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Dissecting the phosphite-induced priming in Arabidopsis infected with 
Hyaloperonospora arabidopsidis

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SUMMARY

Phosphite (Phi), a phloem-mobile oxyanion of phosphorous acid (H₃PO₃) protects plants against diseases caused by oomycetes. Its mode of action is unclear as evidence indicates both direct antibiotic effects on pathogens as well as inhibition through enhanced plant defense responses, and its target(s) in the plants is unknown. Here we demonstrate that the biotrophic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) exhibits an unusual biphasic dose-dependent response to Phi after inoculation of *Arabidopsis* (*Arabidopsis thaliana*), with characteristics of indirect activity at low doses (≤ 10 mM) and direct inhibition at high doses (≥ 50 mM). The effect of low doses of Phi to *Hpa* infection was nullified in salicylic acid (SA) defective plants (*sid2-1*, NahG), and in a mutant impaired in SA signaling (*npr1-1*). Compromised jasmonate- (*jar1-1*), ethylene- (*ein2-1*) signaling or abscisic acid- (*aba1-5*) biosynthesis, reactive oxygen generation (*atrbohD*), or accumulation of the phytoalexins camalexin (*pad3-1*) or scopoletin (*f6’h1-1*) did not affect Phi activity. Low doses of Phi primed the accumulation of SA and *PR1* transcripts and mobilized two essential components of basal resistance, EDS1 and PAD4, following pathogen challenge. Compared to inoculated, Phi-untreated plants, gene expression, accumulation and phosphorylation of the mitogen-activated protein kinase MPK4, a negative regulator of SA-dependent defenses, were reduced in plants treated with low doses of Phi. We propose that Phi negatively regulates MPK4, thus priming SA-dependent defense responses following *Hpa* infection.
INTRODUCTION

Plants deploy an innate immune system including an array of pre-formed barriers and inducible responses for defense against invading pathogens. A type of induced resistance (IR) is the systemic acquired resistance (SAR) found in adjacent and distal plant parts after infection by a necrotizing pathogen, and requires salicylic acid (SA) (Delaney et al., 1994) and the presence of the defense regulatory protein NPR1 (Nonexpressor of Pathogenesis-Related protein 1) (Durrant and Dong, 2004). An other type of IR is induced by non-pathogenic growth-promoting rhizobacteria and is called induced systemic resistance (ISR) that requires jasmonate (JA) and ethylene (ET) (Van Loon et al., 1998).

IR is often associated with the priming phenomenon, the augmented capacity to mobilize cellular defense responses following challenge by a broad spectrum of pathogens (Conrath et al., 2002; Conrath, 2011). Thus, inoculation of Arabidopsis thaliana leaves with the avirulent strain of Pseudomonas syringae pv. tomato (Pst) DC3000 carrying the AvrRpm1 gene, primes defense responses to subsequent challenge by the virulent strain Pst DC3000 (Kohler et al., 2002). Priming is also effective in plants after root inoculation with beneficial rhizobacteria (Van Wees et al., 2000; Conrath et al., 2002; Verhagen et al., 2004). Similarly, treatments with natural or synthetic compounds enhance resistance responses only after pathogen challenge. Treatments of plants with SA, riboflavin (vitamin B2), thiamin (vitamin B1), menadione, 2,6-dichloroisonicotinic acid (INA), benzo(1,2,3)thiadiazole-7-carbothioc acid S-methyl ester (BTH), the non-protein amino acid β-aminobutyric acid (BABA) or phosphite (Phi) have been shown to prime defenses for augmented responses to pathogens (Eshraghi et al., 2011; Kauss et al., 1992; Kauss and Jeblick, 1995; Katz et al., 1998; Zimmerli et al., 2000; Ahn et al., 2007; Borges et al., 2009; Zhang et al., 2009). Numerous studies have attempted to decipher the molecular components of defense priming (Zimmerli et al., 2000; Kohler et al., 2002; Ton et al., 2005; Zhang et al., 2009). BABA has been shown to prime defenses against Hyaloperonospora arabidopsis (Hpa) (formerly Peronospora parasitica or Hyaloperonospora parasitica) through an SA- and NPR1-independent signaling pathway (Zimmerli et al., 2000; Ton et al., 2005). BABA-IR involves increased deposition of callose at the site of attempted infection (Zimmerli et al., 2001; Ton and Mauch-Mani, 2004). However, the biochemical and molecular mechanism(s) of priming remained poorly understood until fairly recently. Beckers et al. (2009) demonstrated that defense priming resulted from the accumulation of inactive mitogen-activated protein kinases (MAPKs) following pathogen challenge, leading to development of systemic immunity (Beckers et al., 2009). The MAPK cascade involves three functionally linked protein kinases, a MAP kinase
kinase kinase (MEKK), a MAP kinase kinase (M KK), and a MAP kinase (MPK) (Colcombet and Hirth, 2008). The elevated accumulation of inactive forms of the MAP kinases MPK3 and MPK6 in Arabidopsis exposed to BTH was proposed as a possible priming mechanism (Beckers et al., 2009). Chromatin modifications and alteration of primary metabolism have also been shown to be linked to priming (Jaskiewicz et al., 2011; Schmitz et al., 2010).

Phi ($\text{HPO}_3^{2-}/\text{H}_2\text{PO}_3^-$), an oxyanion of phosphorous acid ($\text{H}_3\text{PO}_3$), is the reduced form of phosphate (Pi), and as such may be considered as a structural analog of Pi. Phi, a phloem-mobile molecule, is especially effective against oomycete diseases (Guest and Bompeix, 1990; Guest and Grant, 1991). The mode of action of Phi is still unknown and debated. At high concentrations, Phi inhibits mycelial growth of Phytophthora spp. through direct toxicity (Fenn and Coffey, 1984) by inhibiting key phosphorylation reactions (Niere et al., 1994). Phi also activates plant defense responses (Saindrenan and Guest, 1994). Phi-induced resistance in cowpea (Vigna unguiculata) infected with P. cryptogea was suppressed by the application of a competitive inhibitor of phenylalanine-aminon glycine and isoflavonoid phytoalexin biosynthesis (Saindrenan et al., 1988). Similarly, Phi-induced resistance and localized cell death are inhibited by quenchers of superoxide anion ($\text{O}_2^-$) in Phi-pretreated Arabidopsis seedlings inoculated with zoospores of P. palmivora (Daniel and Guest, 2006). Phi was recently shown to directly enhance expression of defense genes and to prime callose deposition and hydrogen peroxide ($\text{H}_2\text{O}_2$) accumulation in Arabidopsis infected with P. cinnamomi (Eshraghi et al., 2011). All these results only provide correlative evidence for an indirect mode of action of Phi and there is still no conclusive evidence on the mode of action of Phi, and its potential target(s) in the plant.

The Hpa-Arabidopsis pathosystem is a suitable model to unravel the physiological and molecular mechanism(s) underlying Phi-IR. Effector-triggered immunity (ETI) in Arabidopsis against Hpa is mediated by the isolate-specific resistance genes RPPs through SA-dependent or -independent pathways depending on the isolate used (McDowell et al. 2000; van der Biezen et al., 2002). Among the early signaling events and cellular responses in plants acting downstream of pathogen-associated molecular pattern (PAMPs) or avirulence gene is the activation of the MAPK cascade (Asai et al., 2002; Nakagami et al., 2005). Thus, resistance to the Hpa avirulent isolate Hiks 1 was compromised in MPK6-silenced Arabidopsis Col-0 plants (Menke et al., 2004) and overexpression of the M KK7 gene enhanced resistance to the Hpa isolate Noco2 (Zhang et al., 2007). Reactive oxygen species (ROS) are part of the early components of defense responses in Arabidopsis. The plasma
membrane NADPH oxidase enzyme ATRBOHD was shown to be responsible for a stronger oxidative burst in ETI triggered by the Hpa isolate Emco5 (Torres et al., 2002). SA is a component essential in ETI and in basal resistance as the SA-deficient mutant sid2-1 (Nawrath and Métraux, 1999) and the NahG plants are impaired in resistance to virulent and avirulent isolates of Hpa. In addition, ETI as well as basal defense against Hpa in Arabidopsis involves EDS1 (Enhanced Disease Susceptibility 1) and its interactor PAD4 (Phytoalexin Deficient 4), two lipase-like proteins that are required for SA accumulation (Jirage et al., 1999; Feys et al., 2001), as well as NPR1 activation and accumulation of the Pathogenesis-Related protein, PR1 (Durrant and Dong, 2004).

This study aims to dissect the mechanism(s) underlying Phi-IR in Arabidopsis against Hpa. Here we show that Phi exerts a dual activity in a concentration-dependent manner with either a priming-inducing activity in the plant or direct inhibition of the pathogen. At low concentrations (≤ 10 mM), Phi primes defense responses against infection of Arabidopsis Col-0 by the normally virulent Hpa Noco2. Phi-induced priming depends on SA signaling and mobilizes EDS1-PAD4 and NPR1, but is independent of other known pathways of hormone signal transduction. Finally, we identify Phi as a negative regulator of the MAP kinase MPK4 and present evidences that decreased gene expression, accumulation and phosphorylation of this kinase are part of Phi-induced priming against Hpa in Arabidopsis.

RESULTS

Phosphite pretreatment renders Arabidopsis more resistant to H. arabidopsidis

We inoculated Phi-pretreated A. thaliana ecotype Col-0 with the virulent Hpa isolate Noco2 to assess the protective effect of Phi. The avirulent isolate Emwa1 interacts with Col-0 through the RPP4 resistance gene (van der Biezen et al., 2002) and was used to compare genetic and chemical-induced resistance. Plants were treated by soil-drenching with Phi (5 to 100 mM) or with MES (mock) 72 h prior to inoculation with Hpa spores. Susceptibility was assessed by counting the number of pathogen spores formed on the leaf surface and by microscopically examining hyphal growth in inoculated leaves 7 days post-inoculation (dpi). There was no difference in the germination rate of inoculated spores on leaves of Phi-pretreated plants compared to mock-pretreated plants (data not shown). Sporulation of Hpa was not affected by 5 mM Phi treatment, whereas 10 mM and 25 mM Phi-treatments proportionately decreased spore numbers, and 50 mM Phi or more inhibited sporulation as much as in the incompatible interaction with the avirulent Emwa1 (Fig. 1A). The effect of
Phi-treatments on hyphal growth in leaf tissues closely matched the reduction in *Hpa* spore production (Fig. 1B), indicating that spore number gives a valid estimate of susceptibility. Soil-drenching with 10 mM Phi as soon as 24 h before *Hpa* inoculation reduced spore number by 35% compared to mock-inoculated plants, but the same concentration applied simultaneously to inoculation or 24 h post-inoculation (hpi), had no effect (Supplemental Fig. S1). This indicates that Phi does not exhibit any curative effect once the infection is established in leaf tissues.

**The inhibition of *H. arabidopsidis* by Phi exhibits a biphasic dose-dependent response curve**

The effectiveness of Phi on *Hpa* Noco2 infection was analysed in more detail at concentrations of 5 to 50 mM. Plants were inoculated 72 h after soil-drenching with Phi and spore number determined 7 dpi. Response curves were derived from percentage inhibition of sporulation at each concentration of Phi relative to mock-pretreated plants. The dose-dependent response curve appears biphasic (Fig. 2). In the first phase, sporulation was inhibited linearly from 5 to 12.5 mM Phi, then stabilised between 12.5 to 22.5 mM at ~ 43% inhibition across the range of doses. In the second phase, inhibition increased linearly to 97% from 22.5 to 50 mM Phi, consistent with a direct dose-related response to a toxicant. This biphasic dose-response suggests the additive effect of independent responses to Phi. Thus, in contrast to most conventional fungicides, Phi exhibits a dual mode of action.

**Phi effect at 10 mM is abolished in Arabidopsis plants defective in SA signaling, but not in mutants impaired in jasmonate-, ethylene-dependent signals and abscisic acid biosynthesis**

Mutants or transgenic plants impaired in signal transduction and biosynthesis pathways involved in IR were used to investigate the role of Phi-induced plant defenses in the inhibition of *Hpa* Noco2. The effectiveness of Phi against *Hpa* was first tested in the SA-deficient mutant *sid2-1* (Nawrath and Metraux, 1999) treated with 10 mM Phi (Fig. 3), a dose that inhibited sporulation by 35% compared to mock-pretreated plants (Fig. 2). Spore number on *sid2-1* mutant was two-fold higher (1200 ±48 spores mg⁻¹ FW) than on wild type (WT) plants (610±30 spores mg⁻¹ FW) (data not shown), confirming that suppression of SA production in Col-0 affects basal resistance to *Hpa* isolate Noco2. More importantly, 10 mM Phi failed to reduce spore production on *sid2-1* mutant, indicating that SA signaling is essential for the 35% reduction in sporulation afforded by Phi in Col-0 WT (Fig. 3).
NPR1 regulates SA-induced defenses downstream of SA and upstream of PR genes (Cao et al., 1994). Phi-treatment reduced spore number by only 4.6% in npr1-1 compared to 35% in WT (Fig. 3). The response to 10 mM Phi treatment in the mutants jar1-1, ein2-1 and aba1-5 impaired in ethylene (ET), jasmonate (JA) signaling and abscisic acid (ABA) biosynthesis, respectively, was not compromised (Fig. 3), indicating that Phi-IR is independent of JA, ET signaling and ABA biosynthesis.

Overall, these findings indicate that 10 mM Phi partly protects Arabidopsis against Hpa through SA- and NPR1-dependent defense mechanisms. In NahG plants, the effect of 10 mM Phi is also totally suppressed, whereas it is only partially abolished at 25 mM (Supplemental Fig. S2), suggesting that protection afforded at this concentration partly resulted from SA-independent factors. The response to increasing Phi was linear until 50 mM (Supplemental Fig. S2), when sporulation was inhibited at similar levels in WT and NahG plants, indicating a direct toxicity to the pathogen. Unless otherwise stated, plants were treated with Phi at 10 mM throughout the following experiments.

**Phi primes SA accumulation and PRI expression**

Priming only becomes apparent after pathogen challenge and may be monitored using markers of defense-associated cellular events (Conrath et al., 2002). As the SA-signaling pathway was shown to be involved in the Phi-mediated protection of Arabidopsis against Hpa Noco2, we analysed the impact of Phi treatment on accumulation of free and total SA, and on the expression of PRI, two important defense responses in our model system. No significant difference was observed in free and total SA levels, between mock- and Phi-pretreated plants before inoculation, (Supplemental Fig. S3). However, the levels of free SA detected in Phi-pretreated plants were significantly higher than in mock-pretreated plants 24, 48 and 72 hpi (Fig. 4A). Total SA contents in Phi-pretreated plants were also significantly higher than those in mock-pretreated plants 24 and 48 hpi (Fig. 4B). Phi-pretreated plants showed higher PRI transcription earlier than in mock-pretreated plants, but levels were similar to mock-pretreated plants 72 hpi (Fig. 4C). PDF1-2, a biochemical marker of JA- and ET-induced defense responses against necrotrophs (Penninckx et al., 1996; Thomma et al., 1998) was weakly but not differentially induced in mock- and Phi-pretreated plants (Supplemental Fig. S4), confirming that Phi-IR is independent of JA/ET signaling in this pathosystem. Overall, these results indicate that Phi primes SA-dependent defenses for augmented responses against Hpa.

**Phi-induced priming is independent of phytoalexins and ATRBOHD-dependent ROS**
ROS produced by the membrane-associated ATRBOH NADPH oxidases play important roles in plant defense and are an early response to penetration of the Arabidopsis epidermis by an avirulent isolate of *Hpa* (Torres et al., 2002; Slusarenko and Schlaich, 2003). However, *atrbohD* mutants were equally responsive to 10 mM Phi as WT plants (Fig. 5A), suggesting that Phi-IR is independent of ROS produced by the membrane-bound ATRBOHD. As phytoalexins have been shown to be essential to Phi activity in other plant-oomycete interactions (Nemestothy and Guest, 1990; Saindrenan et al., 1988), we then examined the involvement of camalexin and scopoletin in Phi-induced priming using *pad3-1* (*phytoalexin deficient3-1*; Böttcher et al., 2009) and *f6′h1-1* (*feruloyl-CoA 6′hydroxylase1-1*; Kai et al., 2008) mutants. Phi-IR was not compromised in *pad3-1* and *f6′h1-1* mutants as in Col-0 WT plants (Fig. 5A), nor were levels of camalexin and scopoletin accumulation affected by Phi-treatment, either before or after inoculation with *Hpa* (Fig. 5B and C). These results indicate that ATRBOHD-dependent ROS, camalexin or scopoletin are not components of Phi-induced priming against *Hpa* infection in Arabidopsis.

**Phi primes enhanced expression of PAD4 and EDS1**

The interacting PAD4 and EDS1 proteins function upstream of SA and are required for SA signaling in ETI and basal resistance (Falk et al., 1999; Jirage et al., 1999; Brodersen et al., 2006), particularly in response to *Hpa* (Parker et al., 1996). Mutation in *PAD4* impaired response of Arabidopsis to Phi treatment after pathogen challenge indicating that *PAD4* is necessary for full Phi-IR (Supplemental Fig. S5). We monitored *PAD4* and *EDS1* expression in mock- and Phi-pretreated plants before and after inoculation with Noco2 using Emwa1-inoculated plants as positive controls. Levels of *PAD4* and *EDS1* transcripts were calculated relative to their expression at the time of Phi treatment, i.e. 72 h before inoculation with *Hpa* (Fig. 6A and B). *EDS1* and *PAD4* were similarly expressed at the time of inoculation (0 hpi) in mock- and Phi-pretreated plants. Phi treatment enhanced expression levels of *PAD4* and *EDS1* 24 hpi, similar to those observed in Arabidopsis inoculated with *Hpa* Emwa1. The data indicate that *PAD4* and *EDS1* genes are primed upon Phi treatment.

**Phi negatively regulates MPK4 transcription, MPK4 protein accumulation and phosphorylation**

A genetic interaction between EDS1-PAD4 and the mitogen-activated protein kinase MPK4 regulates defenses against biotrophs (Brodersen et al., 2006). Loss-of function *mpk4* mutants exhibit high levels of SA and *PRI* transcripts, and have increased resistance to the virulent
pathogens *Pst* DC3000 and *Hpa* Noco2 (Brodersen et al., 2006). *MPK4* expression was the same in mock- and Phi-pretreated plants 8 and 24 h before inoculation and at the time of inoculation (Supplemental Fig. 6). Inoculation with *Hpa* induced similar levels of *MPK4* expression 8 hpi in mock- and Phi-pretreated plants (Fig. 7A), but by 10 hpi *MPK4* expression had declined in Phi-pretreated plants relative to mock-pretreated plants (Fig. 7A). Accumulation and phosphorylation state of *MPK4* were monitored by western blotting leaf extracts with α-MPK4 and α-p44/42-ERK antibodies, respectively. *MPK4* was present in similar levels in Phi- and mock-pretreated plants at 0 hpi (Fig. 7B). Importantly, upon challenge with the virulent pathogen, *MPK4* levels significantly decreased 8 and 10 hpi in Phi-pretreated plants. Phosphorylation of *MPK4* was enhanced after inoculation in both mock- and Phi-pretreated plants (Fig. 7B), but to a lesser extent in Phi-pretreated plants (Fig. 7B and Supplemental Fig. S7). The signal that corresponds to *MPK4* protein was not detected in extract prepared from the *mpk4* mutant.

*MPK3* was recently shown to be the major component for full priming of stress responses in Arabidopsis (Beckers et al., 2009). The involvement of *MPK3* in Phi-induced priming to *Hpa* was tested using the *mpk3* mutant. Mutation in *MPK3* did not impair response of Arabidopsis to Phi treatment after pathogen challenge providing evidence that *MPK3* is not required for Phi-IR (Fig. 8). Moreover, Phi did not modify *MPK3* transcription, protein accumulation or the phosphorylation state of *MPK3* following pathogen challenge (Supplemental Fig. S8). Altogether, these results suggest that Phi specifically down-regulates *MPK4* gene expression, *MPK4* protein accumulation and phosphorylation during the response of Arabidopsis to infection with *Hpa* Noco2.

**DISCUSSION**

Phi is extensively used to protect plants against diseases mainly caused by oomycetes (Lobato et al., 2010). The mode of action of Phi is unknown and remains controversial given evidence for both direct and indirect modes of action (Guest and Grant, 1991). Here we have dissected Phi-induced defense responses in the Arabidopsis-*Hpa* pathosystem to decipher the molecular mechanisms of Phi-IR. We show that Phi exerts a dual activity on *Hpa* infection in a concentration-dependent manner. Phi directly inhibits mycelial growth at high concentrations (≥ 50 mM), but induces at low concentrations (≤ 10 mM) partial protection against *Hpa* by priming SA-dependent defenses and down-regulating the MAP kinase 4.

**Phi-induced protection is strictly dependent on SA signaling**
Soil-drenching Col-0 WT plants with 10 mM Phi 72 h before inoculation with \textit{Hpa} Noco2, reduced sporulation by 35% compared to mock-treated plants. Importantly, 10 mM Phi was completely ineffective in \textit{sid2-1} mutant (Fig. 3), indicating that Phi inhibits pathogen growth via SA-dependent plant defenses at this concentration. It could be argued that Phi uptake and translocation in the leaves is modified by the absence of a functional SID2 protein, resulting in a lower concentration of the chemical at the site of infection. However, transgenic NahG plants also failed to respond to 10 mM Phi (Supplemental Fig. S2), independently confirming the involvement of SA-dependent defenses. Moreover, the \textit{npr1-1} mutant is insensitive to 10 mM Phi (Fig. 3), indicating that a functional \textit{NPR1} gene is required for IR, and underscores the importance of SA signaling in Phi-IR. Fosetyl-Al (aluminium tris-O-ethyl phosphite; Aliette\textsuperscript{\textregistered}), an agrochemical that releases Phi, ethanol and aluminium ions was shown to protect Arabidopsis against \textit{Hpa} when sprayed at high concentrations, and its effectiveness was only partially impaired in NahG plants and \textit{nim1/npr1} mutant (Molina et al., 1998). However, fosetyl-Al directly induced \textit{PR1} expression as consequence of the chemical toxicity (Molina et al., 1998) confounding the identification of the precise function of Phi as SAR activator.

ET, JA and ABA were shown to be involved in activating certain defense responses in Arabidopsis (Van Loon et al., 1998; Ton et al., 2009; Ballaré, 2011). Mutants of Arabidopsis affected in JA, ET perception and ABA biosynthesis were not impaired in their responsiveness to 10 mM Phi treatment after \textit{Hpa} inoculation (Fig. 3). Moreover, transcriptional analysis of the JA- and ET-inducible \textit{PDF1.2} gene did not reveal any correlation with Phi-induced responses (Supplemental Fig. S4). Therefore, in this pathosystem, the effectiveness of Phi is strictly dependent of SA signaling.

**Phosphite exhibits a dual function in a concentration-dependent manner**

The inhibition of \textit{Hpa} Noco2 by Phi exhibited an unusual biphasic dose-response relationship suggesting that two independent factors contribute to pathogen restriction in this pathosystem. The first sigmoid in Figure 2 reflects the SA-dependent indirect mode of action of the chemical below 12.5 mM. Interestingly, Phi effect is abolished in \textit{sid2-1} and NahG plants (Fig. 3 and Supplemental Fig. S2). The second sigmoid comprised between 22.5 and 50 mM Phi, reflects the direct toxicity of Phi to the pathogen, as Phi effectiveness is not compromised in NahG plants (Supplemental Fig. S2). Phi treatments between 12.5 and 22.5 mM induce a complex interaction combining indirect and direct modes of action, and results in a 43% inhibition of the pathogen across the range of concentrations. This plateau might result from a saturation of Phi target(s) in the plant and/or in the pathogen, or indicate the maximal
augmented capacity to express the IR (Ahmad et al., 2010). Beyond 22.5 mM Phi, the direct inhibition of mycelial growth overshadows the contribution of Phi-induced plant defenses to pathogen inhibition. Contrasting conclusions on the mode of action of Phi can be explained by understanding this bimodal activity. Phi exerts a dual activity in a concentration-dependent manner through either an SA signaling-dependent activity in the plant or a direct action on the pathogen.

**Phosphite-induced resistance to* H. arabidopsidis* is accomplished by priming of a subset of defense responses**

Some chemicals do not trigger molecular defense responses *per se* although they confer resistance to virulent pathogens by enhancing or priming plant capacity to express defense responses. Thus, the synthetic compounds INA, BTH, BABA and the natural compound SA, are all potent inducers of priming and augmented defense-related gene expression and disease resistance at low concentrations (Kauss et al., 1992; Mur et al., 1996; Katz et al., 1998; Zimmerli et al., 2000). Noteworthy, Phi alone at 10 mM did not induce SA accumulation before inoculation with Hpa (Supplemental Fig. S3). However, SA accumulation and PR1 expression were augmented following pathogen challenge (Fig. 4). Thus, this clearly indicates that Phi primes defense responses in Arabidopsis, resulting in enhanced disease resistance to Hpa.

Phi was shown to induce an oxidative burst and phytoalexin accumulation associated with an HR-like response in cowpea and Arabidopsis infected with *P. cryptogea* and *P. palmivora*, respectively (Saindrenan et al., 1988; Daniel and Guest, 2006). The response of atrbohD mutants infected with Hpa to Phi was similar in the Col-0 WT (Fig. 5) indicating that ROS produced from the plasma membrane NADPH oxidase do not contribute to the Phi-IR to Hpa in Arabidopsis. Camalexin and scopoletin are two phytoalexins that accumulate in Arabidopsis in response to pathogen challenge (Glawischnig, 2007; Simon et al., 2010) through PAD3 and F6’H1 activities, respectively (Kai et al., 2008; Böttcher et al., 2009). Recently, it was shown that disease resistance of Arabidopsis to *P. brassicae* is established by the sequential action of indole glucosinolates and camalexin (Schlaeppi et al., 2010). However, it is not clear whether camalexin accumulation is either a cause or a consequence of IR in the Arabidopsis-Hpa pathosystem (Mert-Türk et al., 2003). Expression of Phi-IR was unaffected in pad3-1 and f6’h1-1 mutants (Fig. 5A) and did not correlate with phytoalexin levels (Fig. 5B and C) demonstrating that neither phytoalexin contributes to Phi-IR. Recently, it was reported that Phi elicited accumulation of PR proteins associated with SA and JA/ET
signaling pathways in non-inoculated leaves of Arabidopsis and primed callose deposition and H$_2$O$_2$ accumulation after inoculation with _P. cinnamomi_ (Eshraghi et al., 2011). It remains puzzling how Phi can prime for SA-inducible _PRI_ expression but not for ROS production in the _Hpa_-Arabidopsis interaction and enhanced production of H$_2$O$_2$ in the _P. cinnamomi_-Arabidopsis interaction. The differences observed between Phi-IR to _Hpa_ and to _P. cinnamomi_ might reflect the different life styles of biotrophic and hemibiotrophic oomycete pathogens and/or the recognition of different PAMPs by the plant leading to activation of different signaling pathways (Baxter et al., 2010).

**Phosphite mobilizes EDS1 and PAD4 expression for priming**

EDS1 and PAD4 proteins are essential components of basal resistance and ETI to biotrophic pathogens (Parker et al., 1996; Jirage et al., 1999) and are required for SA accumulation following pathogen challenge (Falk et al. 1999; Jirage et al., 1999; Feys et al., 2001). Our data reveal that _PAD4_ and _EDS1_ expression is primed by Phi (Fig. 6). _EDS1_ and _PAD4_ are transcriptionally and post-transcriptionally regulated (Feys et al., 2001). Both proteins have been shown to be present in healthy plants (Feys et al., 2001), and after pathogen infection, gene expression changes dependent on EDS1 and PAD4 take place at early time points (Bartsch et al., 2006) before any reported protein up-regulation (Feys et al., 2001; Bartsch et al., 2006). This implies the activation of pre-existing EDS1-PAD4 protein complexes and the existence of translational regulatory protein mechanisms. A common regulatory post-translational modification is phosphorylation (Peck, 2003). However, changes in the phosphorylation state of either PAD4 or EDS1 in challenged plants have not yet been reported. It could be hypothesized that Phi activates pre-existing EDS1 and/or PAD4 protein. Thus, a possible mechanism of Phi-induced priming could be to modify normal EDS1 and/or PAD4 re-localization or redistribution between cytoplasmic and nuclear compartment after pathogen challenge (Feys et al., 2005; Rietz et al., 2011) as signaling through relocalization may be central to EDS1-PAD4 function (Feys et al., 2005; Bartsch et al., 2006). Phi treatment reduced sporulation by only 10% in the _pad4_ mutants compared to 35% in Col-0 WT (Supplemental Fig.5), underlining that this lipase-like protein is a component of the Phi-signal transduction pathway leading to IR. However, the delayed effect of Phi on enhanced expression of _EDS1_ and _PAD4_ after pathogen challenge suggests a more likely target(s) upstream of _EDS1-PAD4_.

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Phi-induced priming involves the negative regulation of the mitogen-activated protein kinase MPK4

The MAP kinase MPK4 is required for normal plant growth and functions in a variety of physiological processes (Gao et al., 2008; Kosetsu et al., 2010). MPK4 is a negative regulator of SAR that is upstream of, but dependent on EDS1 and PAD4 (Petersen et al., 2000). EDS1 and PAD4 are up-regulated in mpk4 mutants (Cui et al., 2010) that are also fully resistant to Hpa and Pst DC3000 (Brodersen et al., 2006; Gao et al., 2008). However, loss-of-function mpk4 mutants exhibit a dwarf phenotype, making difficult to more directly address the role of MPK4 in Phi-induced priming. The Arabidopsis genome encodes more than 20 MPKs, including MPK3, MPK4 and MPK6 that are involved in innate immunity (Petersen et al., 2000; Asai et al., 2002; Menke et al., 2004). While Phi has no effect on MPK4 expression before inoculation (Supplemental Fig. S6), our data show that Phi induces the down-regulation of MPK4 gene expression, protein level and MPK4 phosphorylation 10 hpi underlying that Phi-IR is regulated at the transcriptional level (Fig. 7A and B). It is of note that Phi does not affect MPK4 transcript accumulation 8 hpi while MPK4 protein levels and protein phosphorylation decreased (Fig. 7A and B; Supplemental Fig. S7). It is possible that Phi induces immediate degradation of MPK4 protein following pathogen inoculation that superimposes to later down-regulation of MPK4 gene expression. As MPK4 represses the expression of defense against Hpa (Brodersen et al., 2006), we propose that Phi-induced priming of Arabidopsis against Hpa involves the specific negative regulation of MPK4. BTH-priming against the virulent Pst DC3000 strain was shown to be associated with increased activity of MPK4 and MPK6 (Beckers et al., 2009), two closely related proteins exhibiting a high level of functional redundancy (Colcombet and Hirth, 2008). It was assumed that MPK3 was the major component in primed defense gene activation by BTH, while MPK6 served a minor role (Beckers et al., 2009). MPK4 has opposing effect to MPK3/MPK6 in the regulation of plant defense responses (Nakagami et al., 2005). MPK4 negatively regulates biotic stress signaling while MPK3 and MPK6 act as a positive component of defense responses (Pitzschke et al., 2009). Noteworthy, Phi-IR was not compromised in mpk3 mutant (Fig. 8) and Phi did not modify MPK3 transcription, protein accumulation or the phosphorylation state of MPK3 following pathogen challenge (Supplemental Fig. S8), suggesting that Phi-IR is rather linked to MPK4 activity. Future analyses with plants overexpressing MPK4 should help to specify the precise role of this MAP kinase in Phi-IR.

Phi-Induced resistance to H. arabidopsidis involves major components of SA signaling
Our data suggest a model of how Phi primes defense responses against *Hpa* (Fig. 9). Phi-IR involves EDS1 and PAD4 downstream of MPK4 (Fig. 6). MPK4 negatively regulates SA accumulation (Petersen et al., 2000) and the repression of MPK4 expression/MPK4 activity by Phi following pathogen infection results in enhanced accumulation of *PAD4* and *EDS1* transcripts leading to augmented levels of SA and enhanced expression of *PRI* (Fig. 4). Moreover, Phi primes SA-related defenses in response to *Hpa* infection through a NPR1-dependent signaling pathway (Fig. 3). Although NPR1-independent defense responses involving the transcription factor WHY1 have been described (Desveaux et al., 2004), we could not detect any change in *WHY1* transcript accumulation in Phi-pretreated plants 24 hpi (data not shown). While we suggest that Phi-induced priming results from the repression of an active MPK, an alternative, but not exclusive explanation, would be that priming could originate from one or more factor(s) upstream of MPK4. MPK4 functions in a cascade that includes the MAP kinase kinase kinase MEKK1 and the MAP kinase kinases M KK1 and M KK2 (Ichimura et al., 1998). Hence, it remains conceivable that Phi may target one of these components of the MAP kinase module. The single *mkk1* and *mkk2* T-DNA insertion mutants were as susceptible as the WT plants to the virulent strain *Hpa Noco2*, while *mkk1/mkk2* double mutants were found to be highly resistant to the virulent oomycete pathogen (Qiu et al., 2008). Hence, plants expressing a constitutively active version of M KK1 and/or M KK2 would be more appropriate to improve Phi–IR in such pathosystem.

Phi interferes with Pi sensing machinery in plants and suppresses plant responses to Pi deprivation (Varadarajan et al., 2002), but does not affect cellular metabolism on Pi-sufficient media (Ticconi et al., 2001). As Phi interferes with Pi metabolism (Danova-Alt et al., 2008) this suggests that its effect may be at the plant-pathogen interface where the chemical would disrupt Pi homeostasis. Phosphatase genes are induced by Pi-limitation at transcriptional level (Misson et al., 2005; Thibaud et al., 2010), but also repressed in high Pi. In Arabidopsis, regulated dephosphorylation and inactivation of MAP kinases is mediated by MAPK phosphatases and PP2C-type phosphatases (Andreasson and Ellis, 2009). Inactivation of MPK4 was shown to be mediated either by the tyrosine-specific phosphatase PTP1 (Huang et al., 2000), the dual specificity (Thr/Tyr) protein phosphatase MKP1 (Ulm et al., 2002) or the PP2C-type phosphatase AP2C1 (Schweighofer et al., 2007). Upon pathogen infection, down-regulation of MPK4 by enhanced protein phosphatase activity derepresses the SA-signaling pathway (Fig. 9). Therefore, Phi may prime the plants for augmented defense responses upon biotroph challenge by mimicking Pi starvation and inducing protein phosphatase activity(ies). This possibility can only be resolved by identifying the precise molecular target(s) of Phi.
CONCLUSION
It appears that signaling components like MPK4 and MPK3/MPK6 (Beckers et al., 2009) play a role in Arabidopsis in the priming phenomenon induced by Phi and BTH, respectively. In addition, it was shown that the Arabidopsis ibs1 mutant affected in a cyclin-dependent kinase-like protein was impaired in BABA-induced priming of SA-dependent defenses (Ton et al., 2005). Overall, this underscores the importance of protein phosphorylation cascades in priming for enhanced defense responses in Arabidopsis.

MATERIAL AND METHODS

Plant materials
Arabidopsis thaliana ecotype Columbia (Col-0) and ecotype Wassilewskija-0 (Ws) were used in this work. The mutants sid2-1 (Wildermuth et al., 2001), jar1-1 (Staswick et al., 2002), ein2-1 (Guzman and Ecker, 1990), aba1-5 (Koornneef et al., 1982), npr1-1 (Cao et al., 1994), pad3-1 (Glazebrook and Ausubel, 1994), atrbohD (Torres et al., 2002), f6’h1-1 (Kai et al., 2008), and transgenic NahG plants (Delaney et al., 1994) were all in Col-0 background. Plants were grown in controlled environment chambers under 8h/16h day/night regime with temperature of 20°C/18°C respectively; light intensity was 100 µE m⁻² s⁻¹ and relative humidity 65%. Plants were grown in 12-well plates filled with soil (2.5 mg seeds/well). Two week-old plants were used for experiments and pathogen maintenance.

Chemical treatment
Seedlings were treated with potassium phosphite (Phi) or MES [(2-(N-morpholino) ethanesulfonic acid, 27 mM)] (Sigma-Aldrich®, http://www.sigmaaldrich.com/) as control (2 ml/well) three days prior to pathogen inoculation. Phosphorous acid (Sigma-Aldrich®, 99%) used in this study was partially neutralized with potassium hydroxide prepared in 27 mM MES to yield Phi (HPO₃²⁻/H₂PO₃⁻) at pH 6.3.

Pathogen maintenance
H. arabidopsidis isolates Noco2 and Emwa1 were obtained from Harald Keller (INRA, Sophia-Antipolis, France) and maintained weekly by transferring spores onto healthy seedlings of Col-0 and Ws-0 accessions, respectively. Spores were harvested by vortexing
infected seedlings in water. Healthy seedlings were inoculated by spraying with spore suspension.

**Pathogen assay**

Soil-grown seedlings in 12-well plates were inoculated by spraying with 2 ml suspension of 5×10^4 spores ml^-1. Inoculated seedlings were kept under high relative humidity for 1 day post-inoculation (dpi), then returned to normal conditions, and replaced again at high humidity between 5 and 7 dpi. Spore production was evaluated 7 dpi. Seedlings of each well were removed, weighed and then vortexed in 5 ml water for 10 min to liberate pathogen spores. Spores from three samples of each treatment were counted using a Nageotte chamber and the means converted to spore number mg^-1 of fresh weight.

**Histochemical staining**

To visualize pathogen mycelium at the cellular level, infected plants were stained with trypan blue in lactophenol and ethanol 7 dpi as described by Cao et al. (1998). The seedlings were destained overnight in saturated solution of chloral hydrate and imaged using a light macroscope (AZ100 NIKON).

**Quantification of SA, camalexin and scopoletin**

Salicylic acid (SA), camalexin and scopoletin were extracted and quantified as described by Simon et al. (2010). Standards of SA and scopoletin were from Sigma-Aldrich, whereas authentic camalexin was a kind gift of A.J. Buchala (University of Fribourg, Switzerland).

**Analysis of gene expression**

Total RNA was extracted from seedlings tissue using EXTRACT ALL® Reagent according to the manufacturer’s recommendations (Eurobio®, http://www.eurobio.fr/). Samples were subjected to RNase-free DNase I treatment (DNA-free™ kit, Ambion®, Applied Biosystems, http://www.ambion.com/) for 30 min. Total RNA was determined at 260 nm using a spectrophotometer (Thermo Scientific, NanoDrop™, http://www.nanodrop.com/). Reverse transcription reaction for cDNA synthesis was performed on 2 µg of RNA using oligo-dT primers and ImProm-II™ kit (Promega, http://www.promega.com/) according to the manufacturer’s instructions. qRT-PCR experiments were performed with 8 µl of a 1:10 dilution cDNA and LightCycler® 480 SYBR Green I Master (Roche, http://www.roche.fr/) according to the manufacturer’s instructions. Relative transcript levels were determined by
normalizing the PCR threshold cycle number of each gene with that of the ACT2 reference gene. The qRT-PCR primer sequences used in this work are supplied in Table S1.

Protein extraction and immunodetection
Fifteen µg of total soluble protein extracted from Arabidopsis seedlings was separated by SDS-PAGE (12% acrylamide) as described (Conrath et al., 1997). Equal loading of protein was confirmed by Ponceau S staining. After transfer to a PVDF membrane, immunodetection of MPK4 and MPK3 was performed with a SNAPid system (Millipore, http://www.millipore.com/) using anti-rabbit polyclonal antibodies raised against MPK4 and MPK3 (a kind gift from H. Hirt, URGV, INRA, Evry, France) at a 1:1500 dilution. To detect phosphorylated MPK4 and MPK3, α-phospho-p44/42-ERK antibodies (α-P-MAPK<sup>ac</sup>) (Cell Signaling Technology®, http://www.cellsignal.com/) were used at 1:1000 dilution (Heese et al., 2007). Antigen-antibody complexes were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibody (Pierce, http://www.piercenet.com/) used at 1:10000 dilution and an ECL kit (Perkin Elmer, http://las.perkinelmer.com/).

Statistical analyses
All experiments were repeated at least two times with similar results. Means of acquired data were compared using ANOVA, Newman-Keuls or Mann-Whitney test as indicated in figure legends.

Supplemental Data

Supplemental Figure S1. Incidence of timing of treatment on Phi effectiveness.
Supplemental Figure S2. Phi effectiveness in Col-0 WT and NahG plants against H. arabidopsidis Noco2.
Supplemental Figure S3. Effect of Phi treatment on SA accumulation in Arabidopsis before inoculation with H. arabidopsidis.
Supplemental Figure S4. Impact of Phi on PDF1.2 transcript accumulation in Arabidopsis in response to H. arabidopsidis Noco2.
Supplemental Figure S5. Phi effectiveness in Col-0 WT and pad4 mutant against H. arabidopsidis Noco2.
Supplemental Figure S6. Impact of Phi on MPK4 expression in Arabidopsis before inoculation with H. arabidopsidis.
Supplemental Figure S7. Quantification of immunodetection signals of MPK4 activity in Figure 7B.

Supplemental Figure S8. Effect of Phi treatment on gene expression, protein accumulation and phosphorylation of MPK3 in response to *H. arabidopsidis* Noco2.

Supplemental Table S1. List of primers used in this work.

ACKNOWLEDGMENTS

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LITERATURE CITED


Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1 and the nudix hydrolase NUDT7. Plant Cell 18: 1038–1051


Susceptibility1 (EDS1) bound to and dissociated from Phytoalexin Deficient4 (PAD4) in Arabidopsis immunity. New Phytol 191: 107-119


FIGURE LEGENDS

Figure 1. Phi effectiveness against H. arabidopsis
Two week-old plants of Col-0 wild type (WT) were treated by soil-drenching with MES (as mock) or Phi (5-100 mM) 72 h prior to being inoculated by H. arabidopsis isolate Noco2 or Emwa1 (5x10⁴ spores mL⁻¹). Level of infection was evaluated 7 dpi by A: quantifying the number of pathogen spores on aerial tissues and B: histochemical staining of pathogen mycelium in leaves with lactophenol-trypan blue. For inoculation with Emwa1 isolate, plants were pre-treated with MES. Values in A are means ± SE of 15 replicates from 5 biological independent experiments. Letters indicate significant differences between values (ANOVA, Newman-Keuls test, P < 0.05). Experience in B was repeated three times with similar results.

Figure 2. Dose-response curve of Phi towards H. arabidopsis
Col-0 WT plants were treated and inoculated by H. arabidopsis isolate Noco2 as described in legend of Figure 1. Effectiveness of Phi was calculated as a percentage of reduction in spore number caused by Phi treatment. Values are means ± SE of 9 replicates from 3
biological independent experiments. Letters indicate significant differences between values (ANOVA, Newman-Keuls test, \( P < 0.05 \)).

**Figure 3.** Phi effectiveness against *H. arabidopsidis* in different Arabidopsis-deficient signaling mutants.

Col-0 WT plants as well as *sid2-1, jar1-1, ein2-1, aba1-5* and *npr1* mutants were treated with MES (as mock) or Phi (10 mM) and inoculated as described in legend of Figure 1. Effectiveness of Phi was calculated as in legend of Figure 2. Values are means ± SE of 15 replicates from 5 biological independent experiments. Letters indicate significant differences between values (ANOVA, Newman-Keuls test, \( P < 0.05 \)). Means ± SE of spore number quantified on mock-pretreated plants of WT and *sid2-1, npr1, jar1-1, ein2-1, aba1-5* mutants were 610 ± 18, 1200 ± 25, 625 ± 12, 610 ± 16, 595 ± 15 and 554 ± 13 spore mg\(^{-1}\) FW, respectively.

**Figure 4.** Effect of Phi treatment on the accumulation of SA and *PR-1* transcripts in Arabidopsis in response to *H. arabidopsidis*.

Col-0 WT plants were treated and inoculated as described in legend of Figure 3. A, Accumulation of free SA; B, Accumulation of total SA; C, Accumulation of *PR-1* transcripts normalized to transcript level of the internal control gene *ACTIN2*. Levels of SA and *PR-1* transcripts were quantified by HPLC and qRT-PCR, respectively. Values of graphs are means ± SD of three replicates. The experiment was repeated twice with similar results. Asterisks indicate data that are significantly different between Phi and MES treatments (Mann-Whitney test, \( P < 0.05 \)).

**Figure 5.** Relationship between Phi effectiveness and ROS, camalexin and scopoletin accumulation.

Plants were treated and inoculated as described in legend of Figure 3. A, Phi effectiveness in Col-0 WT plants and *atrbohD, pad3-1* and *f6’h1-l* mutants. Phi effectiveness was calculated as described in legend of Figure 2; B, Levels of camalexin; C, Levels of total scopoletin. Values are means ± SE of 15 replicates from 5 biological independent experiments in A and means ± SD of three replicates in B and C. No significant differences were found between values in A, B and C (ANOVA, Newman–Keuls test, \( P < 0.05 \)). The experiments in B and C were repeated twice with similar results. Means ± SD of spore number quantified on mock-
pretreated plants of Col-0 WT plants and *atrbohD, pad3-1* and *f6’h1-1* mutants were 610 ± 75, 575 ± 46, 615 ± 40 and 680 ± 48 spore mg⁻¹ FW, respectively.

**Figure 6.** Effect of Phi treatment on *PAD4* and *EDS1* expression in Arabidopsis in response to *H. arabidopsidis*.

A, *PAD4* expression. B, *EDS1* expression. Plants were treated and inoculated as described in legend of Figure 3. Samples were harvested at 0 h (before chemical treatment), 0 hpi (72 h post-treatment), 10 and 24 hpi. Levels of *PAD4* and *EDS1* transcripts were quantified by qRT-PCR, normalized to level of internal control gene *ACTIN2*, and expressed relative to their levels at 0 h. For inoculation with Emwa1, plants were pre-treated with MES. For each time point, values are means ± SD of 3 replicates. Asterisks indicate significant differences between values (ANOVA, Newman–Keuls test, *P* < 0.05). Experiment was repeated three times with similar results.

**Figure 7.** Effect of Phi treatment on gene expression, protein accumulation and phosphorylation of MPK4 in response to *H. arabidopsidis*.

A, *MPK4* expression B, Accumulation and phosphorylation state of MPK4 protein. Plants were treated and inoculated as described in legend of Figure 3. Samples were harvested at 0, 8 and 10 hpi. Two aliquots of leaf tissues were used for quantification of *MPK4* transcripts by qRT-PCR as for *PR1* transcripts in legend of Figure 4. Another aliquot of leaf tissues was used for protein extraction and SDS-PAGE. A polyclonal α-MPK4 was used for the immunodetection of MPK4 protein. α-phospho-p44/42 ERK antibody was used to check phosphorylation state of MPK4. Leaf protein extract of *mpk4* mutant was used as a negative control for MPK4. The experiment was repeated three times with similar results. Letters indicate data that are significantly different between Phi and MES treatments (Mann-Whitney test, *P* < 0.05).

**Figure 8.** Phi effectiveness in *mpk3* mutant and Col-0 WT plants against *H. arabidopsidis*.

Phi treatment, pathogen inoculation and calculation of Phi effectiveness were performed as described in legend of Figure 3. Values are means ± SE of 15 replicates from 5 biological independent experiments. Phi effectiveness was not different between Col-0 and *mpk3* plants (Mann-Whitney test, *P* < 0.05). Means of spore number quantified in mock-pretreated plants of Col-0 WT and *mpk3* mutant, were 600 ± 75 and 612 ± 62 spore mg⁻¹ FW, respectively.
Figure 9. Model for priming by phosphite for augmented defense responses in Arabidopsis infected with *H. arabidopsidis*

Treatment with Phi inhibits accumulation and phosphorylation of MPK4 after *H. arabidopsidis* (*Hpa*) inoculation. The exact target(s) of Phi could be MPK4 or upstream components of the MAPK cascade as MEKK1 and MKK1/MKK2. Inactivation of MPK4 may also be mediated by phosphatase protein(s) (Phase). Repression of MPK4 expression/MPK4 activity enhances PAD4 and EDS1 accumulation (dashed line) and triggers SA signaling-dependent priming for enhanced resistance through the defense regulatory protein NPR1.
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Two week-old plants of Col-0 wild type (WT) were treated by soil-drenching with MES (as mock) or Phi (5-100 mM) 72 h prior to being inoculated by *H. arabidopsidis* isolate Noco2 or Emwa1 (5x10^4 spores mL\(^{-1}\)). Level of infection was evaluated 7 dpi by A: quantifying the number of pathogen spores on aerial tissues and B: histochemical staining of pathogen mycelium in leaves with lactophenol-trypan blue. For inoculation with Emwa1 isolate, plants were pre-treated with MES. Values in A are means ± SE of 15 replicates from 5 biological independent experiments. Letters indicate significant differences between values (ANOVA, Newman-Keuls test, \(P < 0.05\)). Experience in B was repeated three times with similar results.
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