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

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Abstract

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

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

INTRODUCTION

Fusarium fujikuroi Niremberg is the anamorph of *Gibberella fujikuroi* (Sawada) Ito in Ito & K. Kimura and is a member of the *G. fujikuroi* species complex.[1] This seed-borne pathogen is causal agent of bakanae disease in rice (*Oryza sativa* L.). Infected rice turns pale yellow and exhibits chlorosis, poor grain ripening, empty panicles, and foot and stem rot.[2] Many of these symptoms are due to exposure to gibberellins, a large family of isoprenoid compounds originally characterized as plant hormones, which are produced by the pathogen.[3] Some of them are bioactive growth regulators, controlling seed germination, stem elongation, and flowering. These phytohormones are synthesized in the young vegetative tissues of plants and later transported to other parts for the enhancement of growth and development.[4] Gibberellin A_3 was significantly produced only in cultures of strains of *F. fujikuroi*.[5]

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

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

zones of the internode. Abscisic acid is a potent antagonist of gibberellins action in rice internodes.[10, 11]

The aim of the current work was to elucidate the chemical response of rice to *Fusarium fujikuroi* infection. A sensitive and rapid method to simultaneously quantitate different classes of molecules, i.e. phytohormones and phytoalexins, could facilitate the investigation of rice defense towards pathogens. Therefore, we aimed at developing an easy HPLC-MS/MS method for rapid phytohormones profiling that included: abscisic acid, jasmonic acid, salicylic acid, and gibberellin A_3; additionally, this method permitted the determination of four phytoalexins (naringenin, sakuranetin, momilactone A and momilactone B) in the same analysis.

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

MATERIALS AND METHODS

Plant material and fungal cultures

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

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

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

Disease index

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

Phytohormones and phytoalexins extraction and analysis

Reagents and standards

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

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

Preparation of standard solutions

Stock solutions of each analyte were prepared at 1 mg/mL in CH_3OH. Working solutions were prepared diluting stock solutions in CH_3OH:H_2O (8:2) at different concentrations depending on the range of the calibration curve. A stock solution mixture, made from these individual stock solutions, was prepared in LC mobile phase and matrices, to obtain calibration curves and to determine ion suppression, recovery, limit of detection (LOD) and limit of quantitation (LOQ) for each analyte.

Extraction procedure

Acetone, ethyl acetate, dichloromethane, methanol, acetonitrile, water and two different mixtures of water:methanol (1:1 and 2:8 pure or containing 0.1% CH_3COOH) were tested. In a second set of analysis, the influence of three extraction times (1h, 2h, overnight) and two methods (ultrasonic bath or rotary shaker) on the recovery were evaluated. Analyte-free rice matrix was not available, so for each test two sets of samples, with or without standard additions, were prepared and each extraction was performed twice for each sample.

A few plants of rice were ground and 0.2 g fresh plant material was transferred in 2 mL centrifuge tubes with 1 mL extraction solution (80% CH_3OH acidified with 0.1% CH_3COOH). The samples were frozen in liquid nitrogen and homogenized by using TissueLyser (Qiagen, Venlo, Netherlands), then shaken at 4 °C in the dark overnight. Finally, samples were centrifuged at 15,000 rpm and 4 °C for 2 min and the supernatant was analyzed by HPLC-MS/MS.

HPLC-MS/MS analysis

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

HPLC separation of analytes was performed using a 150 x 2 mm i.d., 3 µm, Luna Phenyl-Hexyl (Phenomenex, Torrance, CA) under a flow of 200 µL/min. The column temperature was set at 25 °C. Solvent A was H_2O with 0.1% of HCOOH, solvent B was CH_3CN. HPLC analysis was programmed

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

Samples were ionized using an electrospray (ESI) ion source operating in negative ion mode for salicylic acid, jasmonic acid, abscisic acid, gibberellic acid, naringenin and sakuranetin; in positive ion mode for momilactone A and momilactone B. For the multiple reaction monitoring (MRM) experiments, the quantification transitions for each compound were: (1) *m/z* 345→239 CE 14V; (2) *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 18V; (6) *m/z* 285→119 CE 30V; (7) *m/z* 331→269 CE 25V; (8) *m/z* 315→271 CE 14V. The collision gas (Ar) pressure was set at 2 mbar for all experiments.

Method validation

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

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

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

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

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

Statistical analysis

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

Molecular identification of *F. fujikuroi*

DNA extraction

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

Design of real-time PCR primers and probe

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

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

TaqMan Real-time PCR assay

The real-time TaqMan PCR assays were conducted using an Applied Biosystems plus detection system. Twenty µL reactions containing 12.5 µL Taqman Master Mix (2×) (Applied Biosystems), PCR primers at a concentration of 10µM, the TaqMan FfujiPq at 5 µM and 5 µl of DNA at the concentration of about 30 ng/µL were prepared in triplicate. Amplification and detection were performed in 96-well optical reaction plates (Applied Biosystems) sealed with MicroAmpTM optical adhesive film (Applied Biosystems) on an ABI Prism 7900HT sequence detection system real time thermal cycler. The amplification conditions were: 2 min at 50 °C, 10 min at 95 °C, followed by 15 s at 95 °C and 40 s at 62 °C for 40 cycles.

A standard curve was prepared by serial dilution of known quantities of *F. fujikuroi* DNA in order to quantitate the fungus inside rice tissues. In the preparation of the standard curve, the initial concentration of DNA was 386 ng /µL.

Primer and probe design, specificity and sensitivity

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

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

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

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

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

The quantity of the fungus in plants was measured in ng 100 mg^{-1} by extraction of DNA from the culm and from the leaves of the cvs. Selenio and Dorella.

RESULTS

Rice germination and disease index

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

Phytohormones and phytoalexins extraction and analysis

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

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

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

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

Accumulation of phytoalexins

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

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.[17, 18] Liu et al.[19] 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.[20] 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.[21] 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[22] or in presence of an increase of jasmonic acid or jasmonic acid related compound.[23]
313 Sakuranetin biosynthesis is rapidly induced by biotic and abiotic stresses and was not found in healthy
314 rice leaves.[24] 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.[25] Hasegawa et al.[26] 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;[27] 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,[28] then it was isolated from rice leaves and
325 characterized as phytoalexin.[29] 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,[30] produced in different concentrations depending on the rice cultivars.[31] Generally, stress could
332 induce an increase of the production of allelochemicals, that play an important role in defense
333 mechanism.[32] In rice, the increased allelopathy with elevated secretion of momilactone B, was
334 recorded in different stress conditions.[33] 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

interactions produce variations in the level of various phytohormones simultaneously but each hormone has a characteristic biological effect.[34]

Various plant hormones are often involved in the same biological process with additive, synergistic or antagonistic action.[35] The rice pathogen *F. fujikuroi* is able to produce large amounts of GAs, especially the bioactive compounds gibberellic acid.[36] Gibberellic acid was significantly detected in cv. Dorella infected with *F. fujikuroi.* At 3 and 4 wpgs, increased levels of gibberellic acid were observed and the plants showed typical bakanae symptoms: infected seedlings were elongated, more slender and slightly chlorotic when compared to healthy seedlings. The increased level of gibberellic acid could be attributed to the presence of the pathogen, which was confirmed by qPCR, only in the susceptible cultivar starting at 2 wpg. GAs production contributes to increase the pathogenicity of *F. fujikuroi* on rice.[37] The resistant cultivar Selenio did not show the typical symptoms of bakanae, despite a slight increase of *F. fujikuroi* being observed in inoculated plants at the 3 and 4 wpgs. Gibberellic acid was observed at low levels in inoculated plants of resistant cultivar only at 4 wpg.

Jasmonic acid levels had a different trend between infected plants and uninoculated control for cv. Dorella. At 3 wpg, a decrease of jasmonic acid was observed in inoculated plants of the susceptible cultivar in correspondence with an increase of gibberellic acid, as previously described by Yang et al..[38] Gene expression studies and pathobiological assays suggested that gibberellic acid controls jasmonic acid-responsive gene expression and jasmonic acid-mediated plant immune responses.[39] Gibberellic acid promotes plant growth by regulating the degradation of a class of nuclear growth-repressing proteins called DELLAs. SLENDER RICE 1 (SR1), the only DELLA protein in rice, inhibits JAZ1, the key repressor of jasmonic acid signaling.[40] The increase of gibberellic acid, produced by the fungus, restricts jasmonic acid signaling that is strongly activated during necrotroph infections.[41]

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

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

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

The analytical method developed in this paper could be used to study the response of rice to other biotic or abiotic stresses, besides *Fusarium fujikuroi*. The pathogen considered in this work is a seedborne pathogen, and the chemical responses of plants have rarely been studied in relationship to seedborne pathogen infection. Some responses are similar to those activated in response to other rice pathogens, such as *Magnaporthe oryzae*, which is a foliar fungal pathogen, with the main difference that gibberelins are typically produced by *F. fujikuroi*. The levels of phytohormones and phytoalexins in rice showed that *F. fujikuroi* may behave differently with respect to the cultivar. In the susceptible cultivar, the pathogen could systemically act as a necrotroph destroying the plant cells. On the contrary, in the resistant cultivar, the pathogen is present on rice, though at lower concentration, 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
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B, and Dr. Giampiero Valè, Rice Research Unit of Consiglio per la Ricerca e la Sperimentazione in Agricoltura, Italy, for providing the rice seeds.

SUPPORTING INFORMATION

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

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

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

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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 ± SD (n=12 independent analysis) in fresh plant tissues (ng/g of fresh weight, FW).

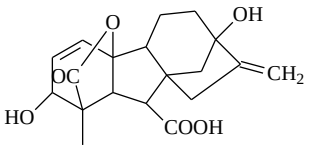
Figure 4. Changes in phytohormones jasmonic acid and salicylic acid accumulation during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means ± SD (n=12 independent analysis) in fresh plant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

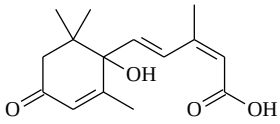
Figure 6. Changes in phytoalexins momilactone A and momilactone B accumulation during 4 weeks after germination in resistant (Selenio, S) and susceptible (Dorella, D) rice cultivar, inoculated (+) or not (-) with *Fusarium fujikuroi*. Values are means ± SD (n=12 independent analysis) in fresh plant tissues (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 ± SD (n=9 independent analysis) in fresh plant tissues [Log_{10}(ng DNA of *Fusarium fujikuroi* / 100 mg plant tissue)].

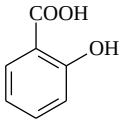
Figure 1



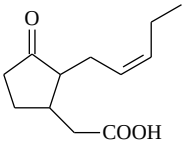
Gibberellic acid, **1**



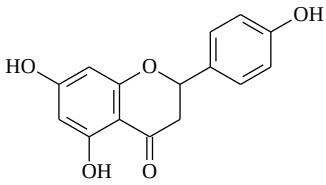
Abscisic acid, **2**



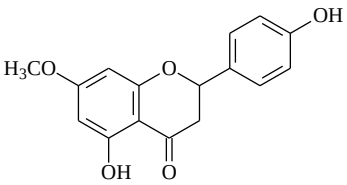
Salicylic acid, **3**



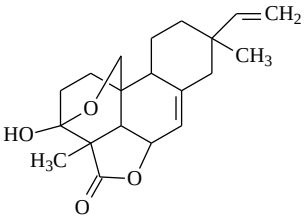
Jasmonic acid, **4**



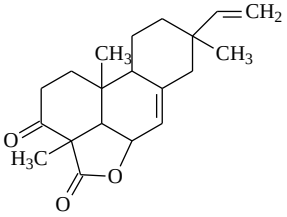
Naringenin, **5**



Sakuranetin, **6**



Momilactone B, **7**



Momilactone A, **8**

Figure 2

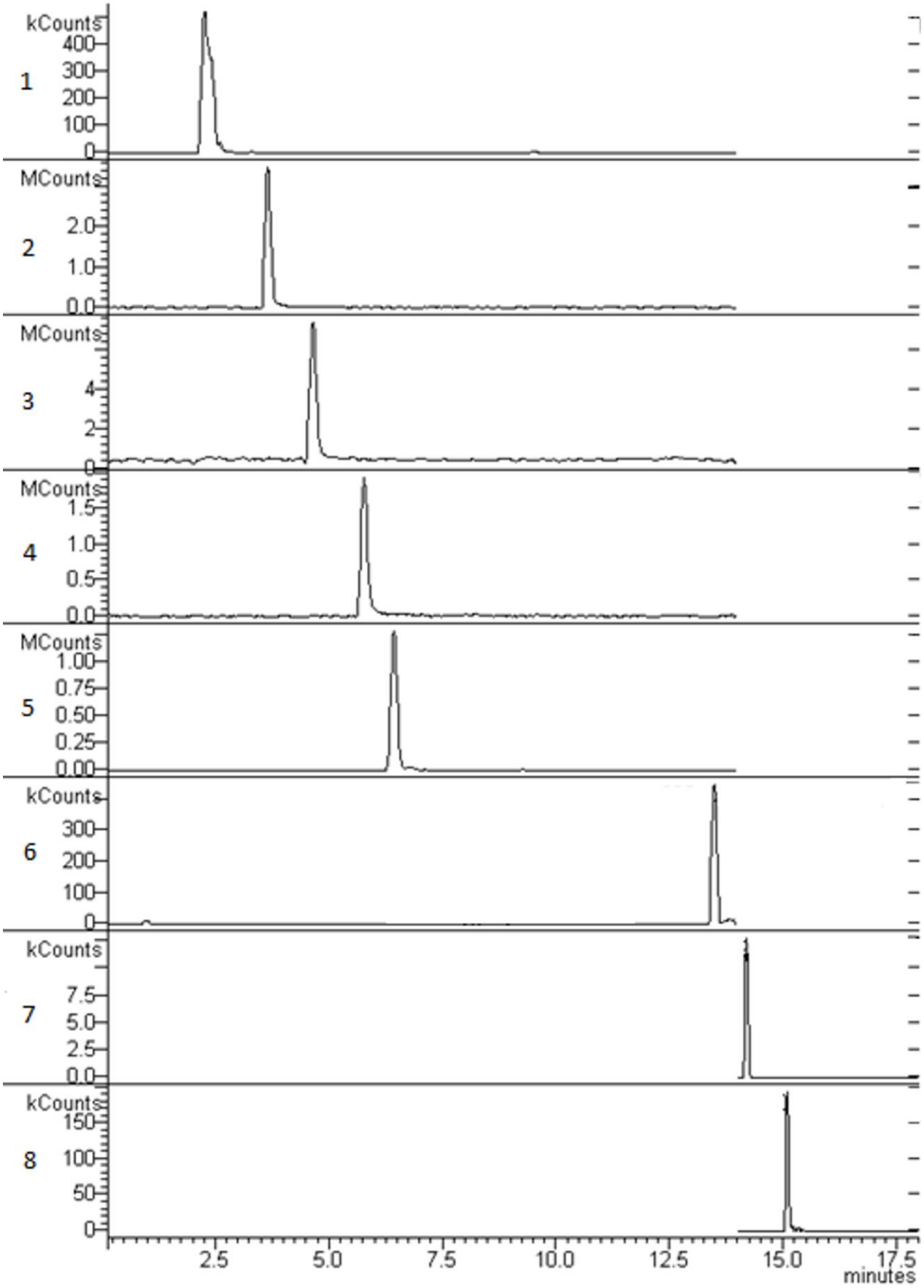

kCounts
1
400
300
200
100
0
MCounts
2
2.0
1.0
0.0
MCounts
3
4
2
0
MCounts
4
1.5
1.0
0.5
0.0
MCounts
5
1.00
0.75
0.50
0.25
0.00
kCounts
6
300
200
100
0
kCounts
7
7.5
5.0
2.5
0.0
kCounts
8
150
100
50
0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
minutes

Figure 3

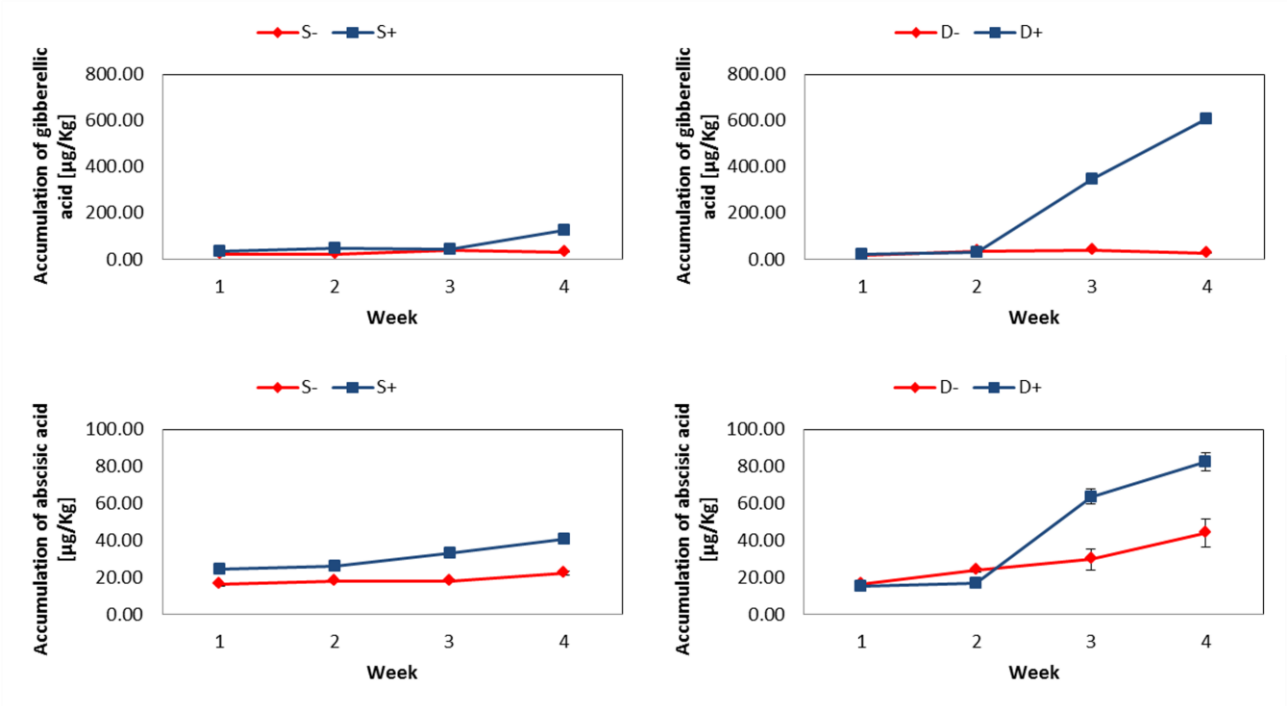

S-
S+
Accumulation of gibberellic acid [µg/Kg]
800.00
600.00
400.00
200.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of gibberellic acid [µg/Kg]
800.00
600.00
400.00
200.00
0.00
1
2
3
4
Week
S-
S+
Accumulation of abscisic acid [µg/Kg]
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of abscisic acid [µg/Kg]
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
Week

Figure 4

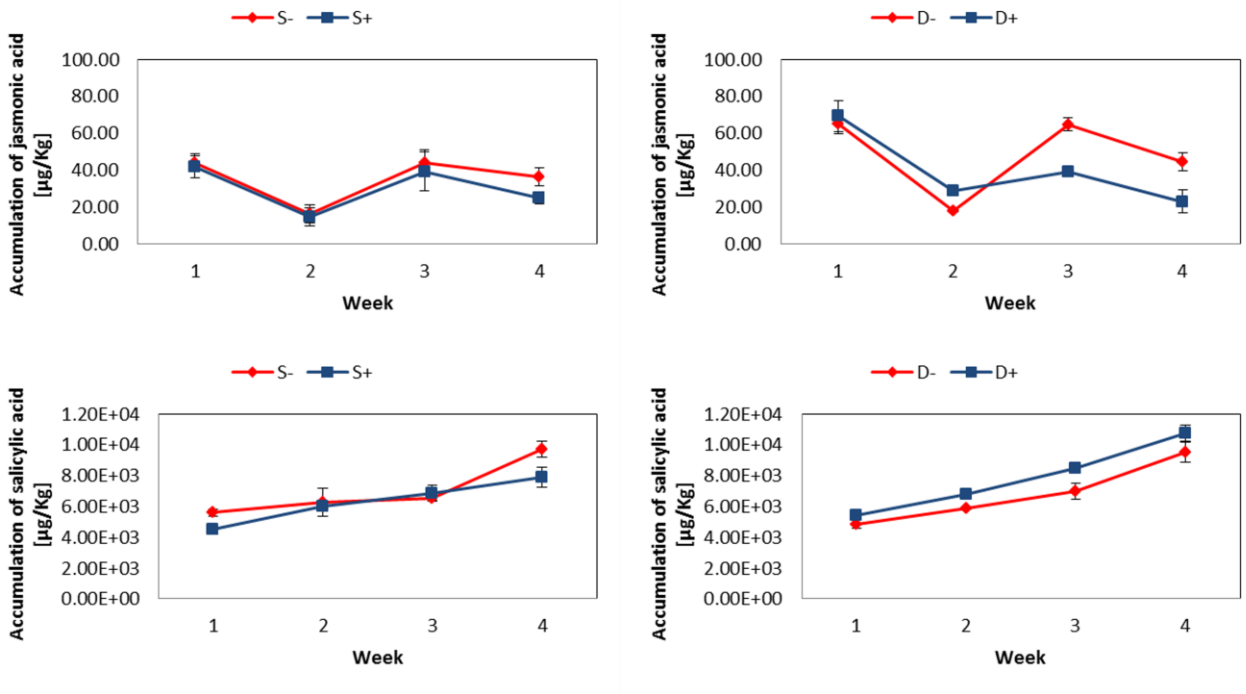

S-
S+
Accumulation of jasmonic acid [µg/Kg]
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of jasmonic acid [µg/Kg]
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
Week
S-
S+
Accumulation of salicylic acid [µg/Kg]
1.20E+04
1.00E+04
8.00E+03
6.00E+03
4.00E+03
2.00E+03
0.00E+00
1
2
3
4
Week
D-
D+
Accumulation of salicylic acid [µg/Kg]
1.20E+04
1.00E+04
8.00E+03
6.00E+03
4.00E+03
2.00E+03
0.00E+00
1
2
3
4
Week

Figure 5

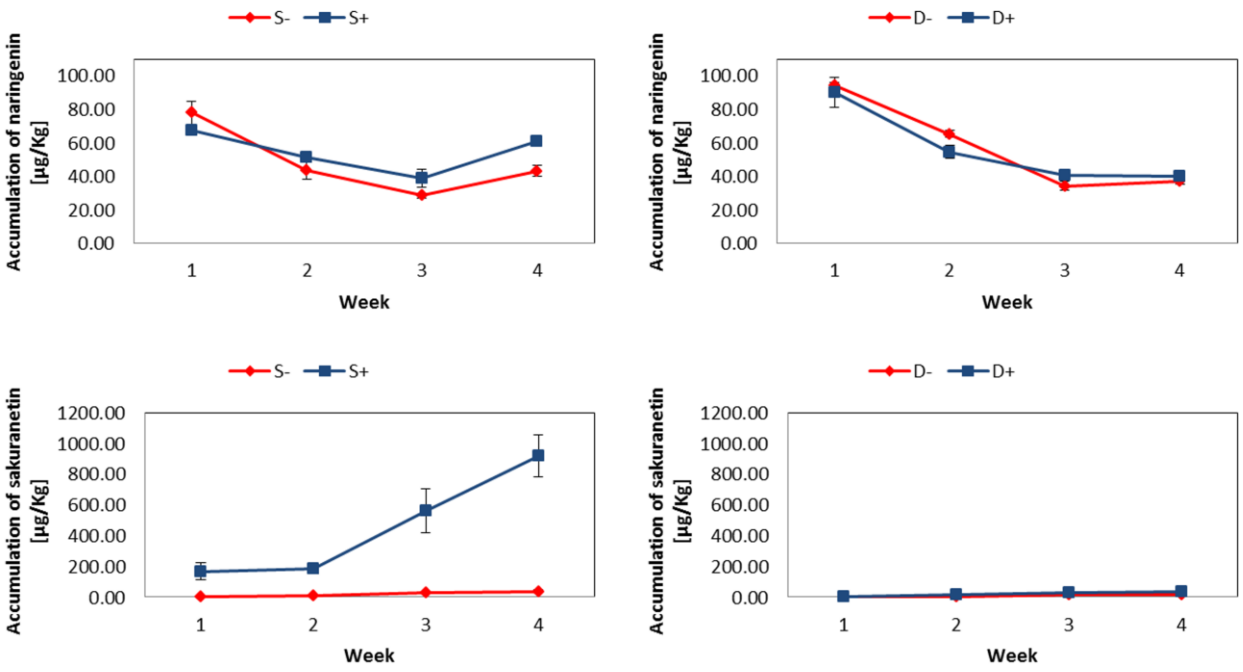

S-
S+
Accumulation of naringenin [µg/Kg]
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of naringenin [µg/Kg]
100.00
80.00
60.00
40.00
20.00
0.00
1
2
3
4
Week
S-
S+
Accumulation of sakuranetin [µg/Kg]
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of sakuranetin [µg/Kg]
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
1
2
3
4
Week

Figure 6

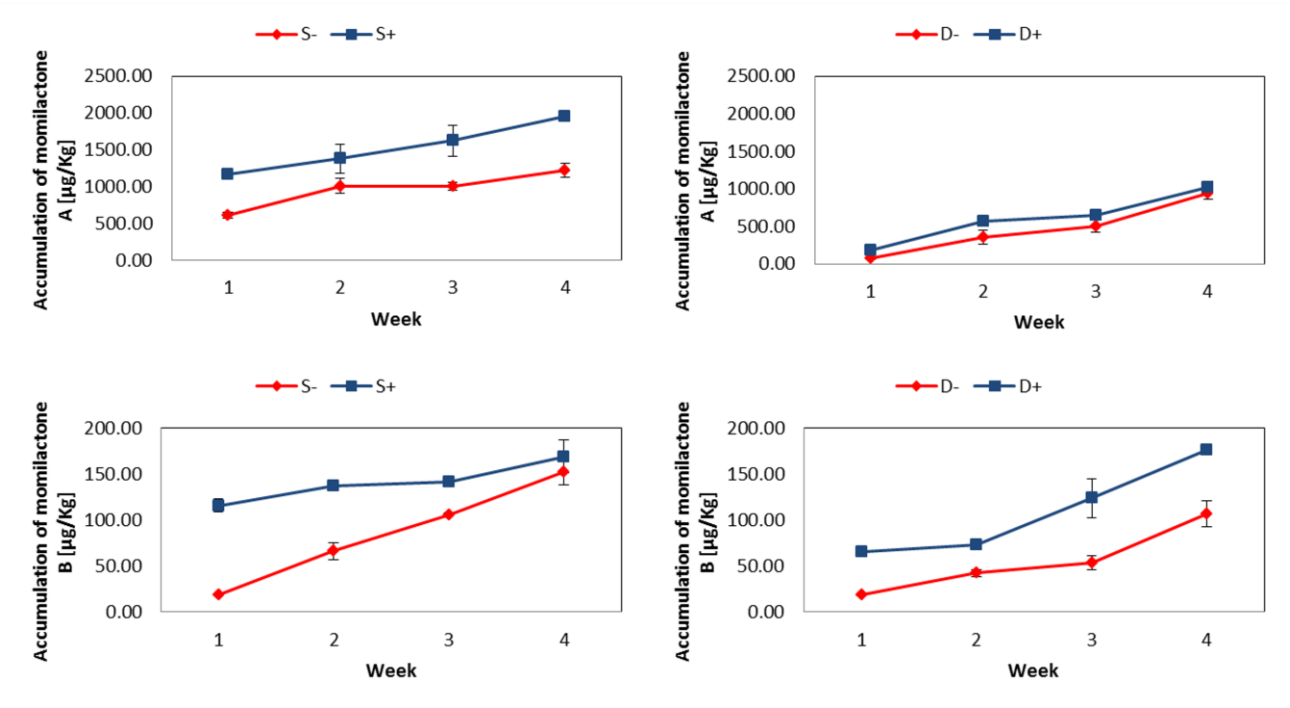
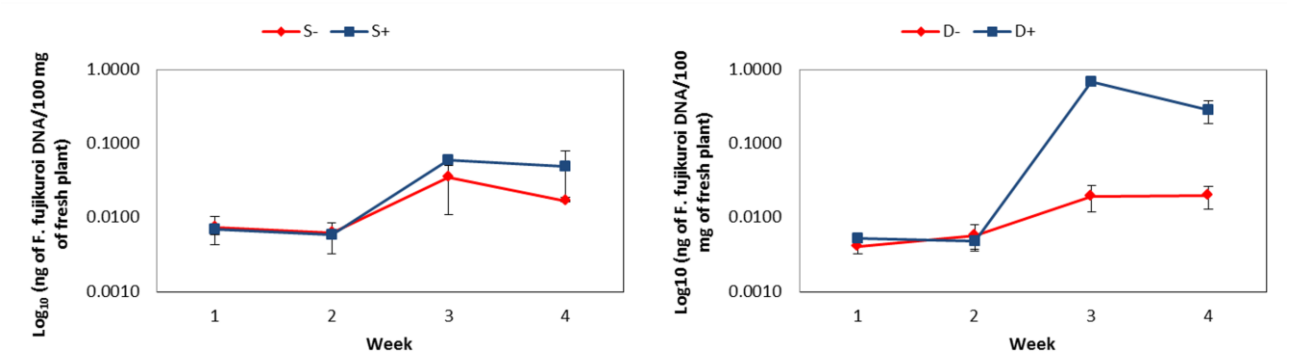
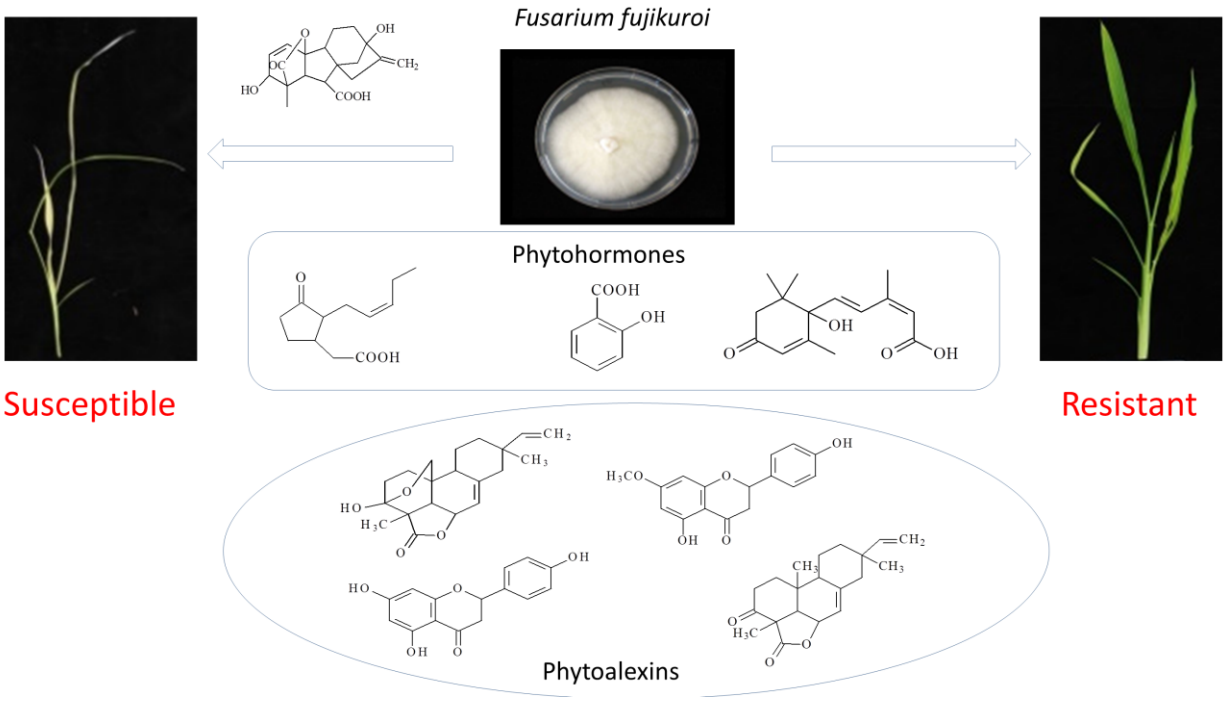

S-
S+
Accumulation of momilactone A [µg/Kg]
2500.00
2000.00
1500.00
1000.00
500.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of momilactone A [µg/Kg]
2500.00
2000.00
1500.00
1000.00
500.00
0.00
1
2
3
4
Week
S-
S+
Accumulation of momilactone B [µg/Kg]
200.00
150.00
100.00
50.00
0.00
1
2
3
4
Week
D-
D+
Accumulation of momilactone B [µg/Kg]
200.00
150.00
100.00
50.00
0.00
1
2
3
4
Week

Figure 7


S-
S+
1.0000
0.1000
0.0100
0.0010
Log10 (ng of F. fujikuroi DNA/100 mg of fresh plant)
1
2
3
4
Week
D-
D+
1.0000
0.1000
0.0100
0.0010
Log10 (ng of F. fujikuroi DNA/100 mg of fresh plant)
1
2
3
4
Week

TOC graphic


Fusarium fujikuroi
Phytohormones
Phytoalexins
Susceptible
Resistant