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Abstract

Plants react to microbial attack with a number of defense mechanisms, including the synthesis of volatile organic compounds (VOCs) and the production of reactive oxygen species (ROS). These responses are triggered by elicitors derived from either the cell surface of pathogens or the incomplete hydrolysis of the plant cell wall. Here we show the response of rice (*Oryza sativa* L., cv *Gigante Vercelli*) cell cultures following treatment with cell wall hydrolysates prepared from the rice blast *Magnaporthe oryzae*. Elicitation prompted the production of several plant VOCs, which were analyzed by stir bar sorptive extraction from both the liquid and head-space phase (SBSE and HSSE, respectively) and gas chromatography coupled to mass spectrometry (GC–MS) analysis. VOCs included alkanes, alkenes and long-chain alcohols as well as cinnamyl alcohol, myristicin, a sesquiterpene alcohol (caryolan-1-ol), 1-butanamide and 2-pentylfuran. The major released compounds, 1-octanol and 1-decanol, were found to induce ROS production in both elicited and non-elicited rice cells and showed fungistatic activity against the pathogen *M. oryzae*. The possible role of induced VOCs and ROS production in the plant–pathogen interaction is discussed.

Abbreviations

- SBSE, stir bar sorptive extraction;
- HSSE, head-space sorptive extraction;
- GC–MS, gas chromatography coupled to mass spectrometry;
- VOC, volatile organic compound;
- MEA, malt extract agar

Keywords

- *M. oryzae*;
- Rice (*Oryza sativa*);
- Volatile organic compounds;
- Reactive oxygen species;
- Long-chain alkanols;
- 1-Octanol;
- 1-Decanol;
- Cell wall hydrolysate

Introduction

M. oryzae (anamorph, *Pyricularia grisea*, formerly *P. oryzae*) previously known as *M. grisea* [T.T. Hebert] Yaegashi and Udagawa, is a filamentous heterothallic Ascomycete causing blast, a disease that affects many species of the grass family (Talbot, 2003). The rice blast is one of the most serious diseases on cultivated rice and it is capable of destroying enough rice to feed 60 million people every year (Zeigler et al., 1994 and Skamnioti and Gurr, 2009). Due to the experimental tractability and socioeconomic impact of rice blast, the fungus has served as an important model organism in studies aimed at understanding the biology of fungal plant pathogens (Jeon et al., 2008), the structure of pathogen population and the breakdown of plant resistance genes (Ebbole, 2007) and transcriptome profiling during invasive plant infection (Mathioni et al., 2011).

The interaction between rice and *M. oryzae* is the focus of extensive studies on plant disease resistance and fungal infection mechanisms (Ribot et al., 2008). One of the fastest plant defense reactions against fungal pathogens is the production of reactive oxygen species (ROS) (Apostol et al., 1989). In a previous study, we showed that *M. oryzae* cell-wall hydrolysate elicits peroxide release from cultured cells of some rice cultivars (Rožkowicz et al., 2003). Moreover, the same elicitor was found to induce a significant increase in phenylalanine ammonia lyase (PAL)-specific activity in the same cultivars (Forlani, 2010). Thus, in addition to being directly active on pathogens, *M. oryzae*-induced ROS may also function as a second messenger in the induction of various plant defense-related genes (see also Guo et al., 2011). In this way, rice blast must survive secondary metabolite defense compounds produced by the host plant (Ebbole 2007). Volatile organic compounds (VOCs)-mediated interactions among plants and microorganisms have been frequently documented (Campos et al., 2010). In the case of the fungi–plant interaction, VOC released by plants are of ecological importance as defense molecules, and the emission of microbial VOC (MVOC) may exert significant effects on plants (Minerdi et al., 2011 and Splivallo et al., 2007b).

By using as elicitor the same *M. oryzae* cell-wall hydrolysate found to elicit peroxide release and PAL activity (Forlani 2010; Rožkowicz et al., 2003), we determined the VOCs profile induced in *O. sativa* cell suspensions by means of head-space analysis coupled to gas chromatography–mass spectrometry (GC–MS) and we assessed the possible relationship with ROS induction. Furthermore, isolated compounds were tested to assess the possible fungitoxic/fungistatic effect on *M. oryzae*.

Materials and methods

Plant material, growth conditions and elicitor treatment

Rice (*Oryza sativa* L., cv *Gigante Vercelli*) cell suspension cultures were grown in a liquid medium consisting of Murashige and Skoog salts and vitamins supplemented with 30 g L^{-1} sucrose and 2 mg L^{-1} 2.4D; 0.2% (v/v) Plant Preservative Mixture solution (Plant Cell Technology, Inc., Washington, DC, USA) was routinely added to reduce the risk of microbial contamination. Cells were grown in 250 mL or 1 L Erlenmeyer flasks containing 62 mL or 250 mL suspensions, respectively. Flasks were incubated under dim light (<50 µmol m^{-2} s^{-1}) on a rotary shaker (100 rpm) at 24 ± 1 °C. Subcultures were made every 14 days by transferring 12 mL aliquots to 50 mL of fresh medium. Cultures to be used for experiments were maintained in continuous balanced growth by subculturing every 7 days. Mycelial wall hydrolysates (100 mg L^{-1}) were added to the culture medium 3 days after the inoculum. Elicitor fractions were obtained from the purified mycelial cell wall of *Magnaporthe oryzae* by thermal hydrolysis, as previously described (

Rožkowicz et al., 2003). Organic matter was quantified as chemical oxygen demand using the Dr. Lange cuvette test LCK 014 (Dr. Lange UK Ltd.), according to the manufacturer's instructions.

Sorption of VOCs on SBSE and HSSE

Stir bars (SBSE, Twister from Gerstel, 0.5 mm thick, 10 mm long, 1 mm polydimethylsiloxane coating) were dropped into 250 mL rice cell cultures in 1 L flasks at the same time at which fungal hydrolysate was added, one SBSE for each flask. After 4 h, SBSE were removed and replaced with freshly conditioned ones. The same was done after 8 and 24 h. The last set was harvested 48 h following elicitation. The experiment was repeated threefold (biological replicates), with three replications each time (technical replicates). VOCs produced by untreated controls were collected with the same protocol but separately in time, in order to avoid drift effects. Stir bars were also suspended on the cultures in order to adsorb volatiles released in the head space (head-space sorptive extraction, HSSE) and the experiment was done as described for SBSE. Twisters were also exposed to increasing concentrations of 1-octanol and 1-decanol dissolved in the medium, both in HSSE and SBSE configuration. A calibration curve was obtained to calculate the relationship between SBSE/HSSE response and the content of the target molecules.

SBSE/HSSE desorption and GC–MS analysis

SBSE/HSSE were introduced in the Gerstel "Thermal Desorption Unit" operating under the following conditions. He flux of 50 mL/min. Splitless mode. Temperature: start 36 °C – hold for 0.5 min, ramp of 25 °C/min to 260 °C – hold for 5 min. Transfer temperature to the "Cooled Injection System" (CIS): 260 °C. CIS conditions: initial temperature −50 °C – hold for 0.2 min, ramp of 12 °C/s to 290 °C – hold for 3 min. Splitless mode for the first 6 min, then split 1:20. Column: HP 5 (HP 19091J-433) – 5% diphenyl, 95% dimethyl siloxane – oven conditions: starting temperature: 50 °C, ramp of 3 °C/min to 200 °C, hold for 10 min; then ramp of 10 °C/min to 290 °C, hold for 10 min. Helium (1.0 mL/min) was used as a carrier gas. Quadrupole detector in autotune mode, ion M+, operating at 70 eV (MS source 150 °C, MS quad 230 °C).

The following authentic standards where purchased from Sigma. 1-Octanol, 1-decanol, 3,7-dimethyl-1-octanol. For calibration, SBSE were stirred in water or medium containing increasing concentrations of standards (from 100 ppb to 100 ppm). Extraction conditions were exactly the same as for the elicitor experiments. Calibration for each standard was repeated at least three times.

VOCs identification was performed by comparison with spectra in mass spectra databases (NIST 98), comparison of Kovats retention indices to the literature data (http://www.pherobase.com/database/kovats/kovats-index.php; http://www.flavornet.org/flavornet.html, and an in-house database), and direct GC–MS comparison with authentic standards. At least nine replicates were performed for each experiment.

Rice culture oxidative burst measurement

The induction of superoxide synthesis in VOCs-treated rice cells was measured *in vivo* by quantifying the corresponding amount of hydrogen peroxide through the oxidation of 3,5-dichloro-2-hydroxybenzensulfonic acid (DCBS) by exogenous peroxidases, and spectrophotometric detection of the product after reaction with 4-aminoantipyrine (AAP), as described previously. Briefly, cultured cells were harvested by vacuum filtration onto a 50 µm nylon mesh, weighed, resuspended at 50 mg (fresh weight) mL^{-1} in 20 mL of 200 mM K phosphate buffer, pH 6.5, and transferred into 100-mL Erlenmeyer flasks on a rotary shaker at 100 rpm. The reaction was started by the addition of 1 mM DCHBS, 0.1 mM AAP and 4 mg L^{-1} horseradish peroxidase (Sigma

P8125). At 10-min intervals and up to 60 min, culture aliquots (1 mL) were withdrawn. Cells were sedimented by centrifugation for 1 min at 12,000 × *g*, and the supernatant was read at 515 nm against time-0 blanks. H_2O_2 concentration was calculated from a molar extinction coefficient of 26,000 M^{-1} cm^{-1}. The rate of hydrogen peroxide release was estimated from the power regression equation of AAP–DCHB complex concentration over time. Each treatment was carried out at least in triplicate, and means ± standard errors were reported.

VOCs inhibition of *M. oryzae* growth

Two strains of *M. oryzae* (MUT 4183 and MUT 4184, isolated from *condor* and *ghibli* rice varieties, respectively) were used. The fungal inoculum consisted of 5 mm diameter discs taken from the margin of actively growing colonies. These disks were placed at the center of 6 cm plates containing 8 mL of malt extract agar (MEA) from which a piece of 5 mm agar was removed and replaced with the fungal inoculum. A small aluminum bin was glued on the lid of each plate with a drop of silicone. After the fungi were inoculated, the plates were inverted and 2 µL of sterile water (control), or either 20 ppm 1-octanol or 1-decanol were placed into the aluminum bins. The plates were immediately sealed with Parafilm® and incubated separately in sealed plastic bags in the dark at 24 °C. The radial growth of fungi was monitored for 14 days. At the end of experiment, several microscopic slides were prepared from each plate to assess the presence of any morphological change of the mycelium. Finally, to verify whether the effect of secondary metabolites was fungistatic or fungitoxic, 5 mm diameter mycelial discs taken from the margin of colonies grown in presence of 1-octanol and 1-decanol were transferred into plates of MEA devoid of alcohols.

Statistical analysis

Data were analyzed by using standard statistical procedures for linear regression, analysis of variance and *t*-test.

Results

VOCs production in rice cell cultures upon M. oryzae hydrolysate elicitation

In order to evaluate the VOCs emission of cultured rice cells, we used two methods: the direct sorption of VOCs released in the liquid medium by SBSE and the sorption of VOCs that were released into the atmosphere from the liquid medium by HSSE. Preliminary tests showed that healthy rice cell suspensions turned brownish when shaken in the same incubator where rice cells were incubated with the *M. oryzae* hydrolysate. Thus, in order to avoid contamination, we always ran experiments at different times and different incubators for controls and elicited plant cells.

The time-course chemical composition of VOCs released in the culture medium by rice cells is described in Table 1. Overall, after elicitation, alkanes, alkenes and long-chain alcohols were the main compounds released in the medium, along with cinnamyl alcohol, myristicin, a sesquiterpene alcohol (caryolan-1-ol), 1-butanamide (or butylamide) and 2-pentylfuran. Aliphatic alcohols (alkanols) ranged from butanol to pentadecanol, whereas alkanes and alkenes were mainly represented by undecane, dodecane and dodecene. The content of alcohols was always significantly higher in elicited plants with respect to controls.

Table 1. Chemical composition of volatile organic compounds in rice cell cultures media in controls and after elicitation with *Magnaporthe oryzae* hydrolysate in four time points from

elicitor inoculation. Values are expressed as ppb; (±SEM); tr, trace amounts; nd, not detected; NC, not computable.

	4 h				8 h				24 h				48 h			
Compounds	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**
Alcohols																
2-Methyl-1-butanol	132.1 (47.2)	280.8 (132.2)	>0.05	2.13	tr	221.1 (100.5)	NC	NC	201.8 (80.3)	230.3 (101.2)	>0.05	1.14	tr	tr	NC	NC
Benzyl alcohol	nd	tr	NC	NC	nd	tr	NC	NC	nd	78.1 (9.9)	NC	NC	41.3 (10.3)	80.2 (49.0)	>0.05	1.94
1-Octanol	22.4 (5.1)	166.4 (29.9)	0.03	7.44	tr	923.6 (175.3)	NC	NC	31.5 (4.2)	804.6 (105.8)	0.05	25.51	28.4 (7.7)	266.3 (14.3)	<0.01	9.37
3,7-Dimethyl-1-octanol	122.4 (56.3)	32.5 (15.3)	>0.05	0.27	tr	52.8 (12.7)	NC	NC	tr	166.6 (104.7)	NC	NC	nd	120.8 (25.7)	NC	NC
1-Decanol	117.3 (61.2)	550.8 (278.5)	>0.05	4.70	tr	3656.8 (659.2)	NC	NC	109.4 (21.1)	3761.0 (399.5)	0.04	34.37	100.6 (11.0)	4392.8 (430.4)	0.01	43.65
1-Undecanol	nd	nd	NC	NC	nd	22.2 (2.3)	NC	NC	nd	118.2 (27.4)	NC	NC	nd	230.2 (34.8)	NC	NC
1-Dodecanol	473.1 (201.4)	379.5 (177.2)	>0.05	0.80	83.3 (9.7)	528.2 (80.3)	0.04	6.34	497.8 (200.5)	1184.0 (687.8)	>0.05	2.38	429.7 (330.1)	930.5 (434.9)	>0.05	2.17
1-Pentadecanol	nd	nd	NC	NC	nd	tr	NC	NC	nd	26.3 (6.2)	NC	NC	nd	nd	nd	NC
Cinnamyl alcohol	tr	38.8 (7.8)	NC	NC	nd	80.3 (22.5)	NC	NC	nd	50.6 (8.4)	NC	NC	nd	25.3 (6.6)	NC	NC
Caryolan-1-ol	nd	85.2 (21.5)	NC	NC	nd	40.2 (6.4)	NC	NC	16.8 (10.4)	40.3 (29.6)	>0.05	2.40	29.6 (12.3)	23.2 (17.1)	>0.05	0.78
Alkanes/alkenes																

Compounds	4 h				8 h				24 h				48 h			
	Control cells (*C*) Mean	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**
4-Methyl-undecane	nd	nd	NC	NC	nd	nd	NC	NC	nd	32.5 (16.9)	NC	NC	nd	124.9 (33.1)	NC	NC
Cis-5-dodecene	142.0 (73.8)	294.7 (112.9)	>0.05	2.08	28.4 (21.5)	30.2 (3.3)	>0.05	1.06	56.7 (31.7)	29.9 (18.5)	>0.05	0.53	51.7 (33.8)	26.0 (17.2)	>0.05	0.50
Cis-3-dodecene	69.9 (30.7)	74.3 (29.9)	>0.05	1.06	tr	28.6 (12.2)	NC	NC	23.7 (20.4)	37.8 (12.5)	>0.05	1.59	30.3 (29.2)	26.7 (9.3)	>0.05	0.88
Trans-3-dodecene	54.7 (34.1)	tr	NC	NC	tr	35.6 (21.6)	NC	NC	33.3 (27.0)	28.7 (17.1)	>0.05	0.86	nd	15.5 (4.0)	NC	NC
Dodecane	nd	nd	NC	NC	tr	90.9 (14.2)	NC	NC	51.9 (32.0)	55.1 (16.3)	>0.05	1.06	58.2 (22.9)	48.5 (36.1)	>0.05	0.83
2,6,10-Trimethyl-dodecane	nd	58.3 (12.1)	NC	NC	tr	nd	NC	NC	21.8 (20.1)	53.4 (12.1)	>0.05	2.45	47.1 (11.4)	45.2 (11.5)	>0.05	0.96
1-Chloro-dodecane	252.1 (133.7)	250.0 (155.3)	>0.05	0.99	47.1 (9.2)	113.0 (19.4)	0.02	2.40	84.0 (29.3)	88.6 (20.4)	>0.05	1.06	131.5 (29.4)	97.7 (44.2)	>0.05	0.74
Other compounds																
1-Butanamide	60.8 (24.6)	tr	NC	NC	tr	244.3 (111.3)	NC	NC	tr	235.2 (85.0)	NC	NC	454.8 (399.2)	tr	NC	NC
2-Pentylfuran	tr	30.5 (7.7)	NC	NC	tr	tr	NC	NC	28.3 (15.9)	30.3 (14.2)	>0.05	1.07	24.6 (4.8)	99.1 (55.1)	>0.05	4.03
Myristicin	nd	tr	NC	NC	nd	69.8 (12.5)	NC	NC	nd	119.6 (25.1)	NC	NC	nd	21.8 (3.8)	NC	NC
2-	nd	19.4	NC	N	nd	43.2	NC	N	nd	149.	NC	NC	nd	110.	NC	NC

Compounds	4 h				8 h				24 h				48 h			
	Control cells (*C*) Mean	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**	**Control cells (*C*) Mean**	**Elicited cells (*E*) Mean**	***P* value**	***E*/*C* ratio**
Tridecanone		(2.1)		C		(22.9)		C		9 (41.5)				8 (38.0)		

After 4 h from elicitor inoculation, five new compounds were produced by elicited rice cells: cinnamyl alcohol, the sesquiterpene caryolan-1-ol, 2,6,10-trimetyl-dodecane, 2-pentylfuran and 2-tridecanone. The content of 1-octanol (7.44-fold) significantly increased, with respect to controls. After 8 h from *M. oryzae* hydrolysate inoculum, most of the identified compounds were only present in elicited cells (Table 1). Significantly higher amounts of 1-chloro-dodecane and 1-dodecanol were present in elicited cells, with respect to controls (2.40- and 6.34-folds, respectively). After 24 h, the content of 1-octanol and 1-decanol was found significantly higher in elicited cultures with respect to controls (25.51-fold and 34.37-fold, respectively), whereas benzyl alcohol, 3,7-dimethyl-1-octanol, 1-undecanol, 1-pentadecanol, cinnamyl alcohol, 1-butanamide, 4-methyl undecane, 2-tridecanone and myristicin were found only in elicited cells. Finally, after 48 h from inoculum, the content of 1-decanol and 1-octanol (43.65-fold and 9.37-fold, respectively) significantly increased in elicited cells, whereas 3,7-dimethyl-1-octanol, 1-undecanol, cinnamyl alcohol, *trans*-3-dodecene, 2-tridecanone and myristicin were found only in elicited cultures. With respect to controls, the content of 1-butanamide was significantly higher at 8 and 24 h in elicited cells and lower at the other data points.

In rice cell cultures elicited with the *M. oryzae* hydrolysate, 1-octanol reached maximum levels after 8 h, 1-decanol showed a progressive increase from 4 to 48 h, whereas 1-dodecanol reached the maximum level after 24 h.

The chemical composition of VOCs released into the atmosphere from the incubation medium is described in Table 2. 1-Decanol was found to be induced about 267 times in elicited cells, whereas 1-octanol and 1-pentadecanol showed a 8.35- and 3.81-fold change with respect to controls. Benzyl alcohol, 3,7-dimethyl-1-octanol, 1-undecanol, caryolan-1-ol, *trans*-3-dodecene, dodecane, myristicin and 2-tridecanone were only found in elicited cells, whereas cinnamyl alcohol, *cis*-3-dodecene, 1-butanamide, and 2-pentylfuran were significantly higher in VOCs emissions of elicited cells, with respect to controls. Even in this case, the overall emission of alcohols was significantly higher in elicited cells than in controls (Table 2).

Table 2. Head space sorptive extraction (HSSE) chemical composition of volatile organic compounds in rice cell cultures in controls and after 48 h elicitation with *Magnaporthe oryzae* hydrolysate. Values are expressed as ppb; (±SEM); tr, trace amounts; nd, not detected; NC, not computable.

Compound	Control cells (*C*) Mean	Elicited cells (*E*) Mean	*P*-value	*E*/*C* ratio
Alcohols				
2-Methyl-1-butanol	168.3 (81.4)	204.8 (77.2)	>0.05	1.22
Benzyl alcohol	nd	75.0 (34.1)	NC	NC
1-Octanol	45.1 (10.2)	376.2 (33.1)	<0.01	8.35
3,7- Dimethyl-1-octanol	nd	33.7 (20.0)	NC	NC
1-Decanol	42.0 (4.4)	11221.2 (497.6)	<0.01	267.09
1-Undecanol	tr	813.4 (300.2)	NC	NC
1-Dodecanol	tr	1270.1 (299.4)	NC	NC
1-Pentadecanol	17.4 (3.3)	66.3 (5.8)	<0.01	3.81
Cinnamyl alcohol	38.2 (4.3)	75.7 (7.0)	0.02	1.98
Caryolan-1-ol	nd	418.3 (117.3)	NC	NC
Alkanes/alkenes				
4-Methyl-undecane	186.7 (100.7)	229.1 (171.3)	>0.05	1.23
Cis-5-dodecene	48.6 (21.6)	50.4 (27.5)	>0.05	1.04
Cis-3-dodecene	25.9 (2.1)	49.3 (3.9)	0.04	1.90
Trans-3-dodecene	nd	81.9 (7.9)	NC	NC
dodecane	nd	36.6 (4.3)	NC	NC
4,8-Dimethyl-undecane	31.5 (19.4)	44.8 (33.7)	>0.05	1.42
2,6,10-Trimethyl-dodecane	nd	nd	NC	NC
1-chloro-dodecane	nd	nd	NC	NC
Other compounds				
Myristicin	nd	58.3 (4.9)	NC	NC
1-Butanamide	226.9 (21.3)	512.9 (31.6)	0.02	2.26
2-Pentylfuran	41.3 (3.6)	800.1 (64.1)	<0.01	19.36
2-Tridecanone	nd	3677.7 (227.0)	NC	NC

Finally, in order to check for possible contamination, we analyzed *M. oryzae* hydrolysate and medium VOCs contents. No detectable amounts of 1-octanol, 1-decanol or 1-undecanol were found in these samples (Supplementary Table S1).

Oxidative burst in rice cell cultures exposed to long-chain alcohols

Having assessed the ability of the fungal hydrolysate to induce VOCs production in rice cell cultures, we also investigated whether some of the main VOCs might be responsible for the browning effect observed on healthy rice cell cultures. We then evaluated the effect 1-octanol and 1-decanol, two main VOCs produced by rice cell cultures in response to *M. oryzae* hydrolysate, on hydrogen peroxide production in rice cell cultures. Incubation of rice cells with 1-octanol was found to induce a ROS burst after 60 min from treatment (Fig. 1). In particular, 10 ppm 1-octanol was found to be more effective as an early elicitor, whereas 50 ppm 1-octanol was able to sustain the ROS burst up to 150 min. When 1-decanol was used as an elicitor, we found that low concentration (2 ppm) was able to induce a ROS burst about 2.5 h after treatment, whereas higher concentrations induced an earlier and proportional ROS burst (10 ppm and 50 ppm, respectively), which was sustained up to about 4 h (Fig. 2). However, treated cultures did not brown thereafter (not shown).

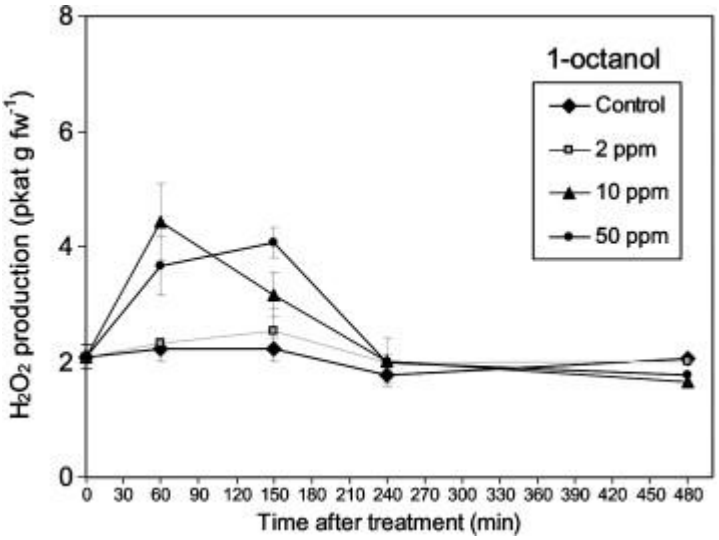

Fig. 1. Time-course analysis of increasing concentration of 1-octanol on H_2O_2 production in rice cell cultures. At all time points, not significant differences were found between controls and 2 ppm 1-octanol. At 10 ppm 1-octanol significant differences in H_2O_2 production were found after 60 min, whereas treatment with 50 ppm 1-octanol prompted a significant increase in H_2O_2 production from 60 to 150 min. After 240 min, no significant difference was found at all concentrations, with respect to control.

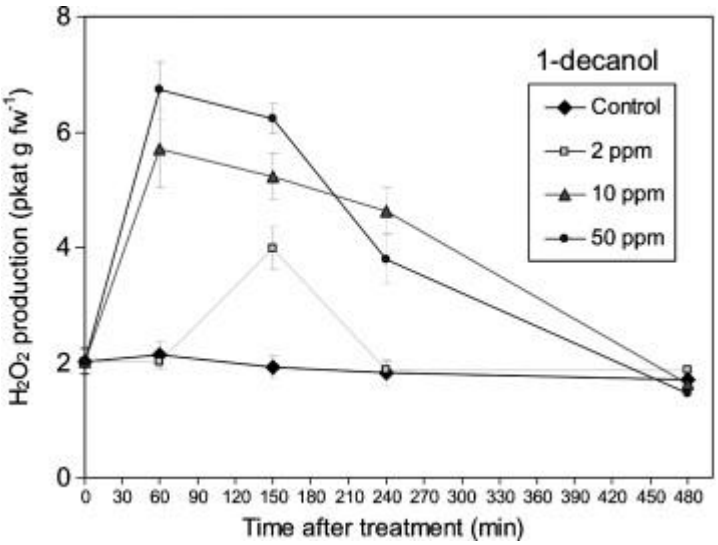

Fig. 2. Time-course analysis of increasing concentration of 1-decanol on H_2O_2 production in rice cell cultures. 2 ppm 1-decanol significantly increased H_2O_2 production of rice cells after 150 min, whereas at higher concentration both 10 ppm and 50 ppm concentration prompted a significant H_2O_2 burst from 60 to 240 min. After 8 h (480 min) no significant difference was found at all concentrations, with respect to control.

Effect of 1-octanol and 1-decanol on M. oryzae

The two strains of *M. oryzae* (MUT 4183 and MUT 4184) always displayed comparable growth rates both in the presence and in absence of 1-octanol and 1-decanol (data not shown). However, the radial growth rate of both strains was significantly inhibited by 20 ppm of both 1-octanol and 1-decanol (Fig. 3). 1-Octanol was found to exert a more pronounced fungus growth inhibiting effect with respect to 1-decanol (about −52% and −35% at the end of the experiment), although no significant differences were recorded between the two plant secondary metabolites.

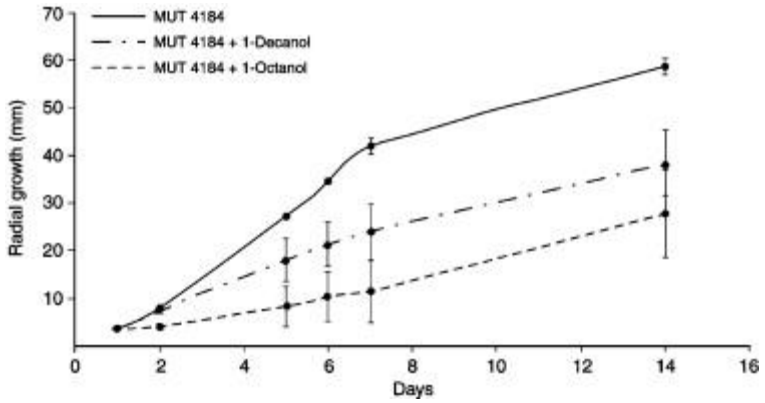

Fig. 3. The two strains of *M. oryzae* (MUT 4183, MUT 4184) are incubated in the presence of 20 ppm 1-octanol and 1-decanol and their growth rate is evaluated. The radial growth rate of both strains was significantly inhibited by both 1-octanol and 1-decanol. Bars indicate standard error.

From a morphological point of view the macroscopic features of fungal colonies in presence/absence of the two metabolites did not show any differences (data not shown). The microscopic analysis revealed a greater presence of chlamydospores in mycelia grown in the presence of 20 ppm 1-octanol and 1-decanol and, exclusively for the strain MUT 4183, conidia production was also inhibited.

Finally, the growth rate of mycelia taken from colonies exposed to 20 ppm 1-octanol and 1-decanol was similar to controls, confirming the fungistatic effect of the two volatiles.

Discussion

The close relationship between plant secondary metabolism and defense response is widely recognized. Plants respond to attack of pathogens and insect herbivores by activating an array of defense mechanisms, including the synthesis of secondary metabolites (Maffei et al., 2007). Resistance of rice plants against *M. oryzae* infections is a complex process and is probably not solely limited to a passive mechanical barrier, or to an induced defense, as previously proposed. Such a resistance is enhanced through both mechanical and biochemical defense reactions (Cai et al., 2008). In plant cultures, many compounds or stimuli that enhance the production of useable secondary metabolites have been identified. In general, elicitors are classified on the basis of their origin and molecular structure. According to its characteristics, each elicitor can induce specific responses that depend on the interaction between the elicitor and plant culture (Vasconsuelo and Boland, 2007). Even though the chemical characterization of the *M. oryzae* cell-wall hydrolysate used in this work is still unknown, our results clearly show a significant eliciting effect on rice cell cultures, comprising ROS generation (Rožkowicz et al., 2003), induction of PAL specific activity levels (Forlani, 2010), and browning of cell cultures. Visible browning of suspension cultured cells following elicitation has often been described in previous studies (e.g., Köhle et al., 1984, Gomez-Vasquez et al., 2004 and Cabrera et al., 2006). It suggests activation/enhancement of phenylpropanoid metabolism, but could be also a simple consequence of phenolic oxidation caused by the induced oxidative burst. Browning has been also associated with cell death during the hypersensitive response (Davis and Ausubel, 1989), and indeed a significant loss of cell viability was found in Gigante Vercelli cultured cells at a late stage following the treatment with fungal hydrolysates (Rožkowicz et al., 2003).

Innovative techniques in plant cell cultures are required for detection of secondary metabolites. Among the most recent are techniques for the detection of VOCs from biological samples. Stir bar

sorptive extraction (SBSE) is based on the same SPME principle (Baltussen et al., 1999 and Demyttenaere et al., 2004). SBSE is typically used for trace and ultra-trace analysis (Splivallo et al., 2007a). The results of this work show that *M. oryzae* hydrolysate induces rice cell culture to produce VOCs, with long-chain alcohols being the most abundant among them. Previous work on induction of rice leaf volatiles upon elicitation with a chitosan oligomer showed the presence of several mono- and sesquiterpenes (Obara et al., 2002), with a pattern of leaf VOC emission completely different from that observed in rice cell cultures. Evidently, tissue differentiation activates different signaling responses to pathogen attacks. However, models in which plants are substituted by cultured cells and pathogens by elicitors can adequately simulate *in vivo* infections (Keller et al., 1996). Such *in vitro* systems provide reliable tools to identify some of the biochemical steps in the rice dynamic response to blast (Kim et al., 2003 and Sengar et al., 2009).

Aliphatic alcohols are among the most widespread compounds found to occur naturally in plants. At higher concentrations, they are known to cause a variety of biochemical responses (Hammond and Kubo, 2000). Long-chain aliphatic alcohols ranging in chain length from 6 to greater than 20 carbon atoms can inhibit the growth of various types of bacteria and fungi (Kato and Shibasaki, 1980, Fujita et al., 2008, Kubo et al., 1995 and Suprapta et al., 1997). Alkanols up to tridecanol act as uncouplers (Hammond and Kubo, 2000) and, at high concentrations, can cause death, showing a potential for use as a means of pest control against larval mosquitoes (Hammond and Kubo, 1999). 1-Decanol and 1-undecanol are VOCs with biological activity, as demonstrated on several microorganisms (i.e., *Saccharomyces cerevisiae*, *Pityrosporum ovale*, *Mucor mucedo*, *Penicillium chrysogenum*, *Zygosaccharomyces bailii* and *Trichophyton mentagrophytes*) (Kubo et al., 1995 and Kubo et al., 2003). The increased amount of alkanols found in rice cells elicited by *M. oryzae* cell-wall hydrolysate might be related to the potential activity of these molecules against pathogenic infection. The biochemical rationale of n-alkanol action on *M. oryzae* is still far from clear. Aliphatic alcohols increase the fluidity of the membrane and are known to inhibit the key enzyme of the regulation of the biosynthesis of saturated fatty acids (Kabelitz et al., 2003 and references cited therein). n-Alkanols like 1-octanol have also been found to interact with proteins and in particular with adenylyl cyclase, the major cAMP generating enzyme (Kou and Yoshimura, 2007).

The antifungal activity of 1-decanol and 1-dodecanol was enhanced in combination with anethole, a compound closely related to myristicin. Myristicin has been shown to exert antimycotic activity against *Aspergillus flavus*, *A. niger*, *A. versicolor*, *A. ochrachloron*, *Penicillium funiculosum* and *Trichoderma viride* (Sokovic et al., 2008). Increased amounts of myristicin after elicitation of rice cells with the *M. oryzae* hydrolysate, along with increased levels of the alkanols, 1-decanol and 1-dodecanol, might be related to the combined fungistatic properties of these molecules.

Another molecule found after *M. oryzae* hydrolysate elicitation is cinnamyl alcohol. Cinnamyl alcohol dehydrogenase (CAD) is the enzyme that catalyses one of the reactions during the production of the lignin monomers, *p*-coumaryl, coniferyl and sinapyl alcohol, from the aromatic amino acid phenylalanine in the monolignol biosynthesis pathway of plants. Reduced CAD plants contain more cinnamylaldehydes and fewer cinnamyl alcohol residues, leading to structural changes that make the polymer more condensed, and yet more easy to extract with alkali or thioglycollic acid (Henault et al., 2006). An important feature of the plant response to invading pathogens is thought to be the rapid production of defense-related enzymes such as peroxidase, which participates in the biosynthesis of lignin. Increased cinnamyl alcohol contents might be related to increased lignin synthesis induced by *M. oryzae* hydrolysate elicitation.

Rice cells responded to *M. oryzae* hydrolysate elicitation by producing an array of toxic fungistatic compounds with potential antimicrobic and antifungal action. However, another interesting aspect of VOCs production is the promotion of direct defense through induction of plant cell ROS. In plant–biotroph interaction, ROS production acts at low concentration as a signaling molecule, while at higher concentration it interacts directly by oxidizing cell membranes (Mithöfer et al., 2009). In rice, antioxidant enzymes participate in the stage-dependent resistance to *M. oryzae* (Hao et al., 2010). In rice cell cultures, 1-octanol and 1-propanol induced production of ROS act synergistically to reinforce plant defense against the pathogen, confirming previous work on peroxide generation and increased overall tolerance of rice cultivars to the pathogen (Rožkowicz et al., 2003). This notwithstanding, and contrary to cultures treated with fungal cell-wall hydrolysates, VOCs-treated cells did not turn brownish, suggesting that multiple molecular signals are required to trigger a full activation of the plant defense response. Indeed, the oxidative burst was found to be neither required nor sufficient to induce the hypersensitive reaction (Dorey et al., 1999).

In conclusion, the results of this work showed that rice cell cultures react to *M. oryzae* by producing VOCs that have a significant effect on rice cell ROS production. VOCs might act synergistically with ROS by increasing the rice cell resistance to *M. oryzae* attack through their ROS-inducing action and by directly inhibiting (through a fungistatic effect) the pathogen growth. At the same time, VOCs emitted by attacked rice cells might elicit healthy rice cells (priming), which start producing ROS. Fig. 4 shows a hypothetic scheme of rice cell responses to fungal hydrolysate elicitation.

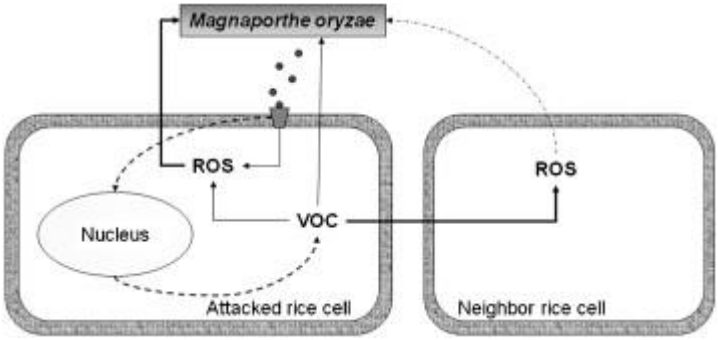

Fig. 4. Hypothetic scheme of rice cell culture responses to fungal elicitation. *Magnaporthe oryzae* elicitors are perceived by rice cells and trigger a cascade of events leading to the production of ROS. Fungal elicitation induces the production of VOCs possibly by gene induction (dotted line). Released VOCs have a fungistatic effect on *M. oryzae*. Furthermore, VOCs released in the cell elicit the production of ROS and induce ROS production in neighbor cells.

Further studies are under way to evaluate the genomic responses of rice cells primed by emission of VOCs from pathogen-attacked cells and to better characterize the chemical composition of the fungal hydrolysate.

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