

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Jasmonic acid, abscisic acid and salicylic acid are involved in the phytoalexin responses of rice to *Fusarium fujikuroi*, a high gibberellin producer pathogen.**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1574709> since 2017-05-12T13:33:22Z

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

Siciliano, I.; AMARAL CARNEIRO, A.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. Jasmonic acid, abscisic acid and salicylic acid are involved in the phytoalexin responses of rice to *Fusarium fujikuroi*, a high gibberellin producer pathogen.. JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY. 63 pp: 8134-8142.

When citing, please refer to the published version.

Link to this full text:

<http://hdl.handle.net/2318/1574709>

1 **Jasmonic Acid, Abscisic Acid and Salicylic Acid Are Involved in the Phytoalexin Responses of**  
2 **Rice to *Fusarium fujikuroi*, a High Gibberellin Producer Pathogen**

3

4 **Ilenia Siciliano<sup>†</sup>, Greice Amaral Carneiro<sup>†</sup>, Davide Spadaro<sup>†,‡,\*</sup>, Angelo Garibaldi<sup>†</sup>, Maria**  
5 **Lodovica Gullino<sup>†,‡</sup>**

6

7 <sup>†</sup>Centre of Competence for the Innovation in the agro-environmental Sector (AGROINNOVA),  
8 University of Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

9 <sup>‡</sup>Dept. Agricultural, Forestry and Food Sciences (DISAFA), University of Torino, Largo P. Braccini  
10 2, 10095 Grugliasco (TO), Italy.

11

12 \*Corresponding author (Phone: +39-0116708942; Fax: +39-0112368942; E-mail:  
13 [davide.spadaro@unito.it](mailto:davide.spadaro@unito.it))

14

15 **Abstract**

16 *Fusarium fujikuroi*, causal agent of bakanae disease, is the main seedborne pathogen on rice. In order  
17 to understand the basis of rice resistance, a quantitative methods to detect simultaneously  
18 phytohormones and phytoalexins was developed by using HPLC-MS/MS. With this method dynamic  
19 profiles and possible interactions of defense-related phytohormones and phytoalexins were  
20 investigated on two rice cultivars, inoculated or not with *F. fujikuroi*. In the resistant cultivar Selenio  
21 the presence of pathogen induced high production of phytoalexins, mainly sakuranetin, and symptoms  
22 of bakanae were not observed. On the contrary, in the susceptible genotype Dorella, the pathogen  
23 induced the production of gibberellin and abscisic acid, inhibited jasmonic acid production,  
24 phytoalexins were very low and bakanae symptoms were observed. Results suggested that a wide  
25 range of secondary metabolites are involved in plants defense against pathogens and phytoalexin  
26 synthesis could be an important factor for rice resistance against bakanae disease.

27

28 **Keywords:** *Fusarium fujikuroi*, bakanae disease, rice, phytoalexins, phytohormones, HPLC-  
29 MS/MS.

## 30 INTRODUCTION

31

32 *Fusarium fujikuroi* Nirenberg is the anamorph of *Gibberella fujikuroi* (Sawada) Ito in Ito & K.  
33 Kimura and is a member of the *G. fujikuroi* species complex.<sup>1</sup> This seed-borne pathogen is causal  
34 agent of bakanae disease in rice (*Oryza sativa* L.). Infected rice turns pale yellow and exhibits  
35 chlorosis, poor grain ripening, empty panicles, and foot and stem rot.<sup>2</sup> Many of these symptoms are  
36 due to exposure to gibberellins, a large family of isoprenoid compounds originally characterized as  
37 plant hormones, which are produced by the pathogen.<sup>3</sup> Some of them are bioactive growth regulators,  
38 controlling seed germination, stem elongation, and flowering. These phytohormones are synthesized  
39 in the young vegetative tissues of plants and later transported to other parts for the enhancement of  
40 growth and development.<sup>4</sup> Gibberellin A<sub>3</sub> was significantly produced only in cultures of strains of *F.*  
41 *fujikuroi*.<sup>5</sup>

42 Plants respond with a rapid series of events called hypersensitive response to protect themselves from  
43 pathogens.<sup>6</sup> Low molecular-weight antifungal compounds called phytoalexins are produced in host  
44 plants in response to infection or stress. Salicylic acid and jasmonic acid rapidly accumulate in plants  
45 that are attacked by pathogens, resulting in the activation of signaling defense. These two molecules  
46 are known to be involved in the expression of defense genes that eventually produce pathogenesis  
47 related proteins and are known to be involved in inducing phytoalexins.<sup>7</sup>

48 Phytoalexins produced by rice can be divided into two classes based on their chemical structure, the  
49 flavonoid-type phytoalexins (naringenin and sakuranetin) and the diterpenoid phytoalexins  
50 (momilactone A and B, oryzalexins A-F and S and four phytocassanes).<sup>8</sup> Experiments on rice plants,  
51 susceptible and resistant to *Magnaporthe oryzae* Cav., suggest that sakuranetin and momilactone A  
52 are the major phytoalexins.<sup>9</sup> Moreover, the plant hormone abscisic acid is an important signal  
53 molecule for abiotic stress adaptation, but it can also act as developmental signal. Internodal  
54 elongation is based on increased cell-division activity and enhanced cell elongation in well-defined

55 zones of the internode. Abscisic acid is a potent antagonist of gibberellins action in rice internodes.<sup>10</sup>

56 <sup>11</sup>

57 The aim of the current work was to elucidate the chemical response of rice to *Fusarium fujikuroi*  
58 infection. A sensitive and rapid method to simultaneously quantitate different classes of molecules,  
59 i.e. phytohormones and phytoalexins, could facilitate the investigation of rice defense towards  
60 pathogens. Therefore, we aimed at developing an easy HPLC-MS/MS method for rapid  
61 phytohormones profiling that included: abscisic acid, jasmonic acid, salicylic acid, and gibberellin  
62 A<sub>3</sub>; additionally, this method permitted the determination of four phytoalexins (naringenin,  
63 sakuranetin, momilactone A and momilactone B) in the same analysis.

64 In this study, two rice cultivars, Selenio and Dorella, infected with *F. fujikuroi* were analyzed at four  
65 time points of inoculation by chemical quantification of four phytohormones and four phytoalexins.  
66 Both cultivars were selected after phenotypic and transcriptomic studies as the most resistant and  
67 susceptible to bakanae disease, respectively.<sup>12</sup> In order to better evaluate the rice response to the  
68 pathogen, a molecular method based on a TaqMan quantitative PCR, was developed to quantitate the  
69 fungal presence into the plant tissues.

70

## 71 **MATERIALS AND METHODS**

72

### 73 **Plant material and fungal cultures**

74 Two rice (*Oryza sativa* L.) cultivars, Selenio and Dorella, were used throughout the experiments.  
75 Seed lots of the rice cultivars were provided by the Rice Research Unit of Consiglio per la Ricerca e  
76 la Sperimentazione in Agricoltura.

77 The monoconidial strain VE13 of *Fusarium fujikuroi*,<sup>13</sup> used for artificial inoculation of rice seeds,  
78 was stored in potato dextrose agar (PDA) (Merck, Darmstadt, Germany) with 27 mg/L streptomycin  
79 (Merck). A small plug was transferred into 500 mL potato dextrose broth (PDB) (Liofilchem, Roseto  
80 degli Abruzzi, Italy) and grown under stirring at 90 rpm and 23 °C for 10 d. Fungal culture was

81 filtered through a sterile gauze to get a conidial suspension at  $10^6$  conidia/mL. Rice seeds were  
82 surface-disinfected in 1% sodium hypochlorite for 2 min and rinsed in sterile distilled water. For each  
83 group, 40 seeds per replicate and three replicates per experiment were tested. Seeds were soaked in  
84 100 mL spore suspension and shaken for 30 min at room temperature, while uninoculated seeds for  
85 each genotype were soaked in sterile distilled water. The seeds were placed in a sterilized soil in  
86 greenhouse and grown at 24-26 °C during the day and 16-18 °C during the night. Leaves and culms  
87 were collected every week for 4 w after seed germination (around 7 days after pathogen inoculation).  
88 The phenological rice stages at wpg 1, 2, 3 and 4 were V1, V2, V3 and V4, corresponding to the  
89 number of leaves with collars on the main stem during vegetative development.<sup>14</sup>

90

#### 91 **Disease index**

92 Germination rate and disease index, for three biological replication, were evaluated 28 d after  
93 germination using a scale of five distinct classes of symptoms. Each symptom corresponds to a  
94 reference number: 0: asymptomatic plants; 25: plants with yellow leaves; 50: plants with long  
95 internodes; 75: necrotic plants; 100: dead plants.<sup>15</sup>

96

#### 97 **Phytohormones and phytoalexins extraction and analysis**

##### 98 *Reagents and standards*

99 LC–MS grade acetonitrile and formic acid used as mobile phase were purchased from Sigma-Aldrich  
100 (St Louis, MO). Methanol, acetonitrile, acetone, ethyl acetate, dichloromethane, formic acid HPLC  
101 grade used for extraction procedures were purchased from Merck (Darmstadt, Germany).

102 Salicylic acid (purity  $\geq$  99%), jasmonic acid (purity  $\geq$  95%), abscisic acid (purity  $\geq$  98.5%),  
103 gibberellic acid (purity  $\geq$  95%), naringenin (purity  $\geq$  95%) and sakuranetin (purity  $\geq$  95%) were  
104 purchased from Sigma-Aldrich. Momilactone A and momilactone B were prepared by Prof. Morifumi  
105 Hasegawa, (College of Agriculture, Ibaraki University, Japan).

##### 106 *Preparation of standard solutions*

107 Stock solutions of each analyte were prepared at 1 mg/mL in CH<sub>3</sub>OH. Working solutions were  
108 prepared diluting stock solutions in CH<sub>3</sub>OH:H<sub>2</sub>O (8:2) at different concentrations depending on the  
109 range of the calibration curve. A stock solution mixture, made from these individual stock solutions,  
110 was prepared in LC mobile phase and matrices, to obtain calibration curves and to determine ion  
111 suppression, recovery, limit of detection (LOD) and limit of quantitation (LOQ) for each analyte.

#### 112 *Extraction procedure*

113 Acetone, ethyl acetate, dichloromethane, methanol, acetonitrile, water and two different mixtures of  
114 water:methanol (1:1 and 2:8 pure or containing 0.1% CH<sub>3</sub>COOH) were tested. In a second set of  
115 analysis, the influence of three extraction times (1h, 2h, overnight) and two methods (ultrasonic bath  
116 or rotary shaker) on the recovery were evaluated. Analyte-free rice matrix was not available, so for  
117 each test two sets of samples, with or without standard additions, were prepared and each extraction  
118 was performed twice for each sample.

119 A few plants of rice were ground and 0.2 g fresh plant material was transferred in 2 mL centrifuge  
120 tubes with 1 mL extraction solution (80% CH<sub>3</sub>OH acidified with 0.1% CH<sub>3</sub>COOH). The samples  
121 were frozen in liquid nitrogen and homogenized by using TissueLyser (Qiagen, Venlo, Netherlands),  
122 then shaken at 4 °C in the dark overnight. Finally, samples were centrifuged at 15,000 rpm and 4 °C  
123 for 2 min and the supernatant was analyzed by HPLC-MS/MS.

#### 124 *HPLC-MS/MS analysis*

125 All analyses were carried out by using a 1260 Agilent Technologies (Santa Clara, CA) system  
126 consisting of a binary pump and a vacuum degasser, connected to a Varian autosampler Model 410  
127 Prostar (Palo Alto, CA) equipped with a 20 µL loop coupled with a Varian 310-MS TQ mass  
128 spectrometer.

129 HPLC separation of analytes was performed using a 150 x 2 mm i.d., 3 µm, Luna Phenyl-Hexyl  
130 (Phenomenex, Torrance, CA) under a flow of 200 µL/min. The column temperature was set at 25 °C.

131 Solvent A was H<sub>2</sub>O with 0.1% of HCOOH, solvent B was CH<sub>3</sub>CN. HPLC analysis was programmed



132 as follows: 40% solvent B for 7 min, followed by a linear gradient from 40-100% B in 5 min, and  
133 finally held at 100% B for 4 min.

134 Samples were ionized using an electrospray (ESI) ion source operating in negative ion mode for  
135 salicylic acid, jasmonic acid, abscisic acid, gibberellic acid, naringenin and sakuranetin; in positive  
136 ion mode for momilactone A and momilactone B. For the multiple reaction monitoring (MRM)  
137 experiments, the quantification transitions for each compound were: (1)  $m/z$  345→239 CE 14V; (2)  
138  $m/z$  263→153 CE 12V; (3)  $m/z$  137→93 CE 16V; (4)  $m/z$  209→59 CE 14V; (5)  $m/z$  271→151 CE  
139 18V; (6)  $m/z$  285→119 CE 30V; (7)  $m/z$  331→269 CE 25V; (8)  $m/z$  315→271 CE 14V. The collision  
140 gas (Ar) pressure was set at 2 mbar for all experiments.

141

#### 142 **Method validation**

143 HPLC-MS/MS method was validated in terms of accuracy, linearity, LOD and LOQ. The validation  
144 was performed on both matrices using non-inoculated samples collected 1 w after germination.

145 *Linearity and Range.* The linearity of measurement was evaluated by analyzing different  
146 concentrations of the standard solutions of the analytes. Calibration standards were prepared by  
147 diluting the stock solutions to obtain specific concentrations.

148 *LOD and LOQ.* Usually LOD and LOQ were established using matrix samples spiked after the  
149 extraction procedure with the low amount of standards. Free-analytes matrix was not available so  
150 LOD and LOQ were determined in solvent. The detection and quantification limits were determined  
151 based on standard deviation of the response ( $\sigma$ ) and the slope of calibration curve (S) ratio in  
152 accordance with ICH Harmonised Tripartite Guideline<sup>16</sup> expressed as:  $LOD=3.3\sigma/S$ ;  $LOQ=10\sigma/S$ .

153 *Accuracy.* Recovery studies were performed with rice samples spiked at 50%, 100% and 150% levels  
154 with all stock solutions prepared. The accuracy of the method was expressed by: [(mean observed  
155 concentration)/(spiked concentration) x 100].

156 Besides validation, the influence of the components of both rice cultivars on the quantification of  
157 every analyte was evaluated. Matrix effect was calculated using the formula:  $(\text{slope}_{\text{matrix}}/\text{slope}_{\text{solvent}})$   
158 x 100.

159

## 160 **Statistical analysis**

161 Rice samples were harvested at 1, 2, 3 and 4 weeks post germination (wpg) in three biological  
162 replicates. The biological replicates were extracted separately in two technical replicates and every  
163 technical replicate was analyzed twice. The data reported are the average of 12 data and standard  
164 deviation is reported as error bar. Statistical analysis among data of the same analyte was performed  
165 using T-student test at 99% confidence.

166

## 167 **Molecular identification of *F. fujikuroi***

### 168 *DNA extraction*

169 DNA extraction was performed on 0.1 g fresh plant material using the commercial E.Z.N.A. Plant  
170 DNA kit (Omega Bio-Tek Norcross, GA), according to the instructions of the manufacturer. For the  
171 final elution of DNA 25  $\mu$ L elution buffer were used.

### 172 *Design of real-time PCR primers and probe*

173 The purpose of this work was to identify regions of variability able to discriminate *F. fujikuroi* from  
174 other species associated with bakanae disease on rice <sup>5</sup> (*Fusarium proliferatum* and *Fusarium*  
175 *verticilloides*). A part of elongation factor 1 $\alpha$  (TEF) gene was sequenced for different strains. Multiple  
176 sequence alignment showed a deletion of six nucleotides in all the strains of *F. fujikuroi* but not in  
177 other species.<sup>17</sup> The deletion of *F. fujikuroi* was used as the basis for design of the following primers  
178 and probe with the Primer Express 3.0.1 software (Applied Biosystems, Foster City, CA): forward  
179 primer FujiF (5'-ATGGGCGCGTTTTGCCCTTT-3'), reverse primer FujiR (5'-  
180 GGCGTACTTGAAGGAACCCT-3') and TaqMan FfujiPq (5'-[FAM]-  
181 TTGTCACGTGTCAAACCTAAACATTCGAC-[TAMRA]-3') labelled with the fluorescent reporter

182 dye FAM at the 5'-end and TAMRA quencher at the 3'-end. The primers and the probe were  
183 purchased from Applied Biosystems.

#### 184 *TaqMan Real-time PCR assay*

185 The real-time TaqMan PCR assays were conducted using an Applied Biosystems plus detection  
186 system. Twenty  $\mu\text{L}$  reactions containing 12.5  $\mu\text{L}$  Taqman Master Mix (2 $\times$ ) (Applied Biosystems),  
187 PCR primers at a concentration of 10 $\mu\text{M}$ , the TaqMan FfujiPq at 5  $\mu\text{M}$  and 5  $\mu\text{l}$  of DNA at the  
188 concentration of about 30 ng/ $\mu\text{L}$  were prepared in triplicate. Amplification and detection were  
189 performed in 96-well optical reaction plates (Applied Biosystems) sealed with MicroAmp<sup>TM</sup> optical  
190 adhesive film (Applied Biosystems) on an ABI Prism 7900HT sequence detection system real time  
191 thermal cycler. The amplification conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 15  
192 s at 95 °C and 40 s at 62 °C for 40 cycles.

193 A standard curve was prepared by serial dilution of known quantities of *F. fujikuroi* DNA in order to  
194 quantitate the fungus inside rice tissues. In the preparation of the standard curve, the initial  
195 concentration of DNA was 386 ng / $\mu\text{L}$ .

#### 196 *Primer and probe design, specificity and sensitivity*

197 The primer pair FujiF and FujiR was designed to amplify a 117 bp unique sequence found in the TEF  
198 gene of *F. fujikuroi*. For confirmation of the specificity of the amplified product, the TaqMan FfujiPq  
199 probe was designed for inclusion in the real time PCR assay.

200 The combination of primers FujiF and Fuji-R with the dual-labelled probe FfujiPq was then tested in  
201 real-time PCR with a series of DNA extracts from pure cultures of three species of *Fusarium* spp..  
202 Real-time reactions using this primer–probe combination proved to be specific, low  $C_t$  values were  
203 obtained for all *F. fujikuroi* strains and not for isolates of any other *Fusarium* spp..

204 The sensitivity of FujiF/ FujiR and FfujiPq combination was measured with a six dilution series of *F.*  
205 *fujikuroi* DNA in deionized DNA-free water, in a range of concentrations from 386 ng/ $\mu\text{L}$  down to  
206 0.3 fg/ $\mu\text{L}$ . A standard curve was built and the corresponding amplification efficiency was 96.18%.

207 The results showed a linear relationship between Ct values and concentration, the correlation  
208 coefficient ( $R^2$ ) was 0.999.

209 The optimized sequences FujiF/ FujiR and FfujiPq permitted the real-time PCR to be run under high  
210 stringency conditions (annealing or elongation at 62 °C), providing maximum specificity without any  
211 significant effect on the performance of amplification.

212 The quantity of the fungus in plants was measured in ng 100 mg<sup>-1</sup> by extraction of DNA from the  
213 culm and from the leaves of the cvs. Selenio and Dorella.

214

## 215 **RESULTS**

216

### 217 **Rice germination and disease index**

218 In plants of rice cv. Selenio, symptoms of bakanae were not observed, while typical bakanae-diseased  
219 symptoms were observed in plants of cv. Dorella. The disease index calculated for cv. Selenio was  
220 5.0% while for cv. Dorella it was 72.4%. Also the percentage of germination was different, 72.5%  
221 for cv. Dorella and 93.0% for cv. Selenio.

222

### 223 **Phytohormones and phytoalexins extraction and analysis**

224 In the first step, authentic standard mixtures were used to evaluate retention times,  $m/z$  values of  
225 corresponding ions and characteristic MS/MS fragmentation patterns. Each analyte was characterized  
226 by two specific precursor-fragment ion combinations and a characteristic retention time. For each  
227 analyte, quantification relied on the most intense transition, while the less intense one was used to  
228 confirm the assignment.

229 The choice of the appropriate chromatographic system is the most important factor that influence the  
230 sensitivity of HPLC-MS analysis. Samples were analyzed using a phenyl-hexyl column which allows  
231 a good separation of all the analytes in a short time (Figure 2). It was possible to separate the analytes  
232 in order to perform a change of polarity and to detect all the analytes in a single analysis.

233 Plant hormones and phytoalexins are structurally diverse with different physiochemical properties.  
234 The choice of extraction solvent is very important, so during the method optimization the composition  
235 of extraction solution was first evaluated. Different organic solvents/mixtures and the influence of  
236 acidification were tested. Non-polar extraction solvent extracted a large amount of chlorophyll and it  
237 was necessary purification. A mixture of methanol and water provided high extraction efficiency for  
238 all the analytes and did not require purification because of a low chlorophyll amount was extracted  
239 using a 8:2 ratio.

240

#### 241 **Validation of analytical method**

242 Validation procedure was carried out in order to evaluate the applicability of this method. The range  
243 of calibration curves was defined for each compound based on the amount in both rice matrices. The  
244 calibration curves showed a good linearity of correlation coefficient ( $R^2$ ) > 0.9999 for all analytes.  
245 LOD for different analytes ranged from 0.87 ng/g for abscisic acid to 20.7 ng/g for salicylic acid, and  
246 LOQ ranged from 2.90 ng/g for abscisic acid to 69.1 ng/g for salicylic acid calculated on fresh weight.  
247 For sakuranetin in cv. Selenio and abscisic acid in cv. Dorella the recovery was very high (around  
248 98%). For the other analytes, recoveries in both cultivars were always higher than 70%. The matrix  
249 components can affect the analyte stability, extraction and ionization. For both cultivars, positive and  
250 negative trends were similar, only for naringenin the matrix effect did not affect ionization. Matrices  
251 mostly influenced salicylic acid and momilactone A. The components of the matrix affected the  
252 response of each analyte with different intensities. In order to consider all the effects of the matrix on  
253 the quantitative results, a quantitation methods developed in presence of the matrix was used.

254

#### 255 **Accumulation of phytohormones**

256 Abscisic acid and gibberellic acid (Figure 3) showed a very different trend in the susceptible cultivar  
257 Dorella (D) compared to the resistant one Selenio (S). In the susceptible cultivar, for both  
258 phytohormones, it is possible to observe, after *F. fujikuroi* inoculation (D+), an increased

259 concentration in the third (347 ng/g for gibberellic acid and 63.7 ng/g for abscisic acid) and fourth  
260 wpg (607 ng/g for gibberellic acid and 82.5 ng/g for abscisic acid). In the resistant cultivar, abscisic  
261 acid was statistically higher in inoculated plants (S+) compared to uninoculated control (S-) at the  
262 four time points. In Selenio gibberellic acid was not statistically different after pathogen inoculation  
263 for the first three wpgs, while it showed a slight increase at 4 wpg.

264 The trends of jasmonic acid and salicylic acid in the resistant and in the susceptible cultivar are shown  
265 in Figure 4. The trend and the amount of jasmonic acid accumulated in both cultivars were similar  
266 during the four weeks. For cv. Dorella, the quantity of jasmonic acid in inoculated plants significantly  
267 decreased at 3 and 4 wpg compared to the uninoculated control (64.8 ng/g for D- and 39.4 ng/g for  
268 D+ at 3 wpg, 44.8 ng/g for D- and 23.1 ng/g for D+ at 4 wpg). The accumulation of salicylic acid in  
269 both cultivars was quite similar (from 4480 to 7910 ng/g for cv. Selenio, and from 5440 to 10700  
270 ng/g from cv. Dorella), infested rice had similar trend compared to control and the difference of  
271 salicylic acid accumulation between them gradually increased with increasing infestation time. In the  
272 susceptible cultivar, the concentration of salicylic acid was always higher compared to the  
273 uninoculated control.

274

### 275 **Accumulation of phytoalexins**

276 An increase of all the PAs in the resistant cultivar was observed. The major difference between the  
277 two cultivars was found in the accumulation of sakuranetin, which greatly increased (24 times) from  
278 the third week in cv. Selenio (Figure 5), while it increased slightly (twice) in the susceptible cultivar.  
279 The accumulation of momilactone A (from 1170 to 1960 ng/g for cv. Selenio, and from 183 to 1020  
280 ng/g from cv. Dorella) was also higher in the resistant cultivar compared to the susceptible one from  
281 1 wpg (Figure 6). For the other two PAs, differences between the two cultivars are lower: naringenin  
282 ranged from 68 to 61 ng/g in cv. Selenio and from 90 to 40 ng/g in cv. Dorella (Figure 5); momilactone  
283 B ranged from 115 to 168 ng/g in cv. Selenio and from 66 to 176 ng/g in cv. Dorella (Figure 6).

284

## 285 **Quantification of *Fusarium fujikuroi***

286 The results obtained from real time PCR allowed the quantification of the fungal DNA and were used  
287 to calculate the concentration of *F. fujikuroi* in plants during their growth.

288 The results showed different trends between resistant and susceptible cultivar (Figure 7). Inoculated  
289 plants of cv. Dorella showed typical bakanae symptoms expressed by abnormal and excessive growth  
290 compared to healthy plants. Quantity of *F. fujikuroi* increased during plant growth. At 3 wpg ng of  
291 fungus was maximum, afterwards the quantity of *F. fujikuroi* started to decrease (Figure 7).  
292 Conversely, in the inoculated plants of cv. Selenio symptoms of bakanae were not observed, the  
293 quantity of *F. fujikuroi* was steadily lower during four weeks (Figure 7). In the uninoculated control  
294 of both cultivars, amount of *F. fujikuroi* was lower throughout the 4 wpgs.

295

## 296 **DISCUSSION**

297

298 In this study the differences between the response of two rice cultivars (Selenio and Dorella) to *F.*  
299 *fujikuroi* were investigated; different types of analyses, *in vivo* tests and chemical and molecular  
300 analyses, were performed in order to elucidate the rice resistance mechanism involved.

301 Several HPLC-MS/MS methods for separation and analysis of free and conjugate phytohormones  
302 were developed in plants.<sup>17, 18</sup> Liu et al.<sup>19</sup> developed a method for the simultaneous determination of  
303 some hormones and phytoalexins in order to study rice-bacterium interactions. With the same method,  
304 Duan et al.<sup>20</sup> studied rice metabolites involved in disease resistance to *M. oryzae*, agent of rice blast.

305 In this work, a simple and specific method for simultaneous extraction and quantification of  
306 metabolites involved in the interaction between rice and *F. fujikuroi* was developed and validated.  
307 All the analytes were extracted with a good recovery using a minimal amount of sample.

308 After pathogen infection or abiotic stress, phytoalexins are produced and accumulated by plants.  
309 Changes in phytoalexin concentrations over time may be important in the disease resistance of rice  
310 plants.<sup>21</sup> In general, flavonoids in plants have a protective function against stress. Naringenin 7-*O*-

311 methyltransferase (NOMT) catalyzes naringenin methylation to sakuranetin when rice leaves are UV-  
312 irradiated<sup>22</sup> or in presence of an increase of jasmonic acid or jasmonic acid related compound.<sup>23</sup>  
313 Sakuranetin biosynthesis is rapidly induced by biotic and abiotic stresses and was not found in healthy  
314 rice leaves.<sup>24</sup> In this work an increased concentration of sakuranetin was found only in cv. Selenio  
315 inoculated plants, not in the uninoculated control or in any sample of susceptible cultivar. It was  
316 demonstrated that sakuranetin, during rice-pathogen interaction, was induced in higher quantities in  
317 cultivars resistant to blast than in the susceptible ones.<sup>25</sup> Hasegawa et al.<sup>26</sup> established that sakuranetin  
318 has stronger antifungal activity against *M. oryzae* compared with momilactone A, that is the major  
319 rice diterpenoid phytoalexin. The main difference observed between the two cultivars is the increase  
320 of sakuranetin in resistant inoculated plants. In the interaction of rice with *F. fujikuroi*, sakuranetin is  
321 the most important phytoalexin, induced at all the time points.

322 Momilactones are naturally present in rice plants and are spread in all plant tissues;<sup>27</sup> generally the  
323 content of momilactone A is higher than momilactone B. Momilactone A was first isolated from seed  
324 husk of rice and identified as growth inhibitor,<sup>28</sup> then it was isolated from rice leaves and  
325 characterized as phytoalexin.<sup>29</sup> The phytoalexin momilactone A was detected in higher levels in cv.  
326 Selenio compared to cv. Dorella, from the first week of growth, either in inoculated plants or in  
327 uninoculated controls. In inoculated plants the levels of momilactone A was always higher than in  
328 uninoculated controls. MomilactoneA showed a high basal level of production, also without *F.*  
329 *fujikuroi* inoculation, in the resistant rice genotype.

330 Momilactone B is a potent growth inhibitor and is secreted from roots throughout the life cycle of the  
331 plant,<sup>30</sup> produced in different concentrations depending on the rice cultivars.<sup>31</sup> Generally, stress could  
332 induce an increase of the production of allelochemicals, that play an important role in defense  
333 mechanism.<sup>32</sup> In rice, the increased allelopathy with elevated secretion of momilactone B, was  
334 recorded in different stress conditions.<sup>33</sup> The increase in momilactone B in both cultivars inoculated  
335 with *F. fujikuroi* was probably due to a response to stress, but since it also acts as phytoalexin,  
336 momilactone B may play an important role in defense against biotic and abiotic stress. Plant-pathogen



337 interactions produce variations in the level of various phytohormones simultaneously but each  
338 hormone has a characteristic biological effect.<sup>34</sup>

339 Various plant hormones are often involved in the same biological process with additive, synergistic  
340 or antagonistic action.<sup>35</sup> The rice pathogen *F. fujikuroi* is able to produce large amounts of GAs,  
341 especially the bioactive compounds gibberellic acid.<sup>36</sup> Gibberellic acid was significantly detected in  
342 cv. Dorella infected with *F. fujikuroi*. At 3 and 4 wpgs, increased levels of gibberellic acid were  
343 observed and the plants showed typical bakanae symptoms: infected seedlings were elongated, more  
344 slender and slightly chlorotic when compared to healthy seedlings. The increased level of gibberellic  
345 acid could be attributed to the presence of the pathogen, which was confirmed by qPCR, only in the  
346 susceptible cultivar starting at 2 wpg. GAs production contributes to increase the pathogenicity of *F.*  
347 *fujikuroi* on rice.<sup>37</sup> The resistant cultivar Selenio did not show the typical symptoms of bakanae,  
348 despite a slight increase of *F. fujikuroi* being observed in inoculated plants at the 3 and 4 wpgs.  
349 Gibberellic acid was observed at low levels in inoculated plants of resistant cultivar only at 4 wpg.  
350 Jasmonic acid levels had a different trend between infected plants and uninoculated control for cv.  
351 Dorella. At 3 wpg, a decrease of jasmonic acid was observed in inoculated plants of the susceptible  
352 cultivar in correspondence with an increase of gibberellic acid, as previously described by Yang et  
353 al..<sup>38</sup> Gene expression studies and pathobiological assays suggested that gibberellic acid controls  
354 jasmonic acid-responsive gene expression and jasmonic acid-mediated plant immune responses.<sup>39</sup>  
355 Gibberellic acid promotes plant growth by regulating the degradation of a class of nuclear growth-  
356 repressing proteins called DELLAs. SLENDER RICE 1 (SR1), the only DELLA protein in rice,  
357 inhibits JAZ1, the key repressor of jasmonic acid signaling.<sup>40</sup> The increase of gibberellic acid,  
358 produced by the fungus, restricts jasmonic acid signaling that is strongly activated during necrotroph  
359 infections.<sup>41</sup>

360 Free salicylic acid level in rice leaves usually ranges from 5-30 µg/g fresh weight.<sup>42, 43</sup> A large amount  
361 of salicylic acid was accumulated in many plants species (*Arabidopsis*, tobacco) after pathogen  
362 inoculation, on the contrary in rice no significantly induction was reported after bacterial or fungal

363 infection.<sup>44</sup> In both non-inoculated cultivars, SA level was similar, but in cv. Dorella after inoculation  
364 the amount of salicylic acid was higher compared to cv. Selenio, this is probably due to the role that  
365 salicylic acid plays in the modulation of redox balance to protect the plant from oxidative stress and  
366 in the defense against pathogen attacks.<sup>45</sup>

367 Abscisic acid probably compromises rice defense against pathogens. Jiang et al.<sup>46</sup> demonstrated that  
368 abscisic acid suppressed the basal resistance of rice when it interacts with the pathogen *M. oryzae* at  
369 3 and 4 wpgs. Increased concentration of this phytohormone was observed only in diseased plants of  
370 cv. Dorella. High level of abscisic acid in the susceptible cultivar could be related to the response to  
371 biotic stress, as it could antagonize gibberellic acid.<sup>47</sup> Conversely, the concentration of abscisic acid  
372 remained constant in the resistant cultivar Selenio, however the levels of this phytohormone were  
373 higher in the inoculated plants compared to the uninoculated control. The role of abscisic acid in  
374 disease resistance remains unclear due to its multifaceted function in different tissues and  
375 development stage of the plant. Abscisic acid plays an ambiguous role in the rice immune signaling  
376 network; a general pattern suggests that abscisic acid plays a stimulatory role in plant defense during  
377 the earlier stages of the pathogen invasion, vice versa at later colonization stages, it could have a  
378 suppressive role.<sup>48</sup>

379 The analytical method developed in this paper could be used to study the response of rice to other  
380 biotic or abiotic stresses, besides *Fusarium fujikuroi*. The pathogen considered in this work is a  
381 seedborne pathogen, and the chemical responses of plants have rarely been studied in relationship to  
382 seedborne pathogen infection. Some responses are similar to those activated in response to other rice  
383 pathogens, such as *Magnaporthe oryzae*, which is a foliar fungal pathogen, with the main difference  
384 that gibberelins are typically produced by *F. fujikuroi*. The levels of phytohormones and phytoalexins  
385 in rice showed that *F. fujikuroi* may behave differently with respect to the cultivar. In the susceptible  
386 cultivar, the pathogen could systemically act as a necrotroph destroying the plant cells. On the  
387 contrary, in the resistant cultivar, the pathogen is present on rice, though at lower concentration,  
388 without damaging the plants and it triggers salicylic acid and, presumably, a hypersensitive response.

389 Collectively, this information provides important information to elucidate the rice responses  
390 occurring during *F. fujikuroi* infection.

391

#### 392 **ABBREVIATIONS USED**

393 D-, cv. Dorella not inoculated; D+, cv. Dorella inoculated; MRM, multiple reaction monitoring; S-,  
394 cv. Selenio not inoculated; S+, cv. Selenio inoculated.

395

#### 396 **ACKNOWLEDGEMENTS**

397 This work was carried out with grants from the project RISINNOVA “Integrated genetic and genomic  
398 approaches for new Italian rice breeding strategies”, funded by AGER Foundation (grant no. 2010–  
399 2369) and from the project TESTA "Seed health: development of seed treatment methods, evidence  
400 for seed transmission and assessment of seed health", funded by the European Union (Grant  
401 Agreement No 311875). The Authors acknowledge Prof. Morifumi Hasegawa, (College of  
402 Agriculture, Ibaraki University, Japan) for providing the chemical standards of momilactone A and

403 B, and Dr. Giampiero Valè, Rice Research Unit of Consiglio per la Ricerca e la Sperimentazione in  
404 Agricoltura, Italy, for providing the rice seeds.

405

#### 406 **SUPPORTING INFORMATION**

407

408 This material is available free of charge via the Internet at <http://pubs.acs.org>.

409

410 **Supporting Table 1.** Parameters of calibration for each analyte: curve range, regression, limit of  
411 detection (LOD), limit of quantification (LOQ). LOD and LOQ were determined in solvent and  
412 expressed as:  $LOD=3.3\sigma/S$ ;  $LOQ=10\sigma/S$ .

413

414 **Supporting Table 2.** Regression ( $R^2$ ), Matrix effect (ME) and Recovery (RE) for both matrices.

415

416 **REFERENCES**

417

- 418 1. O'Donnell, K.; Cigelnik, E.; Nirenberg, H.I. Molecular systematics and phylogeography of the  
419 *Gibberella fujikuroi* species complex. *Mycologia* **1998**, *90*, 465–493.
- 420 2. Hwang, I.S.; Kang, W.R.; Hwang D.J., Bae S.C.; Yun, S.H.; Ahn, I.P. Evaluation of bakanae  
421 disease progression caused by *Fusarium fujikuroi* in *Oryza sativa* L. *J. Microbiol.* **2013**, *6*, 858-  
422 865.
- 423 3. Wiemann, P.; Brown, D.W.; Kleigrew, K.; Bok, J.W.; Keller, N.P.; Humpf, H.U.; Tudzynski  
424 B. FfVell and FfLae1, components of a velvet-like complex in *Fusarium fujikuroi*, affect  
425 differentiation, secondary metabolism and virulence. *Mol. Microbiol.* **2010**, *77*, 972–994.
- 426 4. Jang, S.W.; Hamayun, M.; Sohn, E.Y.; Shin, D.H.; Kim, K.U.; Lee, B.H.; Lee, I.J. Effect of  
427 elevated nitrogen levels on endogenous gibberellin and jasmonic acid contents of three rice  
428 (*Oryza sativa* L.) cultivars. *J. Plant Nutr. Soil Sci.* **2008**, *171*, 181-186.
- 429 5. Wulff, E.G.; Sørensen, J.L.; Lübeck, M.; Nielsen, K.F.; Thrane, U.; Torp, J. *Fusarium* spp.  
430 associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity.  
431 *Environ. Microbiol.* **2010**, *12*, 649–657.
- 432 6. Hasegawa, M.; Mitsuhashi, I.; Seo, S.; Imai, T.; Koga, J.; Okada, K.; Yamane, H.; Ohashi, Y.  
433 Phytoalexin accumulation in the interaction between rice and blast fungus. *Mol. Plant-Microbe*  
434 *Interact.* **2010**, *23*, 1000-1011.
- 435 7. Kanno, H.; Hasegawa, M.; Kodama, O. Accumulation of salicylic acid, jasmonic acid and  
436 phytoalexins in rice, *Oryza sativa*, infested by the white-backed planthopper, *Sogatella furcifera*  
437 (Hemiptera: Delphacidae). *Appl. Entomol. Zool.* **2012**, *47*, 27–34.
- 438 8. Grayer, R.J.; Kokubun, T. Plant-fungal interaction: the search for phytoalexins and other  
439 antifungal compounds from higher plants. *Phytochemistry* **2001**, *56*, 253-263.
- 440 9. Dillon, V.M.; Overton, J.; Grayer, R.J.; Harborne, J.B. Differences in phytoalexin response  
441 among rice cultivars of different resistance to blast. *Phytochemistry* **1997**, *44*, 599-603.

- 442 10. Weiss, D.; Ori, N.; Mechanisms of cross talk between gibberellin and other hormones. *Plant*  
443 *Physiol.* **2007**, *144*, 1240-1246.
- 444 11. Kende, H.; Knaap, E.V.; Cho, H.T.; Deepwater Rice: A model plant to study stem elongation.  
445 *Plant Physiol.* **1998**, *118*, 1105-1110.
- 446 12. Matic, S.; Bagnaresi, P.; Biselli, C.; Valé, G.; Garibaldi, A.; Gullino, M.L.; Spadaro, D.  
447 Transcriptome analysis of two rice genotypes in response to *Fusarium fujikuroi*. *J. Plant Pathol.*  
448 **2014**, *96*, S4.34-S4.35.
- 449 13. Amatulli, M.T.; Spadaro, D.; Gullino, M.L.; Garibaldi, A. Molecular identification of *Fusarium*  
450 spp. associated with bakanae disease of rice in Italy and assessment of their pathogenicity. *Plant*  
451 *Pathol.* **2010**, *59*, 839-844.
- 452 14. Counce, P.A.; Keisling, T.C.; Mitchell, A.J. A Uniform, Objective, and Adaptive System for  
453 Expressing Rice Development. *Crop Sci.* **2000**, *40*, 436-443.
- 454 15. Matic, S.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. Antagonistic yeasts and thermotherapy as  
455 seed treatments to control *Fusarium fujikuroi* on rice. *Biol. Control* **2014**, *73*, 59-67.
- 456 16. ICH. Validation of analytical Procedures : text and methodology Q2(R1). *Guidance* **1994**, 17.
- 457 17. Amatulli, M. T.; Spadaro, D.; Gullino, M. L.; Garibaldi, A. Conventional and real-time PCR for  
458 identification of *Fusarium fujikuroi* and *Fusarium proliferatum* from diseased rice tissues and  
459 seeds. *Eur. J. Plant Pathol.* **2012**, *134*, 401-408.
- 460 18. Almeida Trapp, M.; De Souza, G.D.; Rodrigues-Filho, E.; Boland, W.; Mithöfer, A. Validated  
461 method for phytohormone quantification in plants. *Front. Plant Sci.* **2014**, *5*, 1-11.
- 462 19. Liu, H.; Li, X.; Xiao, J.; Wang, S. A convenient method for simultaneous quantification of  
463 multiple phytohormones and metabolites: application in study of rice-bacterium interaction.  
464 *Plant Methods* **2012**, *8*, 2.
- 465 20. Duan, L.; Liu, H.; Li, X.; Xiao, J.; Wang, S. Multiple phytohormones and phytoalexins are  
466 involved in disease resistance to *Magnaporthe oryzae* invaded from roots in rice. *Physiol. Plant.*  
467 **2014**, *152*, 486-500.

- 468 21. Nakazato, Y.; Tamogami, S.; Kawai, H.; Hasegawa, M.; Kodama, O. Methionine-induced  
469 phytoalexin production in rice leaves. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 577–583.
- 470 22. Rakwal, R.; Agrawal, G. K.; Yonekura, M.; and Kodama, O. Naringenin 7-O-methyltransferase  
471 involved in the biosynthesis of the flavanone phytoalexin sakuranetin from rice (*Oryza sativa*  
472 L.). *Plant Sci.* **2000**, *155*, 213–221.
- 473 23. Tamogami, S.; Rakwal, R.; and Kodama, O. Phytoalexin production by amino acid conjugates  
474 of jasmonic acid through induction of naringenin-7-O-methyltransferase, a key enzyme on  
475 phytoalexin biosynthesis in rice (*Oryza sativa* L.). *FEBS Lett.* **1997**, *401*, 239–242.
- 476 24. Shimizu, T.; Lin, F.; Hasegawa, M.; Okada, K.; Nojiri, H.; Yamane, H. Purification and  
477 identification of naringenin 7-O-methyltransferase, a key enzyme in biosynthesis of flavonoid  
478 phytoalexin sakuranetin in rice. *J. Biol. Chem.* **2012**, *287*, 19315–19325.
- 479 25. Kodama, O., Miyakawa, J.; Akatsuka, T.; Kiyosawa, S. Sakuranetin, a flavanone phytoalexin  
480 from ultraviolet-irradiated rice leaves. *Phytochemistry* **1992**, *31*, 3807–3809.
- 481 26. Hasegawa, M.; Mitsuhashi, I.; Seo, S.; Okada, K.; Yamane, H.; Iwai, T.; Ohashi, Y. Analysis on  
482 blast fungus-responsive characters of a flavonoid phytoalexin sakuranetin; accumulation in  
483 infected rice leaves, antifungal activity and detoxification by fungus. *Molecules* **2014**, *19*, 11404–  
484 11418.
- 485 27. Fukuta, M.; Dang Xuan, T.; Deba, F.; Tawata, S.; Dang Khanh, T.; Min Chung, I. Comparative  
486 efficacies in vitro of antibacterial, fungicidal, antioxidant, and herbicidal activities of  
487 momilactones A and B. *J. Plant Interact.* **2007**, *2*, 245–251.
- 488 28. Kato, T.; Kabuto, C.; Sasaki, N.; Tsunagawa, M.; Aizawa, H.; Fujita, K.; Kato, Y.; Kitahara, Y.;  
489 Takahashi, N. Momilactones, growth inhibitors from rice, *Oryza sativa* L. *Tetrahedron Lett.*  
490 **1973**, *14*, 3861–3864.
- 491 29. Cartwright, D.W.; Langcake, P.; Pryce, R.J.; Leworthy, D.P.; Ride, J.P. Isolation and  
492 characterization of two phytoalexins from rice as momilactones A and B. *Phytochemistry* **1981**,  
493 *20*, 535–537.

- 494 30. Kato-Noguchi, H.; Ino, T.; Ichii, M. Changes in release of momilactone B into the environment  
495 from rice throughout its life cycle. *Funct. Plant Biol.* **2003**, *30*, 995-997.
- 496 31. Kato-Noguchi, H.; Ino, T. Concentration and release level of momilactone B in the seedlings of  
497 eight rice cultivars. *J. Plant Physiol.* **2005**, *162*, 965–969.
- 498 32. Belz, R.G. Allelopathy in crop/weed interactions-an update. *Pest Manage. Sci.* **2007**, *63*, 308-  
499 326.
- 500 33. Kato-Noguchi, H. Stress-induced allelopathic activity and momilactone B in rice. *Plant Growth*  
501 *Regul.* **2009**, *59*, 153–158.
- 502 34. Santner, A.; Estelle, M. Recent advances and emerging trends in plant hormone signalling.  
503 *Nature* **2009**, *459*, 1071-1078.
- 504 35. Robert-Seilaniantz, A.; Navarro, L.; Bari, R.; Jones. J.D.G. Pathological hormone imbalances.  
505 *Curr. Opin. Plant Biol.* **2007**, *10*, 372-379.
- 506 36. Tudzynski, B.; Biosynthesis of gibberellins in *Gibberella fujikuroi*: biomolecular aspects. *Appl.*  
507 *Microbiol. Biotechnol.* **1999**, *52*, 298-310.
- 508 37. Wiemann, P.; Sieber, C.M.K.; von Bargen, K.W.; Studt, L.; Niehaus, E.M.; Espino, J.J.; Huß,  
509 K.; Michielse, C.B.; Albermann, S.; Wagner, D.; Bergner, S.V.; Connolly, L.R.; Fischer, A.;  
510 Reuter, G.; Kleigrew, K.; Bald, T.; Wingfield, B.D.; Ophir, R.; Freeman, S.; Hippler, M.; Smith,  
511 K.M.; Brown, D.W.; Proctor, R.H.; Münsterkötter, M.; Freitag, M.; Humpf, H.U.; Güldener, U.;  
512 Tudzynski, B. Deciphering the cryptic genome: genome-wide analyses of the rice pathogen  
513 *Fusarium fujikuroi* reveal complex regulation of secondary metabolism and novel metabolites.  
514 *PLoS Pathog.* **2013**, *9*, e1003475.
- 515 38. Yang, D.L.; Yao, J.; Mei, C.S.; Tong, X.H.; Zeng, L.J.; Li, Q.; Xiao, L.T.; Sun, T.; Li, J.; Deng,  
516 X.W.; Lee, C.M.; Thomashow, M.F.; Yang, Y.; He, Z.; He, S. Y. Plant hormone jasmonate  
517 prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proc. Natl.*  
518 *Acad. Sci. U. S. A.* **2012**, *109*, E1192–E1200.



- 519 39. Schawechheme, C. Gibberellin signaling in plants-extended version. *Front. Plant Sci.* **2012**, 2,  
520 1-7.
- 521 40. Sun, T.-P.. The Molecular mechanism and evolution of the review GA–GID1–DELLA signaling  
522 module in plants. *Curr. Biol.* **2011**, 21, R338–R345.
- 523 41. Navarro, L.; Bari, R.; Achard, P.; Lisón, P.; Nemri, A.; Harberd, N.P.; Jones, J.D. DELLAs  
524 control plant immune responses by modulating the balance of jasmonic acid and salicylic acid  
525 signaling. *Curr. Biol.* **2008**, 18, 650-655
- 526 42. Wang, D.H.; Li, X.X.; Su, Z.K.; Ren, H.X. The role of salicylic acid in response of two rice  
527 cultivars to chilling stress. *Biol. Plant.* **2009**, 53, 545-552.
- 528 43. Pál, M.; Kovács, V.; Szalai, G.; Soós, V.; Ma, X.; Liu, H.; Mei, H.; Janda, T. Salicylic acid and  
529 abiotic stress responses in rice. *J. Agron. Crop Sci.* **2014**, 200, 1-11.
- 530 44. Silverman, P.; Seskar, M.; Kanter, D.; Schweizer, P.; Métraux, J.P.; Raskin, I. Salicylic acid in  
531 rice. *Plant Physiol.* **1995**, 108, 633-639.
- 532 45. Yang, Y.; Qi, M.; Mei, C. Endogenous salicylic acid protects rice plants from oxidative damage  
533 caused by aging as well as biotic and abiotic stress. *Plant J.* **2004**, 40, 909-919.
- 534 46. Jiang, C.J.; Shimono, M.; Sugano, S.; Kojima, M.; Yazawa, K.; Yoshida, R.; Inoue, H.; Hayashi,  
535 N.; Sakakibara, H.; Takatsuji, H. Abscisic acid interacts antagonistically with salicylic acid  
536 signaling pathway in rice-*Magnaporthe grisea* interaction. *Mol. Plant-Microbe Interact.* **2010**,  
537 23, 791–798.
- 538 47. Ko, J.H.; Yang S.H.; Han, K.H. Upregulation of an Arabidopsis RING-H2 gene, XERICO,  
539 confers drought tolerance through increased abscisic acid biosynthesis. *Plant J.* **2006**, 47, 343–  
540 355.
- 541 48. Koga, H.; Dohi, K.; Mori, M. Abscisic acid and low temperatures suppress the whole plant-  
542 specific resistance reaction of rice plants to the infection of *Magnaporthe grisea*. *Physiol. Mol.*  
543 *Plant Pathol.* **2004**, 65, 3–9.
- 544

## FIGURE CAPTIONS

**Figure 1.** Chemical structure of analytes: (1) gibberellic acid; (2) abscisic acid; (3) salicylic acid; (4) jasmonic acid; (5) naringenin; (6) sakuranetin; (7) momilactone B; (8) momilactone A.

**Figure 2.** Chromatograms of a standard solution containing 200 ng/mL of analytes under MRM condition.

**Figure 3.** Changes in phytohormones gibberellic acid and abscisic acid accumulation during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means  $\pm$  SD (n=12 independent analysis) in fresh plant tissues (ng/g of fresh weight, FW).

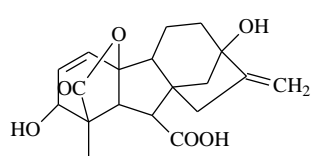
**Figure 4.** Changes in phytohormones jasmonic acid and salicylic acid accumulation during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means  $\pm$  SD (n=12 independent analysis) in fresh plant tissues (ng/g of fresh weight, FW).

**Figure 5.** Changes in phytoalexins naringenin and sakuranetin accumulation during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means  $\pm$  SD (n=12 independent analysis) in fresh plant tissues (ng/g of fresh weight, FW).

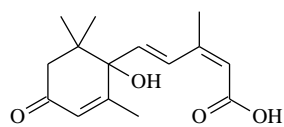
**Figure 6.** Changes in phytoalexins momilactone A and momilactone B accumulation during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means  $\pm$  SD (n=12 independent analysis) in fresh plant tissues (ng/g of fresh weight, FW).

**Figure 7.** Quantification of pathogen with TaqMan real time PCR growth during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means  $\pm$  SD (n=9 independent analysis) in fresh plant tissues [Log<sub>10</sub>(ng DNA of *Fusarium fujikuroi* / 100 mg plant tissue)].

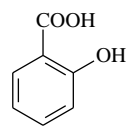
Figure 1



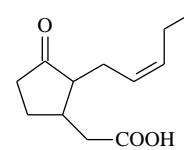
Gibberellic acid, 1



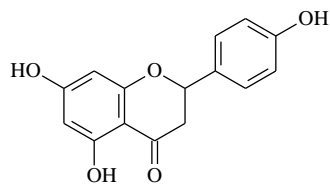
Abscisic acid, 2



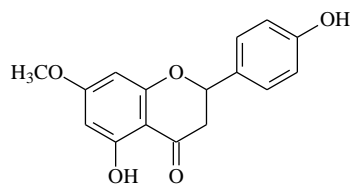
Salicylic acid, 3



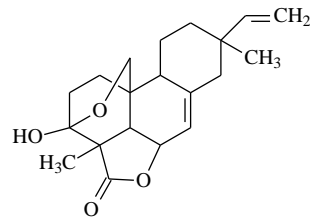
Jasmonic acid, 4



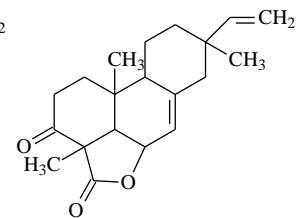
Naringenin, 5



Sakuranetin, 6



Momilactone B, 7



Momilactone A, 8

Figure 2

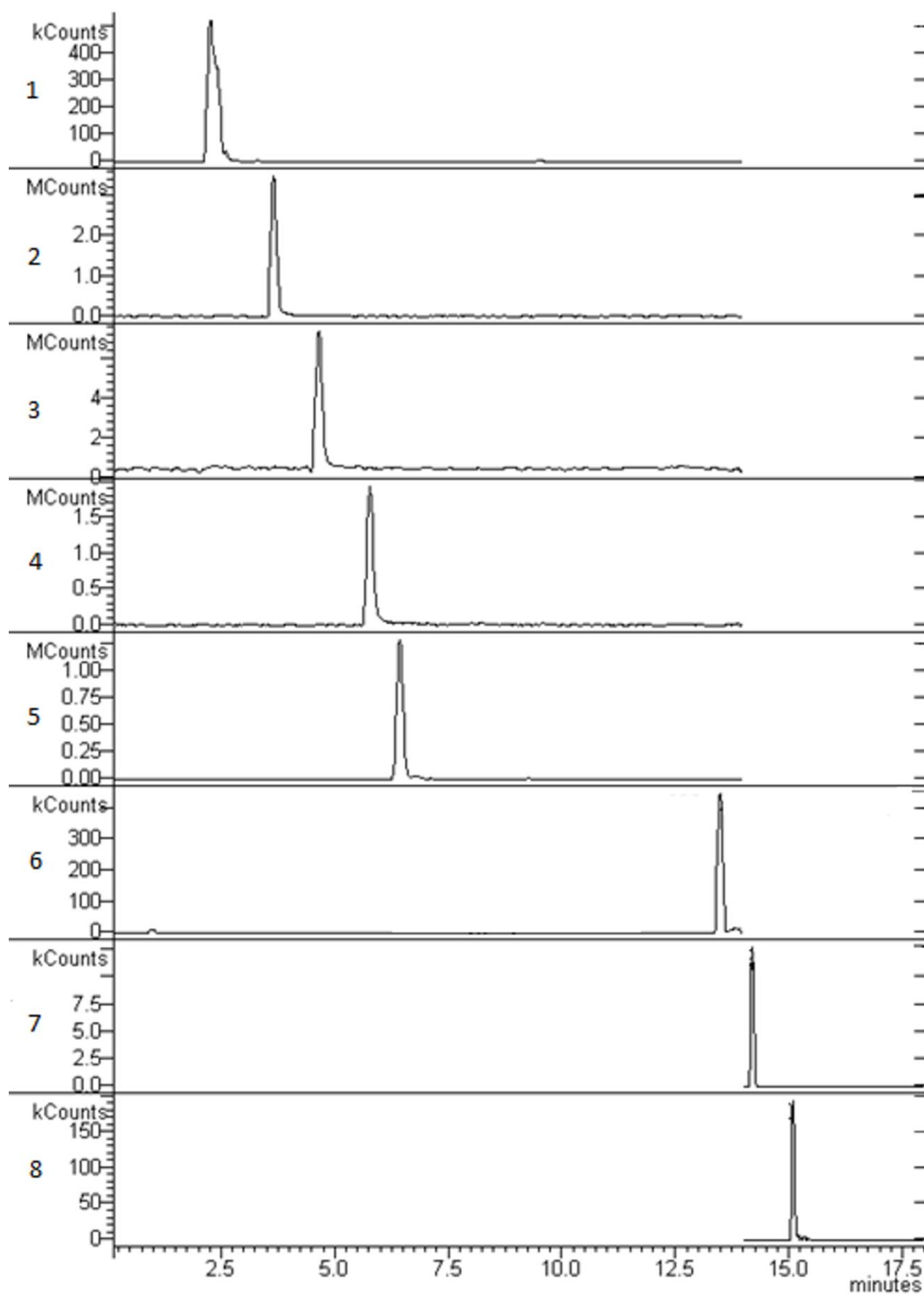


Figure 3

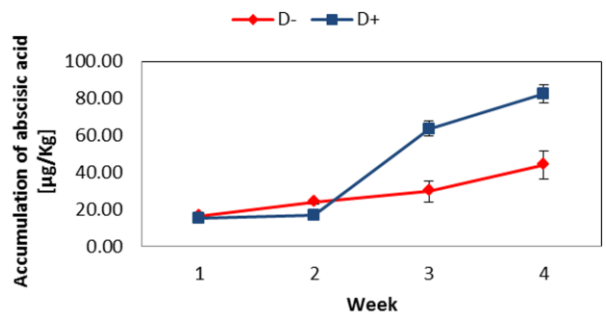
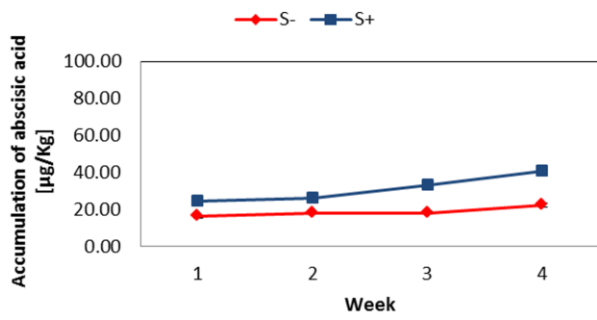
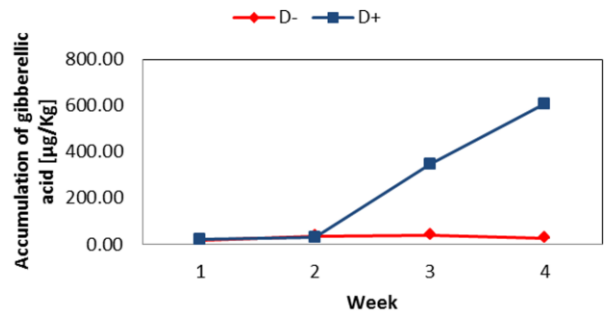
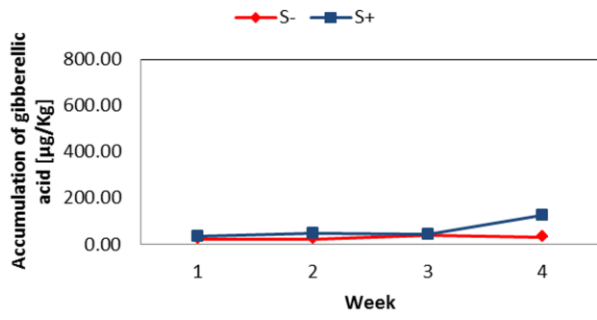


Figure 4

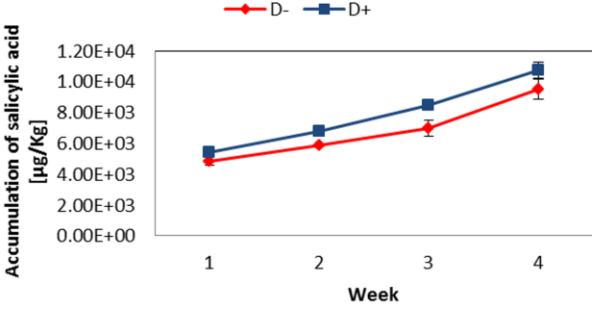
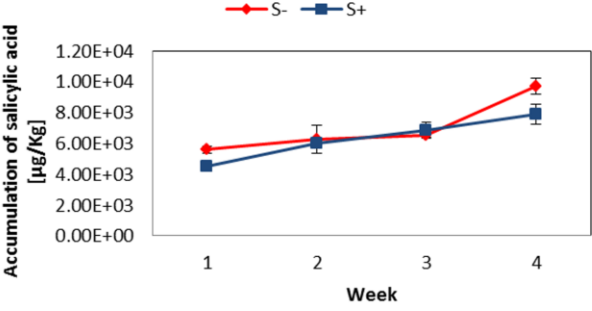
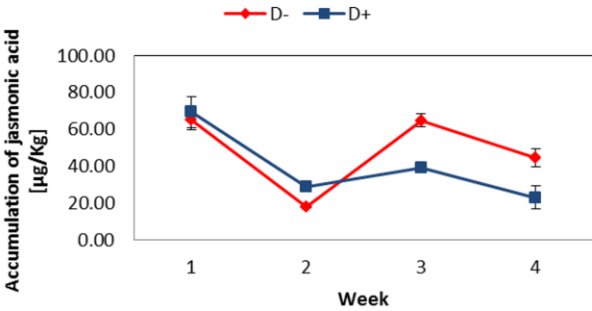
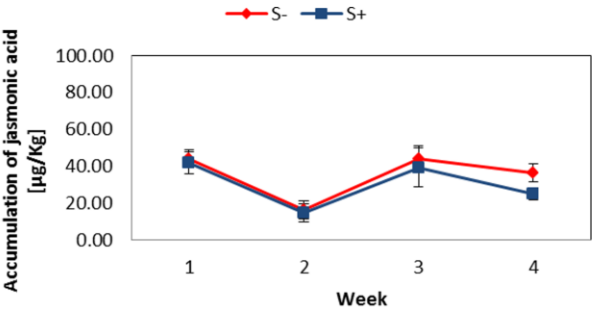


Figure 5

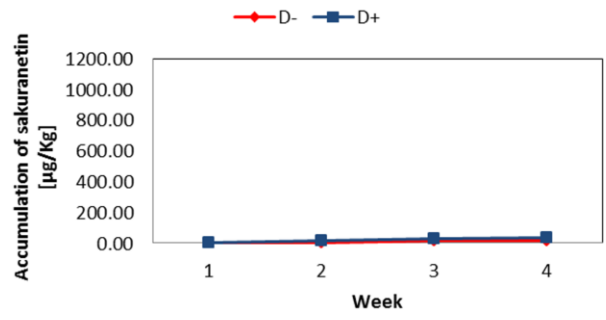
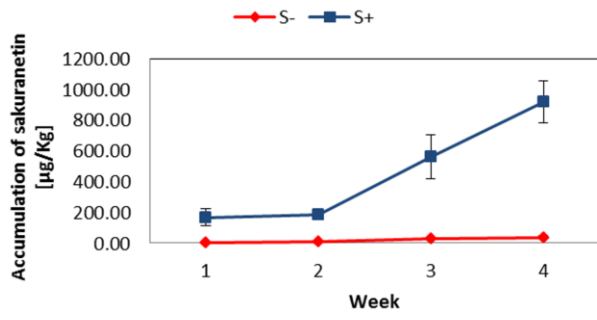
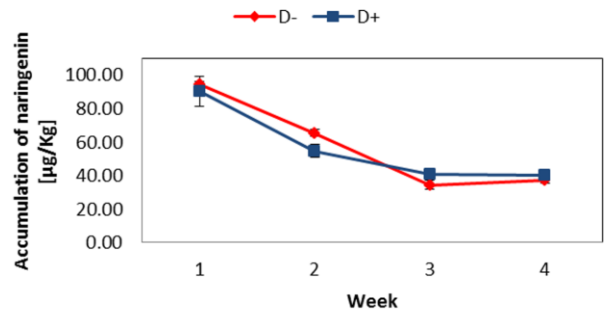
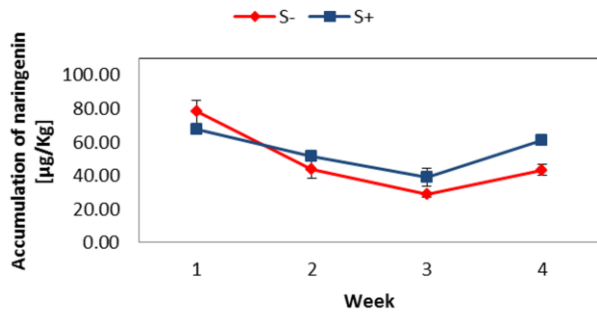




Figure 6

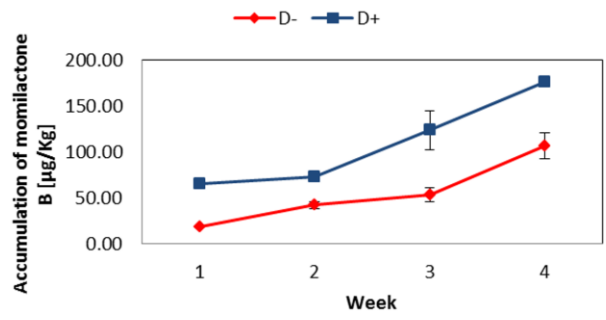
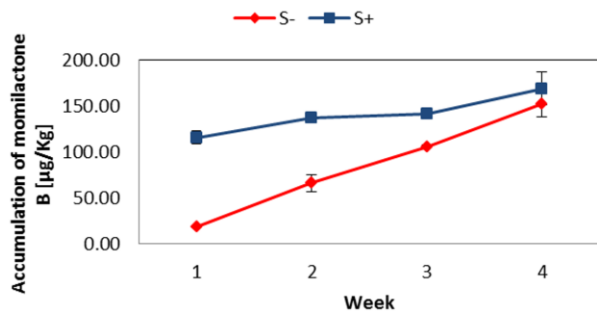
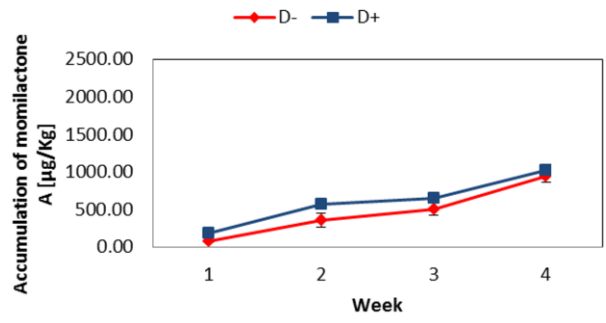
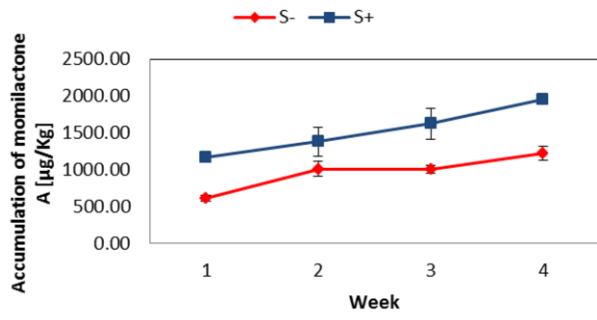
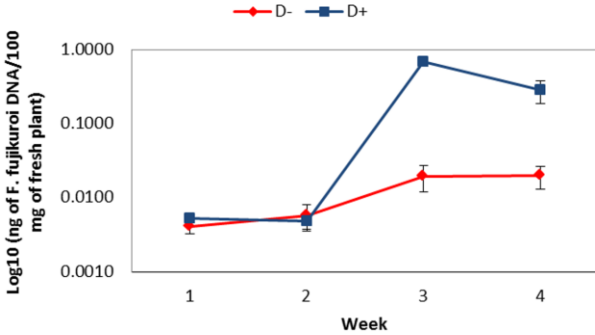
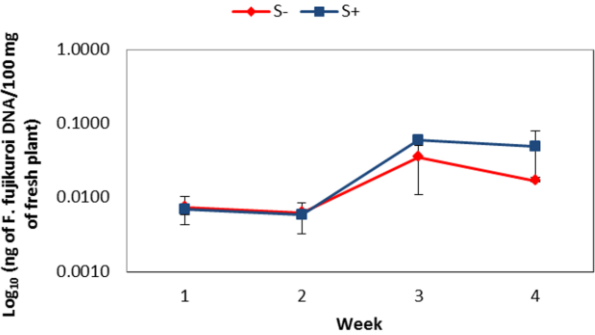
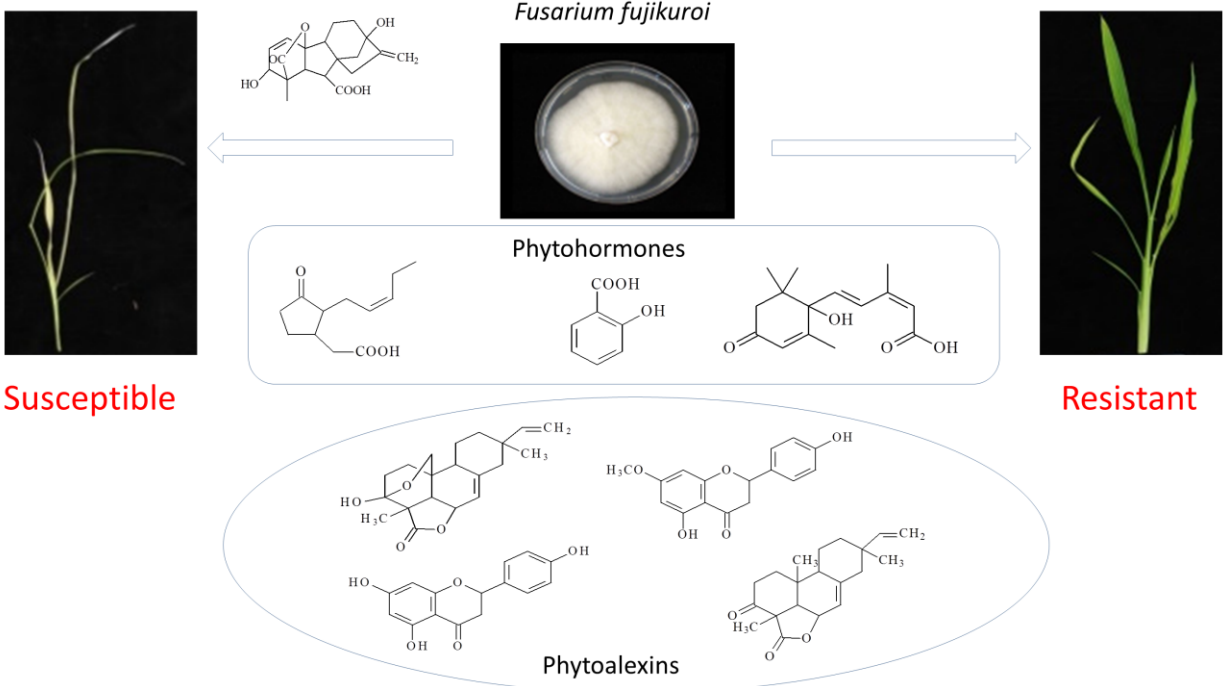


Figure 7



TOC graphic



Susceptible

Resistant