Fusarium Infection Causes Phenolic Accumulations and Hormonal Disorders in *Orobanche* spp.

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Abstract The physiological effects of *Fusarium oxysporum* on in-root parasitic weed, *Orobanche* spp. (broomrape) with references to change in plant hormones and secondary plant constituents were investigated. The levels of IAA, GA, ABA and JA in the experimental group were significantly lower than those in the control group, while the level of SA was higher in the experimental group. In secondary metabolic studies, the quantities of various phenols were measured in the two groups and catechin, syringic acid and p-coumaric acid amounts were significantly higher in the experimental group than in the control group, unlike gallic acid which have a lower amount. Consequently, in the light of all data, it was concluded that *Fusarium oxysporum* (1) causes heavy hormonal disorder, (2) triggered only SA-mediated defense and (3) induced intensively accumulation of phenolic substances in *Orobanche*. *Fusarium oxysporum* causes lethal physiological damage on *Orobanche* spp.

Keywords Broomrape · Parasitic plant · Biocontrol · Mycoherbicide

Introduction

Orobanche spp. (broomrapes) are obligate root parasitic plants that cause severe yield reduction in many important crops such as sunflower, potato, rapeseed, mustard, fava bean, lentil, tomatoes, alfalfa, pepper and tobacco. Methods

for controlling these parasitic plants are crop rotation, soil fumigation and solarization, use of host seed free of the parasite's seeds, manipulating time, use of seeds of varieties genetically resistant to *Orobanche* and chemical herbicide applications [1]. A method not mentioned above is the biological control of *Orobanche* using fungal pathogens. Among these fungal pathogens, *Fusarium oxysporum* Schlecht. f. sp. *orthoceras* (FOO) has been effective against *Orobanche* parasitism, reducing broomrape pathogenicity in tomato by 90% [2]. Pesta granules containing fungal chlamydospores or microconidia as well as different proportions of wheat flour and kaolin (Pesta) mixture have been used effectively for *Orobanche* biocontrol [3]. However *Fusarium* pathogenicity and its mechanisms, are not effective on *Orobanche* currently known. *Fusarium* infection causes irrevocable and fatal damage to broomrape. Recent study [4] indicates that *F. oxysporum* causes heavy ROS damage in *Orobanche*, induced significant irrevocable genotoxic effects on the DNA of *Orobanche*, degraded protein metabolism and synthesis, and finally triggered apoptosis. In this study we examine post-infectional changes in plant hormones and secondary plant constituents.

Materials and Methods

Broomrape seeds were collected from sunflower (cv. HA 89-B) fields in Edirne, in Trakya region of Turkey and were identified as *Orobanche cernua* L. The fungal isolates used in the experiment was *Fusarium oxysporum* Schl Tdl. [Mycobanc no: MB#218372] which was isolated and it was collected from the infected broomrape plants in the same fields (Edirne, 41°45'51"N, 26°59'11"E). Infected broomrape tissue with the fungus was surface-disinfected for

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1 min in a solution containing 1.5% (v/v) sodium hypochlorite, and after rinsing with sterile distilled water, it was immersed in 70% (v/v) ethanol for 1 min and air-dried. Small pieces of tissue cut from the leading edges of lesions were plated directly onto using half-strength potato dextrose agar medium ($\frac{1}{2}$ PDA) and *Fusarium* selective medium [5]. Following incubation at 27.5 °C, the material was transferred to fresh PDA and grown for 7 d at 23 °C, followed by preparation of pure cultures [6]. Fungal culture was preserved in the Technology Research and Development Application and Research Center (TUTAGEM, Edirne, TURKEY) in deep freeze conditions (−80 °C).

Pot experiments were performed according to the method of Müller-Stöver et al. [3] partially modified by Aybeke et al. [7]. Two-thirds of a plastic pot (140 × 130 mm, No. 3) were filled with a 1:1 mixture of clay and sand. *Orobanche* seeds (50 mg per kg of soil; [3]) were placed onto the soil and thoroughly mixed with a mini trowel, and then three sunflower seeds were sown in the pot. After 14 days, the plants were thinned to one plant per pot [7].

Orobanche plants which were post-emergence state, were inoculated with the fungus (fungal mycelial and spor solution) using a sterile forceps at the end of elongation and before flowering. In all experiments, 10-day fungal cultures were used. *Orobanche* uncontaminated by fungi were used as an intact (fungus-free) control group. All trials were performed under greenhouse conditions in a daily temperature range or 15–25 °C or in temperature-calibrated solarium rooms using HQLR lamps (1000 W) to adjust weather conditions to the specific sunflower–*Orobanche* life cycle in 2015 May–June. Necrotic broomrapes after 4–5 day after fungal inoculation, was taken from the pot and immediately stored in a deep freezer at −86 °C for hormonal, and secondary metabolic analyses.

Hormonal Analysis

Healthy control and infected plant tissues (200 mg frozen *Orobanche* samples) were extracted with the Tissuelyser LT (Qiagen, Tissuelyser LT) [8] with minor modifications [9]. After lysis in a 2-ml extraction tube for 2 min, a 100 mg pellet was mixed with 1 ml extraction solvent (methanol/isopropanol, 50:50 (v/v) with 0.5% of ammonium formate) in an Eppendorf tube. This mixture was vortexed rapidly under freezing conditions and then centrifuged at 10,000 rpm for 10 min at 4 °C (Bioer Mixing Block MB-102). The supernatant was filtered through a 0.22 µm PTFE filter. Extracted samples (200 µl) were analyzed by UPLC-ESI-MS/MS (API4000 QTrap; Applied Biosystems).

For authentic hormonal activity analysis, standards of each hormone (indolacetic acid = IAA; gibberellic

acid = GA; abscisic acid = ABA; salicylic acid = SA; jasmonic acid = JA) were loaded onto the MS/MS system to determine fragments and voltage conditions. Then, mixtures of the five hormones were prepared in concentrations ranging from 0.05 to 50 µg/kg to establish five points for calibration. Calibration curves for each hormone were generated using Analyst software (Applied Biosystems). Samples (50 µl) were then analyzed by UPLC-ESI/MS-MS and Spark UPLC system integrated with an Applied Biosystems QTRAP 4000 (Applied Biosystems). Chromatographic separation was performed on a Phenomenex Luna 3 µm C18(2) 100 × 2.0 mm column at 40 °C. The solvent gradient used was 100% A (99.5% H₂O:0.5% ammonium formate) to 100% B (99.5% MeOH:0.5% ammonium formate) over 5 min. The gradient profile for hormones was constructed as follows: ((time in min)/A %): (0/98), (1/2), (3/2), (4/98), (5/98). Hormone analyzes were performed with a Turbo ion spray source in negative ion mode with MRM options in the Analyst software. The curtain gas was set at 10 a.u., the source temperature was 400 °C, and ion source gases 1 and 2 were both 20 a.u. The declustering potential was set at 100 V. The source voltage was 3500 V [9].

Secondary Metabolite Analysis

Chemicals, Standards and Reagents

Formic acid (98–100%), methanol (Hypergrade LC MS), isopropyl alcohol (2-propanol) and DMSO were purchased from Merck, Darmstadt, Germany. Ammonium formate (HPLC grade) was from Sigma-Aldrich, Germany. Reference standards, gallic acid, catechin, 2-5 dihydroxybenzoic acid, trans-caffeic acid, syringic acid, trans-sinapic acid, trans-p-coumaric acid, trans-ferulic acid, resveratrol and salicylic acid were from Fluka and protocatechuic acid was purchased from HWI Analytik GmbH, Germany. MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) was purchased from Biomatik Cambridge, Ontario. Phosphate-buffered saline (PBS) and molecular biology-grade water were from Gibco, Invitrogen, Carlsbad, CA, USA. The resistance of ultra-distilled water used for instrumental analysis was 18.2 MΩ.

Liquid Chromatography and Mass Spectrometry Conditions

Phenol analyzes was performed on an Agilent 1200 infinity LC in combination with the Agilent 6460 Triple Quadrupole MS/MS System, equipped with a Jet Stream Electrospray ionization source (Agilent Technologies, Palo Alto, CA, USA). The analytical column was Agilent Poroshell 120 EC-C18 (4.6 × 50 mm, 2.7 µm particle size) and set

Fig. 1 Hormone analyses of the data set for the Control group and the experimental group (*Fusarium infected*); GA gibberellic acid, ABA abscisic acid, IAA indoleacetic acid, SA salicylic acid, JA jasmonic acid. Double asterisk indicates significant differences between control groups and *Fusarium* infected experimental groups, according to one-

way ANOVA and Duncan's test ($p \leq 0.05$). GA; F 1710.26, df 3, $p = 0.0001$. ABA; F 993.15, df 3, $p = 0.0001$. IAA; F 5819.09, df: 3, $p = 0.0001$. SA; F 55.87, df 3, $p = 0.0001$. JA; F 72.82, df 3, $p = 0.0001$

at 25 °C. Mobile phase A consisted of UPW, 0.2% ammonium formate (v/v), 0.2% formic acid (v/v). Mobile phase B consisted of methanol, 0.2% ammonium formate (v/v) and 0.2% formic acid (v/v). The flow rate was 0.3 ml/min at ambient temperature. The injection volume was 1 μ l and the LC gradient conditions were as follows: 0–1 min, 70% A, 30% B; 3–7 min. 30% A, 70% B; 9–10 min. 50% A, 50% B; 11–12 min; 70% A, 30% B. The run time was 12 min.

The Optimized MS analyzes parameters were as follows: gas temperature was set at 325 °C, the nebulizer gas pressure was set at 45 psi, the nozzle voltage was set at 500 V, the capillary at 3000 V, sheath gas temperature at 400 °C, sheath gas flow at 12 L/min. Multiple reaction monitoring (MRM) was performed on the positive and negative ion mode.

Data acquisition was performed with Mass Hunter (version B.06.01) software. Nitrogen (N₂) was used as the

collision gas at 1.12 mTorr. Calibration standard mixes were prepared in 50% UPW, 25% methanol and 25% isopropanol at calibration concentrations of 1–200 ng/ml.

Statistical Analysis

All tests were repeated independently three times and differences in data of, hormonal, and metabolic tests of control and experimental groups were compared by ANOVA, which means separation by Duncan's test using SPSS 18 software at a significance level of $p \leq 0.05$.

Results

Hormone Analysis

From development-associated hormones, amounts of GA were 77.7 ng/ml in control groups (fungus-free orobanche) but was reduced to 42.6 ng/ml in fungal infected orobanches (experimental groups). Similarly, levels of another developmental hormone, IAA were 93.62 and 13.01 ng/ml respectively in control and experimental groups.

In contrast to all other hormonal trends between the control and the experimental group, SA hormone levels increased fairly in comparison to control groups during fungal infection, from 5.9 to 6.7 ng/ml.

All hormonal differences between the data of the control and experimental groups were statistically significant (Fig. 1).

Secondary Metabolite

Among phenolics, the amounts of catechin, syringic acid, caffeic acid and p-coumaric acid increased significantly, with only gallic acid decreasing relative to the control data (Fig. 2).

Discussion

Fusarium infection of orobanche resulted in significant increases in SA, while the levels of JA, ABA, GA and IAA were reduced. SA is known to have all these developmental and stress hormones [10–12]. Accumulation of SA has been shown to be associated with resistance to biotic stress as well as expression of pathogenesis-related protein genes [4, 13]. *Fusarium* infection induces antioxidant mechanism of *Orobanche* by only significant SA activity, similar to the common necrotrophic stress response of SA [12].

Contrary to conventional knowledge, auxins, IAA and GA function not only in the regulation of plant growth, but also in response to abiotic stresses because of their key

Fig. 2 Data belongs to several phenolic substances in the control groups and *Fusarium* infected experimental groups. Double asterisk indicates significant differences between control and *Fusarium* infected experimental groups, according to one-way ANOVA and Duncan's test ($p \leq 0.05$). Catechin; F 292.92, df 3, p : 0.0001. Gallic acid; F 70.49, df 3, p : 0.0001. Syringic acid; F 503.43, df 3, p : 0.0001. caffeic acid; F 403.27, df 3, p : 0.0001. p-coumaric acid; F 154457.13, df 3, p : 0.0001

signals in plant–pathogen interactions [14, 15]. Stress-induced accumulation of ROS may activate oxidative damage of auxin and decrease expression of genes associated with auxin signal pathway or even inhibit auxin's polar transport [12]. A recent study found a significant reduction in IAA concentration in crop plants, such as rice and tomato, after ROS bursting [16]. Similarly, our recent study indicated highly ROS threat during *Fusarium* pathogenesis on *Orobanche* spp [4].

Based on results of our recent work, expressions of Mn-superoxide dismutase (SOD), Zn-superoxide dismutase (=SOD2, mitochondrial) genes, associated with ROS damage and toxic effects, were significantly higher in the experimental group [4], under *Fusarium* infection. This finding seems very logical to us; because ROS products are rapidly quenched by these antioxidant enzymes, which prevent the accumulation of harmful H_2O_2 levels [13, 17]. In present hormonal data, an antagonistic relationship between the enzymes and ABA was observed. Although this type of ABA hormone–enzyme relationship is common, the opposite case has been seen in some studies. For example, a previous study showed that the activity of CAT stayed constant or even decreased in ABA treated plants [18], and the regulatory mechanism of CAT by ABA treatment varied in different plants [19].

GS (=glutamine synthetase) is another enzyme playing a major role in balance of nitrogen metabolism in several organs and chloroplasts under stress conditions [20]. Because GS expressions occurred by the control of SA to maintain a suitable level of N metabolism under stress conditions [13]. Based on our recent study [4], GS gene

Fig. 3 Control (a) and *Fusarium*-infected (b) *Orobanche* samples, red and yellow arrows indicate Control groups (Fusarium-free) and *Fusarium*-infected *Orobanche* stems experimental groups,

respectively. Please note that infected *orobanches* are flowerless and with *blackened stem* (b), while control group samples are yellow in colour and healthy (a)

expressions fairly increased simultaneously with SA hormone levels in comparison to the control group during the *Fusarium* infection, a clue for the modifier effect of *Fusarium* on N metabolism in *Orobanche*.

These secondary metabolite studies, focused on quantities of several phenolic substances in the control and experimental groups. Secondary metabolites such as phenols and flavonoids defend plants against biotic and abiotic stresses and increases antioxidant activities in plant tissues [21]. Further they have been reported to quench or scavenge reactive oxygen species, chelate metal ions perform as chain-breaking antioxidants by neutralizing lipid peroxy radicals, or incorporate into the lipid bilayer to prevent membrane lipid damage [22]. Similarly, antioxidant effects of caffeic acid, syringic acid and p-coumaric acid isolated from several plants and fungal taxa such as *Smilax*, *Prunus persica* have been reported [23, 24]. Our observation on the enhancements of phenolic compounds due to fungus infections indicates a significant increase in ROS damage of tissues.

In summary, *Fusarium* substantially affects *Orobanche*, by disorders in the hormonal balance (only SA active), and fairly induced phenol synthesis and their subsequent oxidation reasoning out the typical symptoms of tissue browning leading to the death of the parasitic plants (Fig. 3).

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