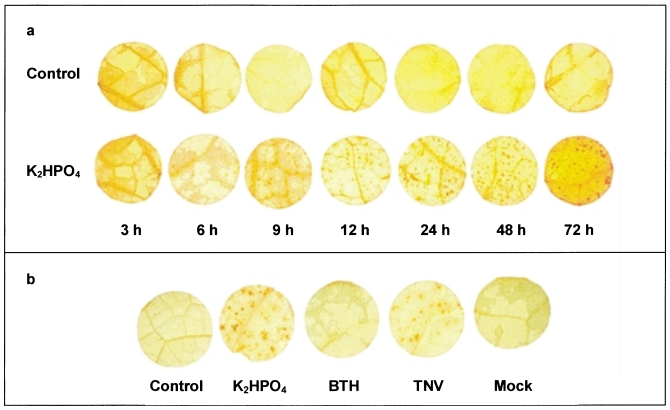

Figure 5. Histochemical detection of H_2O_2 by DAB staining. (a) Time course of H_2O_2 appearance in cucumber tissue after application of 100 mM K_2HPO_4. (b) Staining of H_2O_2 in cucumber tissue 48 h after application of 100 mM K_2HPO_4, 0.05 mM BTH, inoculation with TNV or treatment with abrasive (mock).

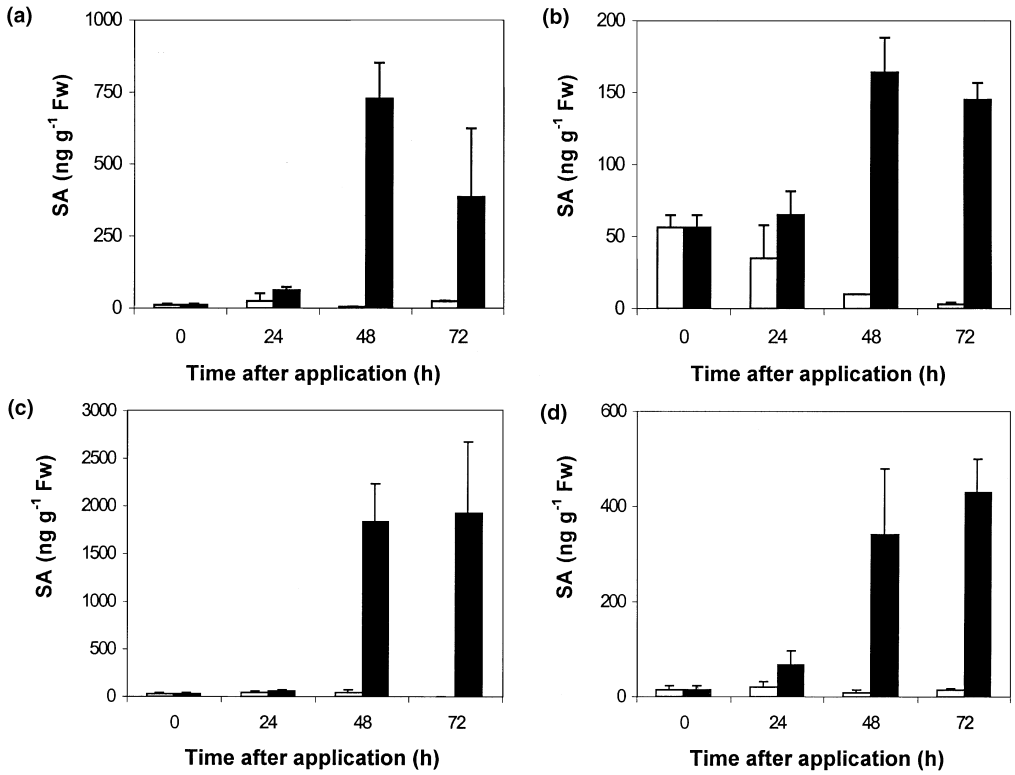

Figure 6. Time course of local and systemic accumulation of SA in cucumber plants sprayed on the first leaf (leaf 1) with 100 mM K_2HPO_4 (■) compared to untreated control plants (□). (a) Contents of free SA in treated leaf tissues (leaf 1). (b) Contents of free SA in untreated leaf tissues (leaf 2). (c) Contents of conjugated SA in treated leaf tissues (leaf 1). (d) Contents of conjugated SA in untreated leaf tissues (leaf 2). Data represent the means ± SD of a typical experiment with five plants per treatment.

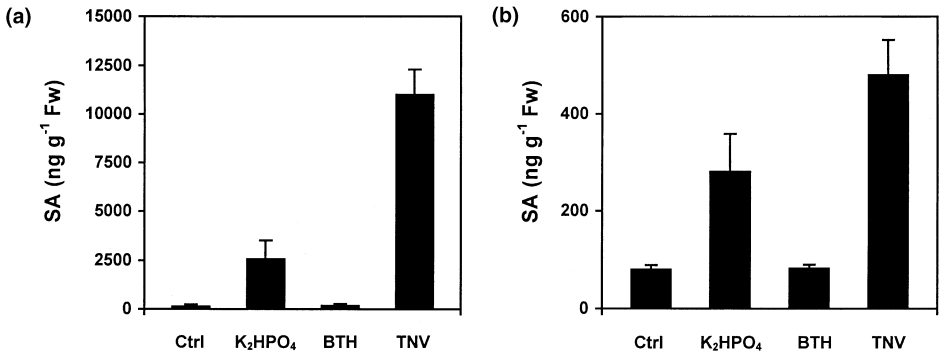

Figure 7. Local and systemic accumulation of SA in cucumber plants sprayed with K_2HPO_4 (100 mM), BTH (0.05 mM) or inoculated with TNV. (a) Total SA contents (free + conjugated SA) in treated leaf tissues (leaf 1), 72 h after inducer application. (b) Total SA contents (free + conjugated SA) in untreated leaf tissues (leaf 2), 72 h after application of the inducers to leaf 1. Data represent the means ± SD of a typical experiment with five plants per treatment.

plants (Sticher et al., 1997; Yamaguchi, 1998) and little is known about their mode of action (Ryals et al., 1996). Phosphate salts are one example of chemicals which exhibit a distinct potency for resistance induction, especially SAR, in a number of plant species against various pathogens. However, most of the reported studies on phosphate-induced resistance were exclusively of a descriptive nature, so that the mechanisms

involved in the initiation of SAR by phosphates are still unknown.

According to Gottstein and Kuć (1989), a pH value of phosphate solutions above 7.0 is required for sucessful induction of SAR in cucumber against *C. lagenarium*. This was demonstrated by applying the acidic phosphate salt KH_2PO_4which even at high concentrations, exhibited no resistance inducing activity (Gottstein and Kuć, 1989). Besides cucumber, induction of resistance by basic phosphates has also been demonstrated in rice (Manandhar et al., 1998) and broad bean (Walters and Murray, 1992) whereas the protection of maize against rust was not related to the pH of the phosphate salt solution (Reuveni et al., 1994). However, even in cucumber, a basic pH solution alone seems to be insufficient for triggering SAR since KOH was not effective in inducing resistance against *C. lagenarium* in this plant (Gottstein and Kuć, 1989).

The first study on phosphate-mediated SAR in cucumber revealed that induction of resistance was closely associated with the appearance of chlorotic stippling or restricted necrosis on the inducer leaves (Gottstein and Kuć, 1989). Moreover, the necrotic reaction was assumed to be a requirement for successful activation of defence in cucumber induced by phosphate (Mucharromah and Kuć, 1991). The results of the present study indicate that the initiation of cell death is a central event in the response of cucumber tissue to K_2HPO_4. This was clearly demonstrated by the close relationship between the dose-dependent appearance of necrotic lesions on phosphate-treated leaves and the level of systemic protection. The rapid initiation of cell death by K_2HPO_4 was accompanied by an oxidative burst indicating that a HR-like response was triggered after application of the chemical inducer. Besides phosphate, other cell death-promoting chemicals with the ability to induce disease resistance in plants have been described. In cucumber, treatment with oxalic acid, a compound known to act as a cell death-inducing, non host-selective toxin (Zhou and Boland, 1999), was also effective in inducing SAR against various pathogens (Mucharromah and Kuć, 1991). In tobacco, enhanced resistance against tobacco mosaic virus induced by β-aminobutyric acid was closely associated with cell death, oxidative burst and lipid peroxidation (Siegrist et al., 2000). In potato, treatment with arachidonic acid induced localized cell death as well as systemic resistance against *Phytophthora infestans* (Coquoz et al., 1995). As a further interesting feature, pro-oxidative herbicides like paraquat and glufosinate ammonium, which also cause local cell death, were found to be effective inducers of SAR in various plants (Strobel and Kuć, 1995; Siegrist et al., unpublished). In contrast, unspecific lesion formation in plant tissues such as a dry ice treatment or mechanical damage, did not result in the activation of plant defense against pathogens (Madamanchi and Kuć, 1991).

Although the primary mode of phosphate action is not known, it can be expected that the rapid initiation of an oxidative burst and of cell death are essential steps for successful activation of SAR in cucumber plants by K_2HPO_4. How these cellular reactions might be triggered is not clear. It was speculated that basic phosphates applied at concentrations in the millimolar range could sequester apoplastic calcium (Gottstein and Kuć, 1989). As a consequence of calcium complexation, membrane integrity might be influenced as well as the activity of apoplastic enzymes, such as polygalacturonases, which could release elicitor-active oligogalacturonides from plant cell walls. Evidence for such a mechanism can be deduced from results published by Walters and Murray (1992). The authors showed that the calcium-chelating agent EDTA was able to induce systemic resistance in broad bean against rust caused by *Uromyces viciae-fabae.* On the other hand, application of $CaNO_3$ after phosphate treatment prevented induction of resistance against the rust fungus (Walters and Murray, 1992).

Besides localized cell death, rapid accumulation of SA was detected in phosphate-treated cucumber leaves. Contents of free and bound SA started to increase when signs of cell death were already noticed. The time course of phosphate-induced local and systemic SA accumulation was comparable to that detected after inoculation of the leaves with TNV (Mölders et al., 1996). This finding provides further evidence that the phosphate-mediated processes are closely related to mechanisms triggered by cell death-inducing viruses, bacteria and fungi during a HR that results in biotic SAR activation. Similar results were recently obtained in tobacco plants which exhibited an enhanced resistance against tobacco mosaic virus after a treatment with β-aminobutyric acid (Siegrist et al., 2000). More arguments for the strong relationship between cell death initiation, accumulation of SA and SAR by certain chemicals can also be deduced from experiments with the herbicides paraquat and glufosinate ammonium. An application of these chemicals to cucumber leaves was found to induce SA accumulation (Orober et al., unpublished results) as well as SAR (Strobel and Kuć, 1995; Siegrist et al., unpublished results). Furthermore, oxygen species-generating agents such

as Rose Bengal also induce cell death, SA biosynthesis and SAR when sprayed on plants (Enyedi, 1999). As another example, harpin, a protein from *Erwinia amylovora* which is known to act as an HR-elicitor (Wei et al., 1992) also induces disease resistance in a SA-dependent fashion (Dong et al., 1999).

In conclusion, our present data strongly suggest that foliar application of K_2HPO_4 to cucumber plants results in the activation of mechanisms resembling those initiated by necrotizing microbes and viruses that trigger SAR.

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