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Methanol extract of mycelia from *Phytophthora infestans*-induced resistance in potato

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ABSTRACT

Plants recognize certain microbial compounds as elicitors in their active defence mechanisms. It has been shown that a series of defence reactions are induced in potato plant cells after treatment with water-soluble hyphal wall components prepared from *Phytophthora infestans*. In this study, a methanol extract from mycelia of *P. infestans* (MEM), which contains lipophilic compounds, was used as another elicitor for the induction of the defence reactions in potato. MEM elicitor induced reactive oxygen species (ROS), especially O₂⁻ and H₂O₂ production, and nitric oxide (NO) generation in potato leaves and suspension-cultured cells. Hypersensitive cell death was detected in potato leaves within 6–8 h after MEM elicitor treatment. The accumulation of phytoalexins was detected by MEM elicitor treatment in potato tubers. In potato suspension-cultured cells, several defence-related genes were induced by MEM elicitors, namely *Strboh*, *Sthsr203J*, *StPVS3*, *StPR1*, and *StNR5*, which regulate various defence-related functions. Enhanced resistance against *P. infestans* was found in MEM-treated potato plants. These results suggested that MEM elicitor is recognized by host and enhances defence activities to produce substances inhibitory to pathogens.

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1. Introduction

Early recognition of the pathogen by the plant is essential, when the plant activates the available biochemical and structural defence to protect itself from the pathogen. Once a particular plant molecule recognizes and reacts with an elicitor derived from a pathogen, alarm signals are transduced to the host cell molecules and to nuclear genes, causing them to become activated so as to produce pathogen-inhibiting substances. Defence reactions include the reinforcement of the cell wall by callose and/or lignin deposition, the production of reactive oxygen species (ROS), hypersensitive reactions (HR), multicomponent responses involving increased expression of defence-associated genes and localized cell death at the site of infection [1,2].

Abbreviations: cPTIO, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide; DAF-2 DA, Diaminofluorescein-2 diacetate; DMSO, Dimethyl sulfoxide; HWC, Hyphal wall components; L-012, 8-Amino-5-chloro-7-phenylpyridol[3,4-d]pyridazine-1,4(2H,3H) dione sodium salt; MES, 2-(N-morpholino)ethanesulfuric acid; MOPS, 3-(N-morpholino)propanesulphonic acid; MS medium, Murashige-Skoog basal medium; NAA, Naphthalene acetic acid; PCD, Programmed cell death; PVS, Potato vetispiradiene synthase; Rboh, Respiratory burst oxidase homolog, RNS, Reactive nitrogen species; ROS, Reactive oxygen species..

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A series of defence reactions are induced in potato plant cells after treatment with hyphal wall components (HWC) prepared from *P. infestans*, including the generation of superoxide anions (O_2^-), induction of HR and phytoalexins production [3]. Sanchez et al. [4] observed that HWC also cause the HR of cells of solanaceous plants. An infiltration of *Phytophthora*-derived oligopeptide elicitor, Pep-13, into potato leaves induces the accumulation of hydrogen peroxide, defence gene expression and the accumulation of jasmonic acid and salicylic acids [5].

A rapid and transient production of ROS, termed “oxidative burst”, is a hallmark of successful recognition of plant pathogens and the oxidative burst could have a direct effect on the pathogen or the defences caused in plant because of its reactivity [6]. ROS could also contribute to the establishment of physical barriers via oxidative cross-linking of precursors during the localized biosynthesis of lignin and suberin polymers [7]. ROS mediate the generation of phytoalexins, antibiotic compounds and secondary metabolites that arrest pathogen growth [8]. ROS are also associated with HR, a localized response at the site of pathogen attack that displays programmed cell death and that could contribute to limit the spread of the pathogens or to be a source of signals for the establishment of further defences [1]. Hypersensitive cell death occurs at the infection site with apoptosis-like features. Despite controversial debate as to the role of ROS in plant HR, they are generally regarded as causes of hypersensitive cell death [9,10].

The most rapid responses of plants to potential pathogens are the congruent production of both nitric oxide (NO) and reactive oxygen intermediates. NO has a key role in plant–pathogen interactions and participates in the early stages of HR induction [11,12]. In an investigation on tobacco cell suspension culture for study of the relations between NO generation and several defence responses induced by INF1 elicitor, cPTIO, a NO scavenger, completely suppressed the activation of a 41-kDa protein kinase and cell death by INF1, which suggests that NO plays a crucial role in the induction of hypersensitive cell death [13]. Potato tubers treated with NOC-18, a NO donor, induced the accumulation of potato phytoalexins [14]. In induced biochemical defence, phytoalexin production is regulated by NO [15].

The synthesis of phytoalexins or phytoanticipins is considered to be an important part of the plant innate immune response to a variety of pathogens [16,17]. ROS within the cell or to the neighbouring cells often generates phytoalexins [18]. Lubimin and rishitin are two of the major sesquiterpenoid phytoalexins produced by potato, and the potato pathogen *Gibberella pulicaris* can degrade both [19]. Eicosapentaenoic and arachidonic acids extracted from the mycelia of *P. infestans* elicit fungitoxic sesquiterpenes in the potato [20]. Substances from germinating spores of *P. infestans* also elicit the accumulation of phytoalexin rishitin in potato tuber slices [21].

In the present study, authors used a liposoluble MEM elicitor derived from *P. infestans* with the aim of examining the effect of the MEM elicitor to ascertain the early reactions and to evaluate the induced resistant reactions in potato plants and potato suspension-cultured cells. This

study also concerns defence strategy in potato – *P. infestans* interactions and defence activities in potato in response to the MEM elicitor.

2. Materials and methods

2.1. Plant materials

Potato suspension-cultured cells, leaves and tubers of cultivar Sayaka, which has resistant genes *R1* and *R3*, were used. Potato suspension-cultured cells are very sensitive to early responses to MEM elicitor, whereas potato leaves were used to observe elicitor responses in more natural conditions. On the other hand, potato tubers gave the best results for phytoalexin production (unpublished data by Monjil and Kawakita). Potato plants were grown at 23 °C and 70% humidity under a 16-h photoperiod in environmentally controlled growth cabinets and the leaves were used for the experiments. Potato suspension-cultured cells (cv. Sayaka) were prepared from callus obtained from potato tuber discs in callus-inducing solid medium [22]. Potato suspension-cultured cells were subcultured every week and used for experiments 4–5 days after subculturing.

2.2. Pathogenic isolate *Phytophthora infestans*

The pathogenic isolate [*P. infestans* (Mont.) De Bary], race 1.2.3.4, was used as a mycelium culture and was maintained at the Plant Pathology laboratory of the Graduate School of Bioagricultural Sciences, Nagoya University, Japan. *P. infestans* strains, used as inocula for liquid culture, were grown on rye-seed extract agar medium in a test tube at 18 °C in the dark for two weeks. Growing of mycelia for methanol extraction was done in rye liquid nutrient [22]. For spray experiment, zoosporangia suspensions from the *P. infestans* isolates were prepared following the method reported by Monjil et al. [23]. Briefly, *P. infestans* isolates were subcultured on rye-media for 7–10 days. Twenty milliliters of water were added to the surface of the *P. infestans* colonies, which were then rubbed with a cotton swab to release zoosporangia. Zoospores from zoosporangia were taken out by keeping the water containing zoosporangia at 16 °C for 3 h. MEM-treated potato leaves were inoculated with 1-mL aliquots of *P. infestans* zoospores (2×10^5 zoospores/mL) and covered with lens paper to keep the suspension of zoospores on the surface of the leaves. The inoculated plants were kept at high humidity at 20 °C for one day after inoculation, and then moved to a growth room at 23 °C.

2.3. Preparation of MEM elicitor from *P. infestans*

MEM elicitor from *P. infestans* was prepared following the method described by Monjil et al. [24]. Frozen mycelia were ground by a mortar and pestle under a freezing temperature using liquid nitrogen. The ground mycelia were suspended in methanol at the rate of 10 mL of methanol per 1 g of mycelia. The mycelia suspension was finely grounded using a polytron-type homogenizer

(HG30, Hitachi Koki, Japan) for 2 min. After centrifugation at 4 °C, 3000 g for 30 min, the supernatant was collected and dried by using an evaporator, and was used as an MEM elicitor. It can be noted that 1 g of mycelia from *P. infestans* yielded 88–90 mg of MEM elicitors.

2.4. Measurement of O_2^- production

The relative intensity of O_2^- generation in potato suspension-cultured cells was measured by counting photons from L-012-mediated chemiluminescence following the method described by Monjil et al. [22]. In plant leaves, O_2^- measurement was done as described by Kobayashi et al. [25]. For the detection of O_2^- production in leaf tissues, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) was infiltrated into potato leaves via a syringe without needle. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in the dark chamber at 20 °C (Aquacosmos 2.5; Hamamatsu Photonics, Shizuoka, Japan) and quantified using the U7501 program (Hamamatsu Photonics).

2.5. H_2O_2 in situ detection

Leaf discs of potato plants infiltrated with elicitors were removed with a cork borer at 3, 6, 12 and 24 h after infiltration. H_2O_2 detection was performed following the DAB staining method described by [26]. Leaf discs were placed in 1 mg/mL 3,3'-diaminobenzidine (DAB-HCl) (Sigma, St. Louis, USA) and incubated at room temperature overnight. DAB reactions were examined in leaves cleared in boiling ethanol (96%) for 10 min. The samples were then stored in ethanol (96%) at room temperature or mounted in PBS (phosphate-buffered saline)/glycerol (50%) and kept at 4 °C for further examination. H_2O_2 was visualized as a reddish-brown coloration.

2.6. Detection of cell death

To detect the cell death on the plant, elicitors were infiltrated by using a syringe without needle from the opposite surface of the leaves. Cell death was measured by monitoring the death areas developed on the infiltrated leaf surface after 6, 12, 24 and 48 h of treatment with elicitors. Cell death was also checked by an electrolyte leakage method, which was adapted from the method previously described by [27]. Leaves were infiltrated by elicitors under the leaf surface, and leaf discs (1 cm in diameter) were collected from the leaf 3, 6 and 12 h after treatment. Seven leaf discs were floated on 7 mL of distilled water for 2 h at room temperature, and electrical conductivity was measured using a conductivity meter (Horiba, Kyoto, Japan).

2.7. Detection of phytoalexins

Potato phytoalexins produced in potato tubers were extracted using ethyl acetate following the method described previously [14], with a little modification. Concave holes (diameter 15 mm, depth 7 mm) were made in the parenchyma of the sliced tuber about 10–15 mm thick by using an electromotive drill. After aging at 20 °C

for 24 h in dark humid chamber, 500 μ L of each treatment were added to the concave hole and were incubated under the same conditions for 48 h. Phytoalexins were extracted from the collected liquid from the treated holes with equal amounts of ethyl acetate. The extract were separated on TLC plates (TLC aluminum sheet of silica gel 60, Merck, Whitehouse Station, USA), which were developed with cyclohexane:ethylacetate (1:1, v/v) and visualized by spraying with 75% sulfuric acid containing 0.5% vanillin, followed by heating at 120 °C. Phytoalexins were quantified by high-performance liquid chromatography (HPLC) analysis. Extracts were concentrated and dissolved in 200 μ L of 70% MeCN in H_2O . A portion (5 μ L) of these solutions was analyzed by an HPLC system (Jasco Co., Tokyo) equipped with two PU-1580 pumps, a DG-2080-54 degasser, and an MD-2010 plus photodiode array detector, under the following conditions. Column: Develsil ODS-UG-5 (4.6 \times 250 mm, Nomura Chemical), solvent: 50% – 50% – 90% – 100% (0 – 5 – 25 – 26 min) MeCN in H_2O , flow rate: 1 mL/min, detection: UV 205 nm. HPLC data were processed by the Borwin program (Jasco Co.).

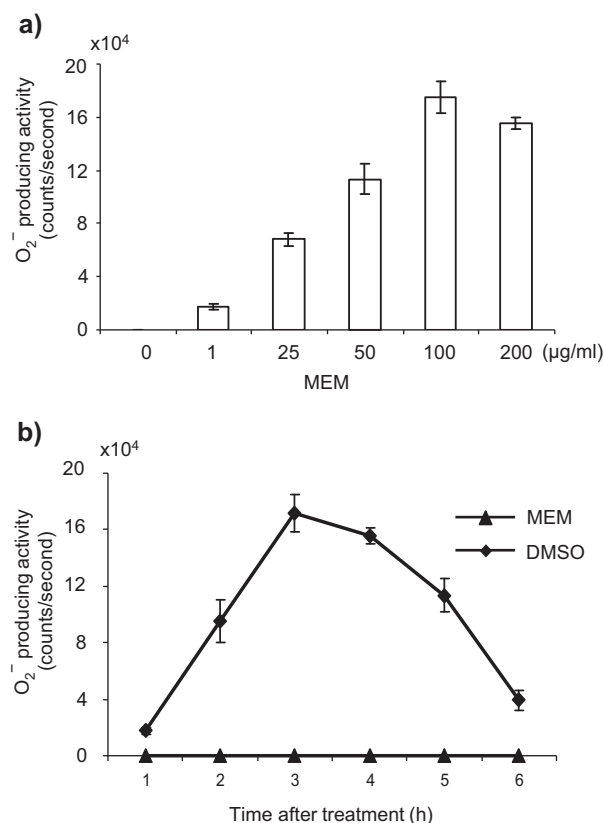


Fig. 1. O_2^- -inducing activity of MEM in potato suspension-cultured cells. a: the dose dependency of MEM was measured in potato suspension-cultured cells treated with 1/10 volume of MEM to get final concentrations 1 μ g/mL, 25 μ g/mL, 50 μ g/mL, 100 μ g/mL, and 200 μ g/mL, respectively. DMSO (3%) was used as a control. The O_2^- -producing activity was measured in a 96-well microtiter plate with L-012 using chemiluminescence plate reader Mithras LB 940; b: time course of the O_2^- producing activity of potato suspension-cultured cells by 100 μ g/ml MEM up to 6 h. Data are means \pm standard deviation from five independent experiments.

The phytoalexins (oxylubimin, 10-*epi*-oxylubimin, rishitin, lubimin, and 10-*epi*-lubimin) were eluted at 5.7, 6.2, 10.3, 12.5, 13.2 min, respectively.

2.8. Measurement of NO production

NO production activity in potato suspension-cultured cells was measured by a fluorometric method using the NO indicator diaminofluorescein-2 (DAF-2 DA; Daiichi Pure Chemicals, Tokyo, Japan) [24]. In potato leaves, NO production was measured by infiltrating 200 mM of a sodium phosphate buffer at pH 7.4 and 12.5 μ M DAF-2DA, using a needleless syringe; leaves were incubated for 1 h in the dark at room temperature before observation. Fluorescence from DAF-2 T, the reaction product of DAF-2DA with NO, was captured using a fluorescence stereomicroscope (MZ16FA, Leica) equipped with a CCD camera (Color 14 bit, AxioCam HRc, Carl Zeiss). The fluorescence intensity of the scanned entire field of the image captured by a CCD

camera was quantified by determining the mean green channel values for the images with the histogram function of Adobe Photoshop 7.0 (Adobe, Seattle) [28]. A fluorescence image was obtained from an inoculated area per leaf, and at least three inoculated areas were analyzed as replicates for each treatment.

2.9. RNA preparation and RT-PCR

Expression of potato genes after the treatment of elicitors was analyzed by RT-PCR [29]. RT-PCR was conducted using a commercial kit (ReverTra-Plus-; Toyobo Co., Osaka, Japan). The cDNA was synthesized from total RNA (1 μ g) with an oligo (dT) primer. After the cDNA synthesis reaction, PCR was performed with denaturing, annealing, and extension temperatures of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s, respectively. Gene-specific primers for each sequence were as follows: StEF-1 α forward primer, 5'-CATTGTGCTCATTGGCCACG-3', StEF-1 α

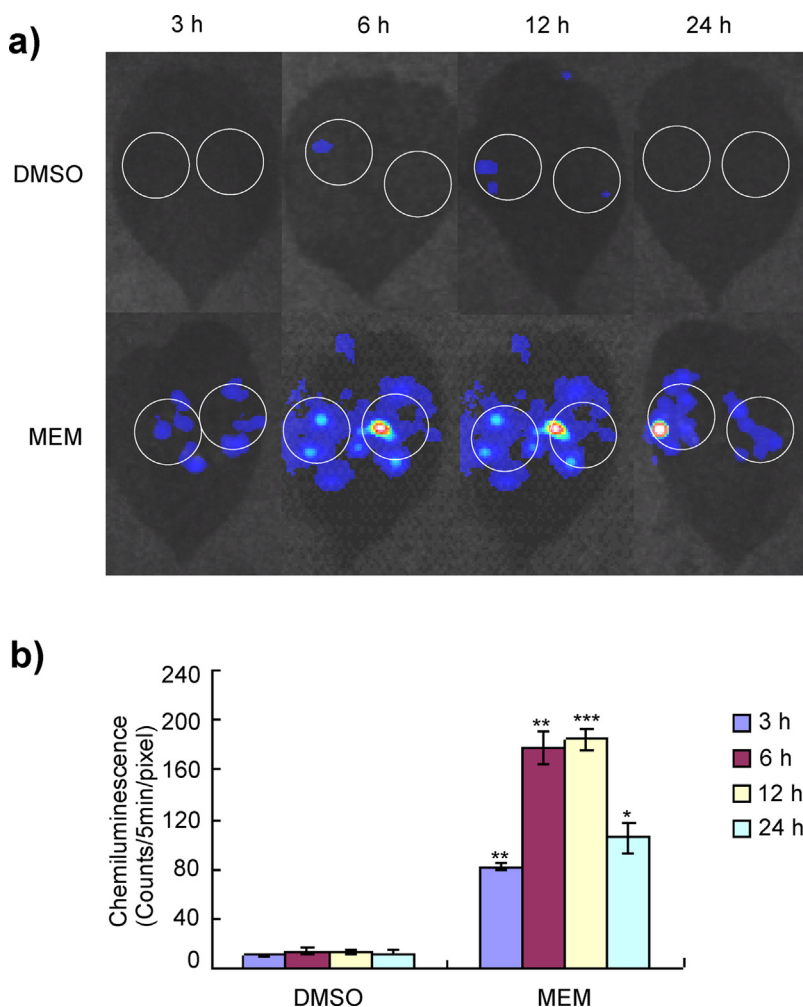


Fig. 2. (Color online.) $O_2^{\cdot-}$ -inducing activity of MEM in potato leaves. a: the $O_2^{\cdot-}$ -producing activity of potato leaves was measured by treatment with MEM elicitors. MEM (0.5 mg/mL) was used for this study. DMSO (3%) was used as a control. Elicitor-treated leaf areas were infiltrated with L-012 solution 3, 6, 12 and 24 h after treatment, and were monitored using a CCD camera. White circles indicate areas infiltrated with L-012; b: chemiluminescence intensities were quantified with a photon image processor. Data are means \pm standard deviation from three independent experiments. Data were subjected to Student's *t*-test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus DMSO-treated control treatment.

reverse primer, 5'-CCAGTCGAGTTGGTAGACC-3', *StrbohA* forward primer, 5'-TTAGCAGCTCGAGCATTACG-3', *StrbohA* reverse primer, 5'-TCCTTCATACCGATGCATTG-3', *StrbohB* forward primer, 5'-ATCGAAAACACGAGGGATTG-3', *StrbohB* reverse primer, 5'-GCACCAGACTTACTCCTATC-3', *StrbohC* forward primer, 5'-CGGAAAATCATCACCCGCAC-3', *StrbohC* reverse primer, 5'-CGGCTCCGGCACTAGTCCG-3', *StPR1* forward primer, 5'-GTGCTAGAGTCAAGTGCAAC-3', *StPR1* reverse primer, 5'-GAATCAAAGTCTGGTTGCTC-3', *StNR5* forward primer, 5'-TACAACATTGGCATTGGCTTG-3', *StNR5* reverse primer, 5'-AACATTTTATACCTAAAGTTAACA-3', *Sthsr203J* forward primer, 5'-AAGCTGATTGGTACATGTACTATGC-3', *Sthsr203J* reverse primer, 5'-TAGCTCCGATTACTTCGCTG-3', *StPVS3* forward primer, 5'-TTGTCTGCTGCTGCTTGTGG-3', and *StPVS3* reverse primer, 5'-TCCATGAGTCCTTACATG-3'.

2.10. Resistance-inducing activity against pathogen infection

Potato leaves were grown and used for resistance induction study. MEMs at the concentration of 100 $\mu\text{g}/\text{mL}$ were sprayed into potato leaves using a hand sprayer. DMSO treatment was used as a control. After 24 h of treatment, challenge inoculation was done by *P. infestans* (2×10^5 zoospores/mL), and potato leaves were kept in dark and humid conditions for 18 h.

3. Results

3.1. MEM elicitor and its O_2^- producing activity

Our MEM elicitor contains relatively low-molecular-weight compounds because of the limited solubility of high-molecular-weight compounds or proteins in methanol. The oxidative burst (rapid and transient production of O_2^-) in potato is an early response induced by elicitor treatment. MEM was added to potato suspension-cultured

cells and O_2^- producing activity was measured by using an O_2^- unique luminous reagent L-012 (Fig. 1). MEM induced rapid O_2^- generation in a dose-dependent manner (Fig. 1a). DMSO (3%) was used as a control because the MEM elicitor was dissolved in 3% DMSO. A time course experiment was done up to 6 h and indicated that O_2^- generation was rapidly induced by MEM, peaked at 3 h after the treatment, and slowly decreased until 6 h (Fig. 1b). In potato leaves, induction of O_2^- production by the MEM elicitor was observed as enhanced L-012 mediated chemiluminescence in infiltrated sites (Fig. 2a). Then, 3, 6, 12 and 24 h after the elicitor treatment, rapid O_2^- production was detected by the MEM elicitor within 3 h, and the maximum elicitor activity was observed 6 or 12 h after treatment; lower O_2^- production was observed after 24 h (Fig. 2b).

3.2. Hydrogen peroxide-producing activity

H_2O_2 generation was detected after MEM treatment and stained with DAB (Fig. 3). DAB captures H_2O_2 and forms a reddish-brown coloration at sites of peroxidase activity. DAB staining was observed in potato leaves at different time points, namely 3, 6, 12 and 24 h after MEM treatment. DAB staining was observed in any time point, indicating that H_2O_2 producing activity is induced by MEM.

3.3. Phytoalexins accumulation

Phytoalexins accumulation in potato tuber was measured by treatment with 0.1, 0.5 and 1 mg/mL of MEM (Fig. 4a). DMSO (3%) was used as a control. Purified rishitin was used as a marker for phytoalexin detection in TLC. HPLC analysis showed that MEM induced several phytoalexins accumulation, particularly rishitin, oxylubimin and lubimin in potato tubers (Fig. 4b and c). 10-*epi*-Oxylubimin and 10-*epi*-lubimin are two stereoisomers that were also observed in HPLC analysis.

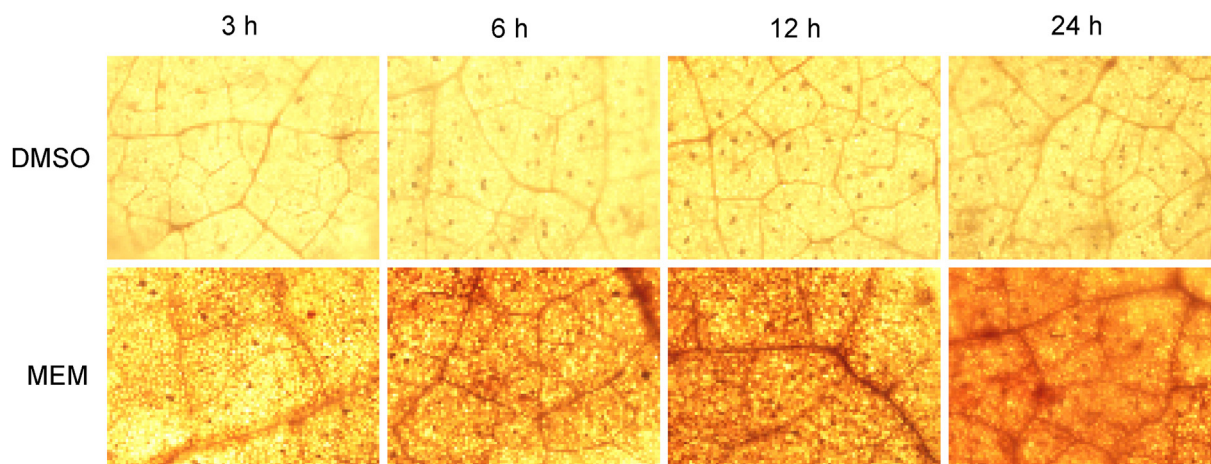


Fig. 3. (Color online.) H_2O_2 accumulation by MEM in potato leaves. Accumulation of H_2O_2 by MEM at the concentration of 0.5 mg/mL in potato leaves after incubation of leaf tissues with the dye DAB were determined after 3, 6, 12 and 24 h of treatment. DMSO (3%) was used as a control. The reddish-brown coloration indicates H_2O_2 accumulation. The results shown are representative of at least three separate experiments.

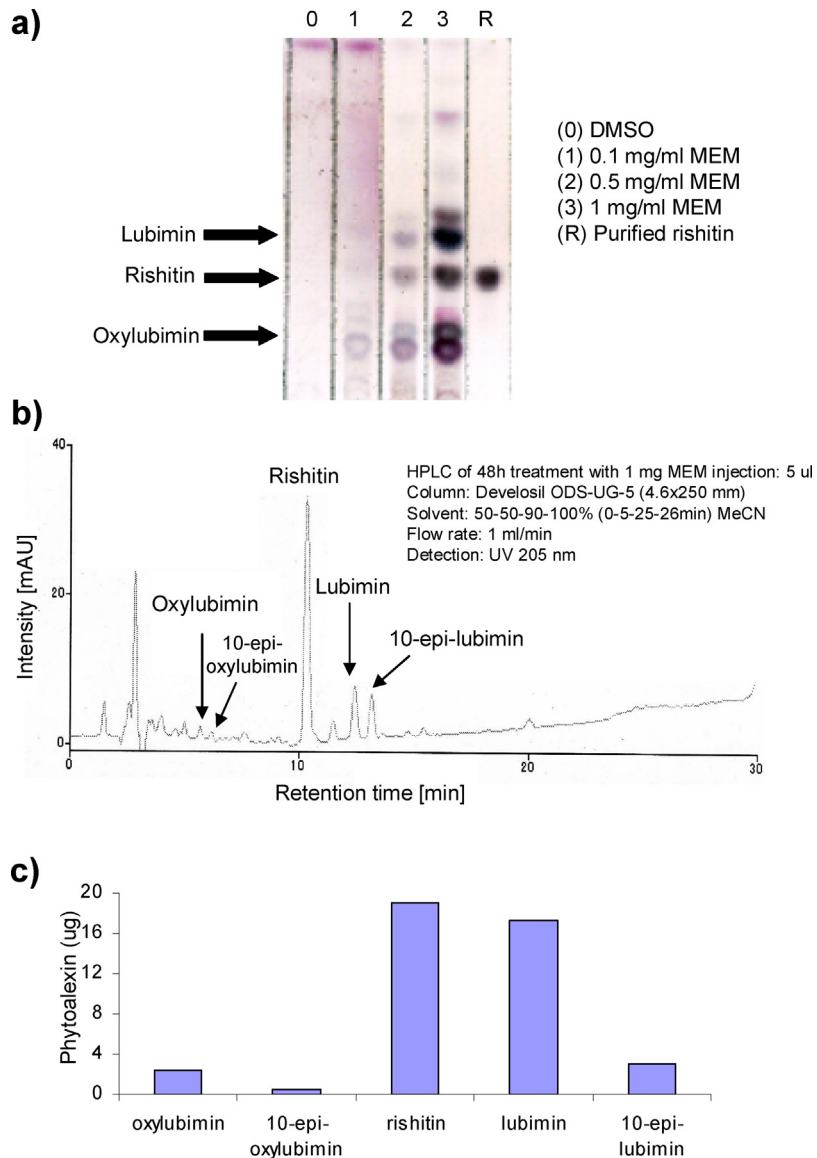


Fig. 4. (Color online.) Production of phytoalexins by MEM in potato tubers. a: for TLC experiment, potato tubers were treated with MEM at three concentrations (0.1, 0.5 and 1 mg/mL), and phytoalexins were extracted 48 h after treatment. DMSO (3%) was used as a control. Equal load of extracted samples and 25 μ g of purified rishitin were separated and developed on a TLC plate. Black arrows indicate the site of rishitin, lubimin, and oxylubimin development in a thin-layer chromatography plate; b: HPLC analysis of rishitin, lubimin and oxylubimin at 1 mg/mL concentration; c: quantification of rishitin, lubimin and oxylubimin from the HPLC peak.

3.4. Induction of cell death

HR-like cell death was examined in potato leaves. In this experiment, average-sized potato leaves (30–40 days after germination) were selected and infiltrated by elicitors by using a syringe without needle from the lower surface of the leaves. Cell death was detected by monitoring the dead areas developed on the leaf's surface 6, 12, 24 and 48 h after treatment with MEM elicitor. As a result, the dead areas were detected within 6 h after treatment with MEM. Elicitor-treated areas became water-soaked like death after 24 h and dried after 48 h (Fig. 5a). Ion leakage has been observed as an indicator of plant cell death [30]. A higher degree of ion leakage was detected in MEM

elicitor-treated leaves after 6 h compared to DMSO-treated leaves (Fig. 5b).

3.5. Nitric oxide-producing activity

NO generation was detected by DAF-2DA-mediated fluorescence in MEM elicitor-treated potato suspension-cultured cells (Fig. 6a). To scavenge NO responsive events in cells, a potent NO scavenger, cPTIO, was used. NO generation in suspension cells treated by MEM was eliminated by cPTIO. A similar experiment was done in potato leaves. The leaf areas were treated with the MEM elicitor and infiltrated with the DAF-2DA solution, and NO production was monitored 1 h later using fluorescence

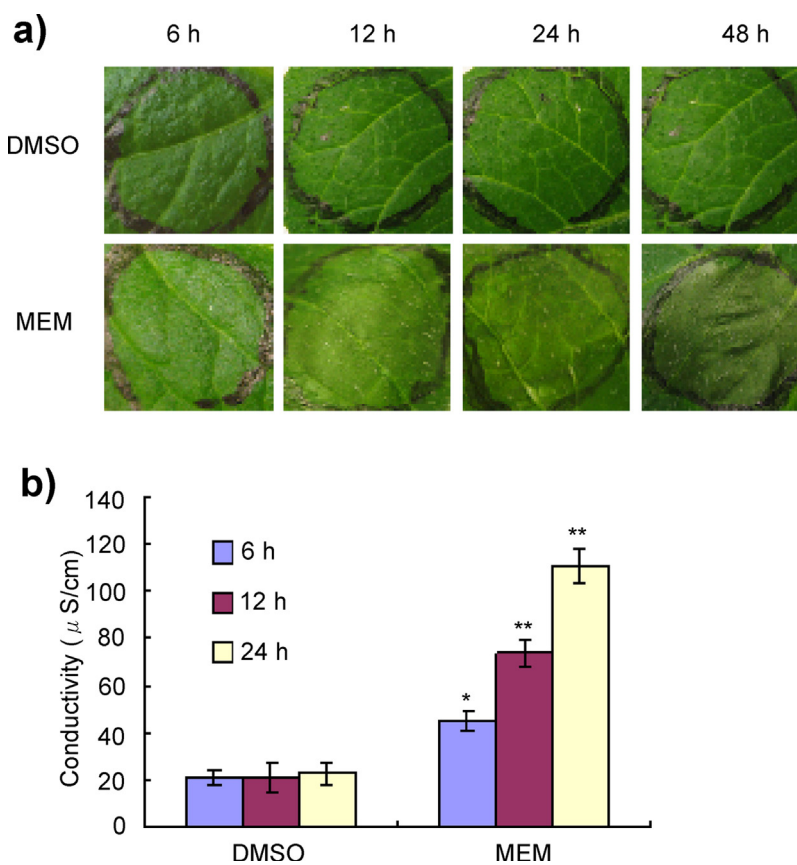


Fig. 5. (Color online.) Induction of cell death by MEM in potato leaves. a: potato leaves were infiltrated with 0.5 mg/mL of MEM and incubated for 48 h. Photographs were taken 6, 12, 24 and 48 h after elicitor treatment. Hypersensitive cell death was observed as a water-soaked lesion 6 h after treatment, which gradually showed increased cell death and by 24 h only dead areas remained in the treated leaf areas. DMSO (3%) was used as a control. The results shown are representative of three separate experiments; b: electrolyte leakage of dead cells of potato leaves. After treatment with elicitors by infiltration into the leaf, leaf discs were floated on distilled water and shaken for 2 h at room temperature. The electrical conductivity was measured using a conductivity meter. Data are means \pm standard deviation from five replications. Data were subjected to Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ versus DMSO control treatment.

stereomicroscopy. Fluorescence was detected 6 and 12 h after elicitor treatment and eliminated by addition of cPTIO (Fig. 6b and c), indicating that the MEM elicitor induced NO in potato leaves.

3.6. Expression of defence-related genes

To investigate whether the defence-related genes are induced by the MEM elicitor, total RNAs from MEM-treated potato suspension-cultured cells were extracted and analyzed by RT-PCR (Fig. 7). MEM elicitor expressed ROS production regulatory genes, *Rboh*s (respiratory burst oxidase homologue). A high expression of *StrbohA* and *StrbohC*, and weak expression of *StrbohB* was observed. The expression of the hypersensitive response marker gene *StHsr203J* indicated that MEM generated ROS-inducing HR. *StPVS3* encoding potato vetispiradiene synthase (PVS) was found highly expressed with MEM treatment. High expression of defence gene *StPR1*, a pathogenesis-related protein 1, was found by MEM elicitor treatment. The MEM elicitor also induced *StNR5* transcript, nitrate reductase 5, a nitric oxide-producing enzyme induced by pathogen or elicitor signals.

3.7. MEM induced disease resistance in potato against *P. infestans*

To investigate the resistance activities during the interaction between MEM-treated potato leaves and *P. infestans*, MEM-treated potato leaves were inoculated with *P. infestans*. MEM (100 $\mu\text{g/mL}$) was sprayed in excised leaves; one day later, inoculation was done by *P. infestans*. Within 3 days after the inoculation of *P. infestans*, DMSO-treated plant leaves showed water-soaked disease symptoms on inoculated leaves (Fig. 8a), and the disease lesions almost extended over entire leaves within 6–7 days. In contrast, few spots were developed in leaves treated with the MEM elicitor (Fig. 8a). The interaction between potato leaves and *P. infestans* was examined using light microscopy in inoculated leaves after staining with lactophenol trypan blue, which is indicative of cell death. As a result, densely stained epidermal and mesophyll cells along with fungal structures were observed in the DMSO-treated leaves six days after inoculation (Fig. 8b). On the other hand, less stained cells were observed in MEM-treated leaves. The percentage of potato leaves with disease severities up to 6 days showed the suppression of disease

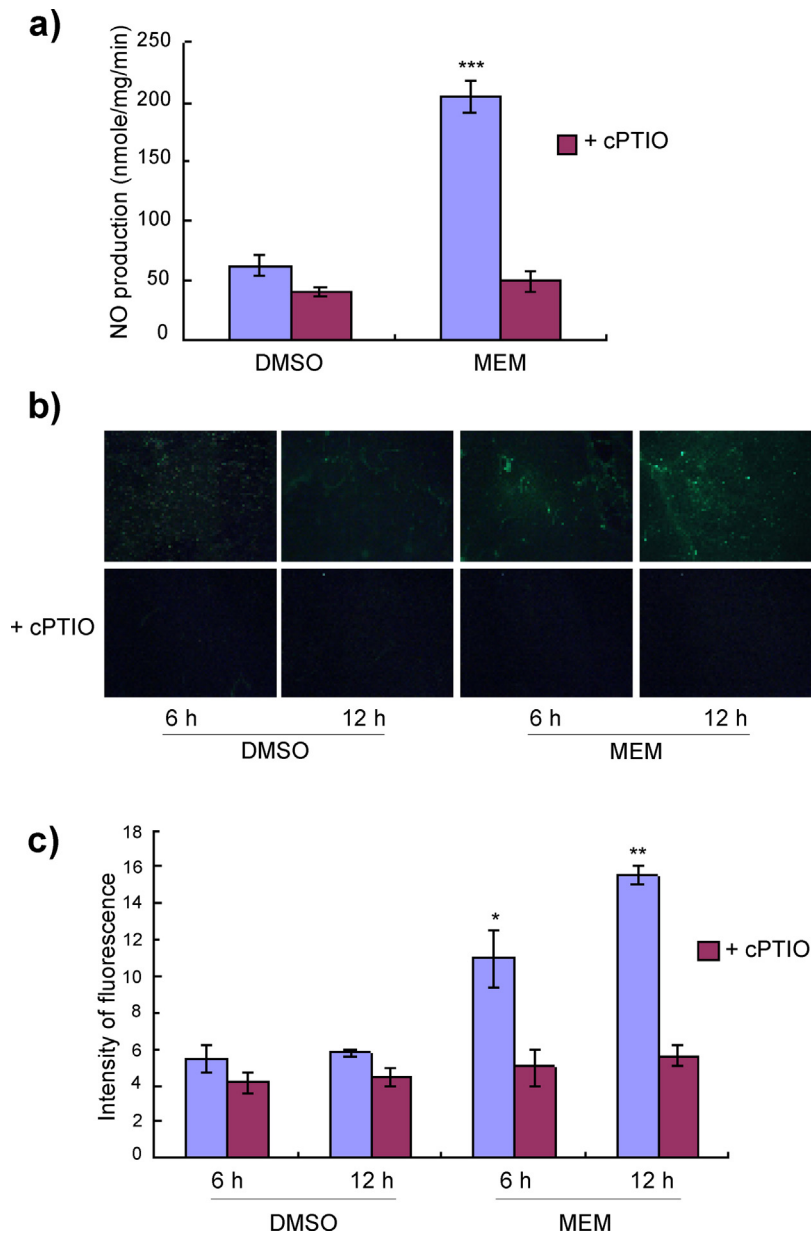


Fig. 6. (Color online.) Induction of nitric oxide by MEM in potato. a: NO generation by the MEM elicitor at the concentration of 100 $\mu\text{g}/\text{mL}$ in potato suspension-cultured cells 3 h after treatment with or without cPTIO. DMSO (3%) was used as a control. The cells were incubated with 10 μM DAF-2 DA for 1 h prior to measurement with a fluorescence spectrophotometer. The excitation and emission wavelengths for DAF-2D were 495 nm and 515 nm (band paths 3 nm and 3 nm), respectively. Each value represents the mean and standard deviation of three replicates; b: NO generation by MEM at the concentration of 0.5 mg/mL in potato leaves 6 h after treatment. The treated leaves were infiltrated with DAF-2 DA and were monitored 1 h later by fluorescence stereomicroscopy. In the case of cPTIO treatment, treated leaf areas were infiltrated with 0.5 mM cPTIO 1 h before infiltration of DAF-2DA; c: signal intensities were quantified by determining the mean channel values for the images 6 or 12 h after treatments, respectively with the histogram function. The results shown are representative of at least three separate experiments. Data were subjected to Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus DMSO control treatment.

in MEM-treated potato leaves (Fig. 8d). From these results, it can be concluded that MEM induces resistance in potato plants against *P. infestans*.

4. Discussion

One of the most rapid defence reactions to pathogen attack is the so-called oxidative burst, which constitutes

the production of ROS, primarily O_2^- and H_2O_2 , at the site of the attempted invasion [31]. In the present study, it was shown that the MEM elicitor induced O_2^- production as well as that of H_2O_2 in potato (Figs. 1, 2 and 3). Doke [32] reported that O_2^- generation is detected in potato tissues as a resistance reaction induced by the invasion of *P. infestans* or treatment of hyphal wall components (HWC) from *P. infestans*. It had been reported that the

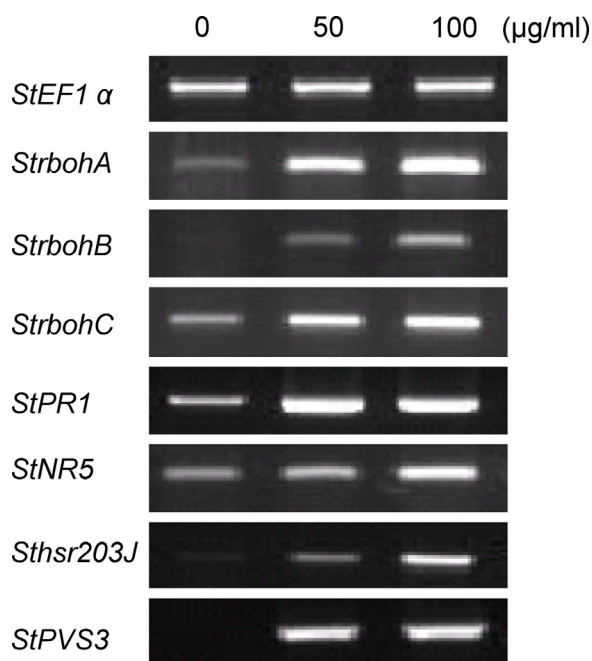


Fig. 7. Gene expression by MEM in potato suspension-cultured cells. Total RNAs were isolated from potato suspension-cultured cells treated for 3 h with 1/10 volume of MEM to get final concentrations of 50 µg/mL and 100 µg/mL, respectively. DMSO (3%) was used as a control. RNAs were analyzed by RT-PCR using specific primers for *StrbohA*, *StrbohB*, *StrbohC*, *StPR1*, *StNR5*, *Sthsr203J*, and *StPVS3*. Equal loads of cDNA were monitored by amplification of constitutively expressed *StEF-1α*. Three repetitions were performed.

oxidative processes were induced in cell suspensions of *Solanum* species by culture filtrate of *P. infestans* [33]. These results, in conjunction with this study, provide evidence that substantial O_2^- generation and peroxidation occurred in MEM-elicited potato suspension-cultured cells as well as in potato leaves.

When O_2^- and H_2O_2 levels are increased in plants in response to an attack by a pathogen, they were followed by a hypersensitive reaction that leads to the death of host cells [34]. MEM showed the induction of HR by cell death on potato leaves (Fig. 5). Kamoun et al. [35] reported that the elicitors, one of the protein elicitors from *Phytophthora* species, were regarded as avirulence factors, as they cause HR-like lesions when infiltrated into tobacco. It was also reported that HWC from *P. infestans* activates O_2^- generation, which is responsible for triggering hypersensitive cell death in the solanaceous host plants, tomato, sweet pepper, and tobacco [36].

There is a good correlation between the degree of host resistance and the level of phytoalexins accumulation. Rishitin and/or solavetivone are phytoalexins that were reported to inhibit the growth of different microorganisms, including *P. infestans* [37,38]. Rishitin was also shown to inhibit *Botrytis cinerea* mycelial growth in both solid and liquid media [39]. The results presented in this article showed that MEM induced the accumulation of phytoalexins, particularly rishitin, lubimin and oxylubimin in potato tubers (Fig. 4). Doke [32] reported that O_2^- generation is closely connected with rishitin production by HWC. ROS can mediate the generation of phytoalexins and of secondary metabolites that arrest the pathogen's growth [8]. It had been reported that *N,N*-dimethylsphingosine induces

phytoalexins production and hypersensitive cell death of Solanaceae plants without generation of ROS [40]. Noritake et al. [14] published that the treatment of potato tuber tissues with NOC-18, a NO donor, induced the accumulation of the potato phytoalexin, rishitin. The MEM elicitor investigated in this study also induced NO generation in potato leaves and suspension-cultured cells (Fig. 6). In addition to phytoalexins production, NO induced other plant defence responses, including cell death [41]. Wilson et al. [42] reported that NO is involved in cell death and defence against biotic and abiotic stresses. Thus, it was suggested that the MEM elicitor induced phytoalexins production on potato tubers due to the activation of superoxide anion and or nitric oxide in plant cells.

In several experiments, ROS- and HR-mediated gene expression was confirmed by some researchers. The treatment of potato tubers with a HWC elicitor caused a rapid but weak transient accumulation of H_2O_2 (phase I), followed by a massive oxidative burst 6 to 9 h after treatment (phase II), to which the expression of *StrbohA* and *StrbohB*, respectively, contributes [43]. The transcript level of *StrbohC* increased drastically and continually in potato leaves after treatment with the HWC elicitor [44]. The treatment of the HWC elicitor from *P. infestans* or infection of *P. infestans* with potato tubers caused transient increases in the transcript level of *PVS3* during, not only incompatible, but also compatible interactions [45]. *Hsr203J* encodes proteins that have homology with esterase, and has been used as a marker of HR [46,47]. Taylor et al. [48] proved that *StPR1* gene is rapidly activated in potato leaves treated with fungal elicitor. It is reported that elicitor-treated NO production in potato tubers induces

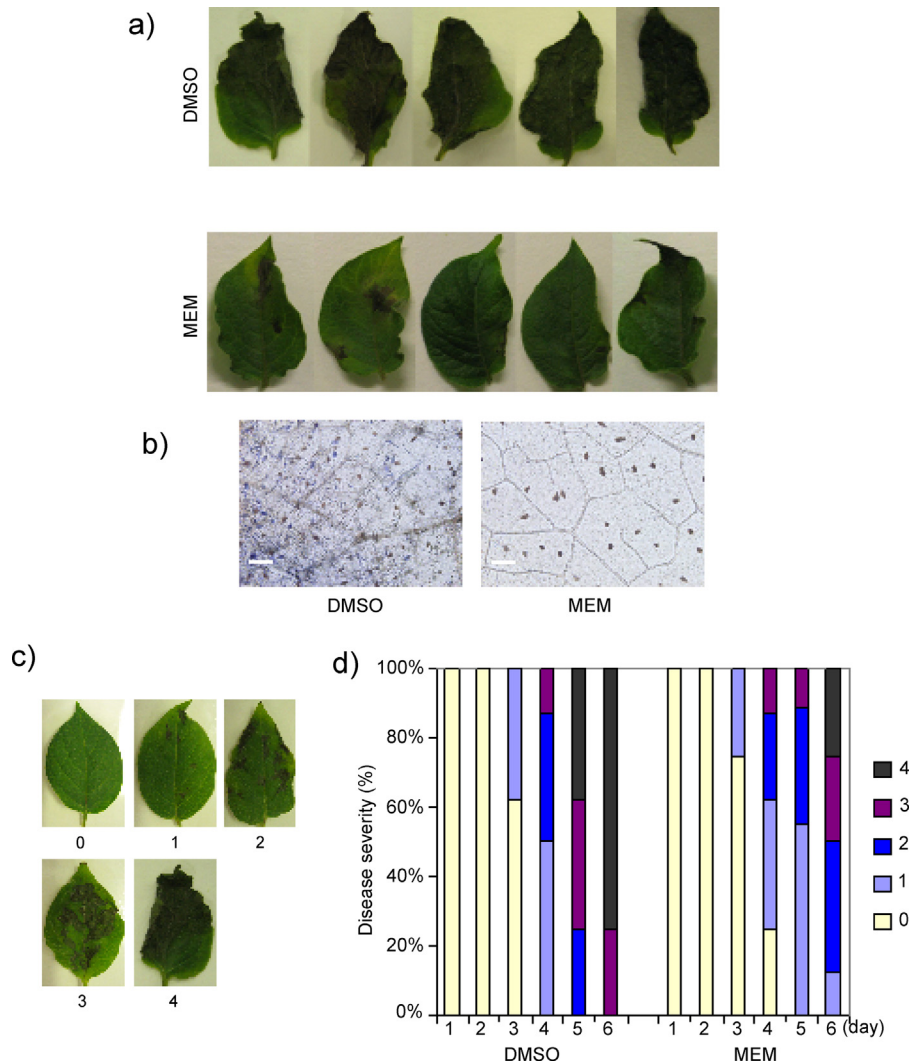


Fig. 8. (Color online.) MEM induced resistance against *Phytophthora infestans* in potato. a: DMSO-treated control plant leaves and MEM potato leaves were kept in an incubator for 24 h and then were inoculated with the spores of *P. infestans*. Photographs were taken from the 6th day after inoculation; b: microscopic observation of potato leaves from the 6th day after inoculation with *P. infestans*. Inoculated leaves were stained with lactophenol trypan blue to visualize the dead plant cells. Bar = 70 μm ; c: appearance of disease symptoms showing differences in severity representative of the five classifications used in D; d: plots showing percentages of potato leaves with disease symptom severities in each of the five classes represented in D, for leaves of DMSO and elicitor-treated plant leaves inoculated with *P. infestans* from 1 to 6 days post-inoculation. At least 10 inoculated leaves from each plant were counted.

StNR5 gene expression [13]. In potato suspension-cultured cells, a group of defence response-related genes, namely *StHsr203j*, *StPVS3*, *StrbohA*, *StrbohB*, *StrbohC*, *StPR1*, and *StNR5* were expressed by the MEM elicitor treatment (Fig. 7). These evidences indicate that the MEM elicitor plays an important role in defence-related gene expression.

For examining the defence role of MEM-treated potato leaves, some of them were inoculated with *P. infestans*. A higher level of resistance in potato was stimulated by MEM against *P. infestans* (Fig. 8). The resistance might be systemic because spraying MEM over a potato leaf surface before challenge inoculation induced resistance along with systemic resistance, inducing *StPR1* gene expression. The activation of systemic-acquired resistance (SAR)

correlates with the expression of the pathogenesis-related 1 (*PR1*) gene [49,50]. Treatment of susceptible potato cultivars with DL- β -aminobutyric acid induced enhanced resistance against *P. infestans* [51,52]. PAMP-triggered defence responses in potato include the accumulation of the signalling molecules salicylic acid (SA) and jasmonic acid (JA), defence gene activation, and hypersensitive cell death [5].

All these results provide a better understanding of the mechanisms by which the MEM elicitor induced defence response in potato. It was shown that MEM from *P. infestans* has the potentiality to induce O_2^- production, hypersensitive cell death, phytoalexins, NO production and defence-related gene expression in potato. Enhanced disease resistance against *P. infestans* might be due to a

MEM-inducing series of responses in plant cells. The authors considered that further purification of MEM is necessary to obtain the compound(s) responsible for O₂⁻ production.

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References

- [1] L.A. Mur, P. Kenton, A.J. Lloyd, H. Ougham, E. Prats, The hypersensitive response; the centenary is upon us but how much do we know? *J. Exp. Bot.* 59 (2008) 501–520.
- [2] M.D. Zurbriggen, N. Carrillo, V.B. Tognetti, M. Melzer, M. Peisker, B. Hause, M.R. Hajirezaei, Chloroplast-generated reactive oxygen species play a major role in localized cell death during the non-host interaction between tobacco and *Xanthomonas campestris* pv. *vesicatoria*, *Plant J.* 60 (2009) 962–973.
- [3] N. Doke, Y. Miura, H.-B. Chai, K. Kawakita, Involvement of active oxygen in induction of plant defense response against infection and injury, in: E. Pell, K. Steffen (Eds.), *Active Oxygen: Oxidative Stress and Plant Metabolism*, American Society of Plant Physiologists, 1991, pp. 84–96.
- [4] L.M. Sanchez, Y. Ohno, Y. Miura, K. Kawakita, N. Doke, Host selective suppression by water-soluble glucans from *Phytophthora* spp. on hypersensitive cell death of suspension-cultured cells from some solanaceous plants caused by hyphal wall elicitors of the fungi, *Ann. Phytopath. Soc. Japan* 58 (1992) 664–670.
- [5] V.A. Halim, A. Hunger, V. Macioszek, P. Landgraf, T. Nurnberger, D. Scheel, S. Rosahl, The oligopeptide elicitor Pep-13 induces salicylic acid-dependent and -independent defense reactions in potato, *Physiol. Mol. Plant Pathol.* 64 (2004) 311–318.
- [6] M.A. Torres, ROS in biotic interactions, *Physiol. Plant.* 138 (2010) 414–429.
- [7] R. Hüelckhoven, Cell wall-associated mechanisms of disease resistance and susceptibility, *Annu. Rev. Phytopathol.* 45 (2007) 101–127.
- [8] I. Thoma, C. Loeffler, A.K. Sinha, M. Gupta, M. Krischke, B. Steffan, T. Roitsch, M.J. Mueller, Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants, *Plant J.* 34 (2003) 363–375.
- [9] M. Delledonne, J. Zeier, A. Marocco, C. Lamb, Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response, *Proc. Natl. Acad. Sci. USA* 98 (2001) 13454–13459.
- [10] M.A. Torres, J.D. Jones, J.L. Dangi, Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*, *Nat. Genet.* 37 (2005) 1130–1134.
- [11] Y.Y. Leshem, Nitric oxide in plants: occurrence, function and use, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2000, p. 154.
- [12] J. Durner, D. Wendehenne, D.F. Klessig, Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose, *Proc. Natl. Acad. Sci. USA* 95 (1998) 10328–10333.
- [13] A. Yamamoto, S. Katou, H. Yoshioka, N. Doke, K. Kawakita, Nitrate reductase, a nitric oxide-producing enzyme: induction by pathogen signals, *J. Gen. Plant Pathol.* 69 (2003) 218–229.
- [14] T. Noritake, K. Kawakita, N. Doke, Nitric oxide induces phytoalexin accumulation in potato tuber tissues, *Plant Cell Physiol.* 37 (1996) 113–116.
- [15] M.J. Xu, J.F. Dong, M.Y. Zhu, Nitric oxide mediates the fungal elicitor-induced hypericin production of *Hypericum perforatum* cell suspension cultures through a jasmonic-acid-dependent signal pathway, *Plant Physiol.* 139 (2005) 991–998.
- [16] H. VanEtten, E. Temporini, C. Wasmann, Phytoalexin (and phytoanticipin) tolerance as a virulence trait: why is it not required by all pathogens? *Physiol. Mol. Plant Pathol.* 59 (2001) 83–93.
- [17] I. Ahuja, R. Kissen, A.M. Bones, Phytoalexins in defense against pathogens, *Trends Plant Sci.* 17 (2012) 73–90.
- [18] R.J. Gray, T. Kokubun, Plant–fungal interactions: the search for phytoalexins and other antifungal compounds from higher plants, *Phytochemistry* 56 (2001) 253–263.
- [19] H.D. VanEtten, D.E. Matthews, P.S. Matthews, Phytoalexin detoxification: importance for pathogenicity and practical implications, *Annu. Rev. Phytopathol.* 27 (1989) 143–164.
- [20] R.M. Bostock, J.A. Kuć, R.A. Laine, Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato, *Science* 212 (1981) 67–69.
- [21] S. Osman, R. Moreau, Potato phytoalexin elicitors in *Phytophthora infestans* spore germination fluids, *Plant Sci.* 41 (1985) 205–209.
- [22] M.S. Monjil, D. Takemoto, K. Kawakita, Defense induced by a bis-aryl methanone compound leads to resistance in potato against *Phytophthora infestans*, *J. Gen. Plant Pathol.* 80 (2014) 38–49.
- [23] M.S. Monjil, Y. Shibata, D. Takemoto, K. Kawakita, Bis-aryl methanone compound is a candidate of nitric oxide producing elicitor and induces resistance in *Nicotiana benthamiana* against *Phytophthora infestans*, *Nitric Oxide* 29 (2013) 34–45.
- [24] M.S. Monjil, Y. Shibata, S. Wada, D. Takemoto, K. Kawakita, Non-host resistance activities of *Arabidopsis thaliana* induced by methanol extract of mycelia from *Phytophthora infestans*, *Int. J. Biosci. Biotechnol.* 1 (2) (2013) 78–85.
- [25] M. Kobayashi, I. Ohura, K. Kawakita, N. Yokota, M. Fujiwara, K. Shimamoto, N. Doke, H. Yoshioka, Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase, *Plant Cell* 19 (2007) 1065–1080.
- [26] H. Thordal-Christensen, Z. Zhang, Y. Wei, D.B. Collinge, Subcellular localization of H₂O₂ in plants, H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction, *Plant J.* 11 (1997) 1187–1194.
- [27] S.I. Yeom, H.K. Baek, S.K. Oh, W.H. Kang, S.J. Lee, J.M. Lee, E. Seo, J.K.C. Rose, B.D. Kim, D. Choi, Use of a secretion trap screen in pepper following *Phytophthora capsici* infection reveals novel functions of secreted plant proteins in modulating cell death, *Mol. Plant Microbe Interact.* 24 (2011) 671–684.
- [28] M. Boccardo, C.E. Mills, J. Zeier, C. Anzi, C. Lamb, R.K. Poole, M. Delledonne, Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host, *Plant J.* 43 (2005) 226–237.
- [29] H. Kato, S. Asai, A. Yamamoto-Katou, H. Yoshioka, N. Doke, K. Kawakita, Involvement of NbNOA1 in NO production and defense responses in INFI-treated *Nicotiana benthamiana*, *J. Gen. Plant Pathol.* 74 (2008) 15–23.
- [30] L. Rizhsky, H. Liang, R. Mittler, The combined effect of drought stress and heat shock on gene expression in tobacco, *Plant Physiol.* 130 (2002) 1143–1151.
- [31] I. Apostol, P.F. Heinstein, P.S. Low, Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Role in defense and signal transduction, *Plant Physiol.* 90 (1989) 106–116.
- [32] N. Doke, Generation of superoxide anion by potato tuber protoplasts during hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity, *Physiol. Plant Pathol.* 23 (1983) 259–367.
- [33] L. Polkowska-Kowalczyk, U. Maciejewska, The oxidative processes induced in cell suspensions of *Solanum* species by culture filtrate of *Phytophthora infestans*, *Z. Naturforsch.* 56c (2001) 235–244.
- [34] K. Apel, H. Hirt, Reactive oxygen species: metabolism, oxidative stress, and signal transduction, *Annu. Rev. Plant Biol.* 55 (2004) 373–399.
- [35] S. Kamoun, M. Young, C.B. Glascok, B.M. Tyler, Extracellular protein elicitors from *Phytophthora* – host-specificity and induction of resistance to bacterial and fungal phytopathogens, *Mol. Plant Microbe Interact.* 6 (1993) 15–25.
- [36] L.M. Sanchez, N. Doke, K. Kawakita, Elicitor-induced chemiluminescence in cell suspension cultures of tomato, sweet pepper and tobacco plants and its inhibition by suppressors from *Phytophthora* spp, *Plant Sci.* 88 (1993) 141–148.
- [37] K. Engström, A.K. Widmark, S. Brishammar, S. Helmersson, Antifungal activity to *Phytophthora infestans* of sesquiterpenoids from infected potato tubers, *Potato Res.* 42 (1999) 43–50.
- [38] P. Stössel, H.R. Hohl, Effect of phytoalexins on hyphal growth and β -glucanases of *Phytophthora infestans*, *Mycopathologia* 73 (1981) 153–159.
- [39] J.A. Glazener, C.H. Wouters, Detection of rishitin in tomato fruits after infection with *Botrytis cinerea*, *Physiol. Plant Pathol.* 19 (1981) 243–248.
- [40] S. Uruma, Y. Shibata, D. Takemoto, K. Kawakita, *N,N*-dimethylsphingosine, an inhibitor of sphingosine kinase, induces phytoalexin production and hypersensitive cell death of Solanaceae plants without generation of reactive oxygen species, *J. Gen. Plant Pathol.* 75 (2009) 257–266.
- [41] D. Wendehenne, J. Durner, D.F. Klessig, Nitric oxide: a new player in plant signalling and defence responses, *Curr. Opin. Plant Biol.* 7 (2004) 449–455.
- [42] I.D. Wilson, S.J. Neill, J.T. Hancock, Nitric oxide synthesis and signalling in plants, *Plant Cell Environ.* 31 (2008) 622–631.

- [43] H. Yoshioka, K. Sugie, H.J. Park, H. Maeda, N. Tsuda, K. Kawakita, N. Doke, Induction of plant gp91 phox homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato, *Mol. Plant Microbe Interact.* 14 (2001) 725–736.
- [44] C. Yamamizo, K. Kuchimura, A. Kobayashi, S. Katou, K. Kawakita, J. Jones, N. Doke, H. Yoshioka, Rewiring mitogen-activated protein kinase cascade by positive feedback confers potato blight resistance, *Plant Physiol.* 140 (2006) 681–692.
- [45] H. Yoshioka, N. Yamada, N. Doke, cDNA cloning of sesquiterpene cyclase and squalene synthase, and expression of the genes in potato tuber infected with *Phytophthora infestans*, *Plant Cell Physiol.* 40 (1999) 993–998.
- [46] P. Czernic, H.C. Huang, Y. Marco, Characterization of hsr201 and hsr515, two tobacco genes preferentially expressed during the hypersensitive reaction provoked by phytopathogenic bacteria, *Plant Mol. Biol.* 31 (1996) 255–265.
- [47] D. Pontier, M. Tronchet, P. Rogowsky, E. Lam, D. Roby, Activation of hsr203, a plant gene expressed during incompatible plantpathogen interactions, is correlated with programmed cell death, *Mol. Plant Microbe Interact.* 11 (1998) 544–554.
- [48] J. Taylor, K. Fritzemaier, I. Hauser, E. Kombrink, F. Rohwer, J. Schroeder, G. Strittmatter, K. Hahlbrock, Structural analysis and activation by fungal infection of a gene encoding a pathogenesis-related protein in potato, *Mol. Plant Microbe Interact.* 3 (1990) 72–77.
- [49] E.R. Ward, S.J. Uknes, S.C. Williams, S.S. Dincher, D.L. Wiederhold, D.C. Alexander, P. Ahl-Goy, J.P. Métraux, J.A. Ryals, Coordinate gene activity in response to agents that induce systemic acquired resistance, *Plant Cell* 3 (1991) 1085–1094.
- [50] K. Lawton, L. Friedrich, M. Hunt, K. Weymann, T. Staub, H. Kessmann, J. Ryals, Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway, *Plant J.* 10 (1996) 71–82.
- [51] L. Eschen-Lippold, S. Altmann, S. Rosahl, DL-beta-aminobutyric acid-induced resistance of potato against *Phytophthora infestans* requires salicylic acid but not oxylipins, *Mol. Plant-Microbe Interact.* 23 (2010) 585–592.
- [52] E. Liljeroth, T. Bengtsson, L. Wiik, E. Andreasson, Induced resistance in potato to *Phytophthora infestans*-effects of BABA in greenhouse and field tests with different potato varieties, *Eur. J. Plant Pathol.* 127 (2010) 171–183.