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Fusarium infection causes genotoxic disorders and antioxidant-based damages in *Orobanche* spp.

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ABSTRACT

This study aims to evaluate the toxic effects of *Fusarium oxysporum* on root parasitic weed, *Orobanche* spp. Comparative genetic and gene expression studies were conducted on uninfected and fungus-infected orobanches. In genetic studies, isolated total DNA was amplified by RAPD PCR. Fragment properties were analysed by GTS test. According to the results, the fragment properties of control and *Fusarium* infected (experimental) groups varied widely; and it has been observed that *Fusarium* has genotoxic effects on the DNA of orobanches. In gene expression studies, the expression levels of genes encoding enzymes or proteins were associated with ROS damage and toxic effects, therefore, gene expressions of Mn-superoxide dismutase (SOD), Zn-superoxide dismutase (=SOD2, mitochondrial), glutamine synthetase (GS), heat shock protein gene (HSP70), BAX, Caspase-3 and BCL2 were significantly higher in the experimental group. In the light of obtained data, it was concluded that *F. oxysporum* (1) caused heavy ROS damage in *Orobanche* (2) induced significant irrevocable genotoxic effects on the DNA of *Orobanche*, (3) degraded protein metabolism and synthesis, and finally (4) triggered apoptosis. The results of this study can be a ground for further research on reducing the toxic effects of Fusarium on agricultural products, so that advancements in bio-herbicide technology may provide a sustainable agricultural production.

1. Introduction

Orobanche (broomrapes) are obligate root parasitic plants, which cause severe yield reduction in a wide range of crops such as sunflower, potato, rapeseed, mustard, fava bean, lentil, tomatoes, alfalfa, pepper and tobacco. Methods for controlling these parasitic plants include crop rotation, soil fumigation and solarisation, using host seeds that are free of parasites, advancing or delaying sowing date, use of seed varieties that are genetically resistant to *Orobanche* and chemical herbicide applications (Kohlschmid et al., 2009). However, some of these control methods are inadequate, difficult to apply and hazardous to the biological environment. For example, soil fumigation involves the application of highly volatile toxic compounds like methyl bromide, which does not only kill *Orobanche* and other beneficial or harmful soil organisms including bacteria, fungi and nematodes, but also harm personnel applying fumigation. The practice of soil fumigation has caused environmental problems (Shabana et al., 2003) and therefore, it was banned by the U.S. Environmental Protection Agency. Another method that is not mentioned above is the biological control of *Orobanche* by using fungal pathogens. Some of the fungal pathogens of *Orobanche* including *Ulocladium*, *Fusarium* spp., *Rhizoctonia*, *Alternaria*, *Sclerotinia* and *Aspergillus* spp. (Kohlschmid et al., 2009; Shabana et al., 2003; Boari and Vurro, 2004; Müller-Stöver and Kroschel, 2005; Aybeke et al., 2014) have been tested. For example, pesta granules prepared with fungal mycelia/spore culture of *Aspergillus alliaceus* Thom & Church as well as different food mixtures at several ratios significantly reduced Orobanche infection in sunflower seedlings (Aybeke et al., 2015; Aybeke, 2016). Another fungal pathogens, *Fusarium oxysporum* Schlecht. f. sp. *orthoceras* (FOO), was also effective against *Orobanche* parasitism, reducing Orobanche pathogenicity in tomato by 90% compared to the control group (Hodosy and Hornok, 1983). Müller-Stöver et al. (2002) used pesta granules containing fungal chlamydospores or microconidia as well as different proportions of wheat flour and kaolin (Pesta) mixture and suggested that the Pesta formulation was effective in *Orobanche* biocontrol. Despite these studies, pathogenicity of any of these fungi and their effective mechanisms on *Orobanche* are not known. For this reason, *Fusarium* toxicity was the first subject of interest addressed in this study.

Several phytopathogenic fungi exert various toxic effects on the host plants during infection, and the responses of plants to these toxic agents may also vary (Alazem and Na-Sheng, 2015). Responses of those plants to biotic stresses such as fungal pathogen infection, induction of reactive oxygen species (ROS) signalling (O'Brien et al., 2012), changes in the metabolism of N and amino acids (Seabra and Carvalho, 2015),

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differences in protein-based repair mechanisms (Augustine et al., 2015), increases in antioxidant enzyme activity (Kang et al., 2014), presence or absence of genotoxic damages (Doganlar, 2012), and local or systemic necrotic are very important to understand the magnitude of stress suffered by the plant and to determine the degree of tolerance. Our hypothesis was that *Fusarium* infection causes irrevocable and fatal damage to Orobanche. In order to test this hypothesis, this study was designed to evaluate the severity of toxic effects of *F. oxysporum* on Orobanche through genetic and gene expression analyses.

2. Materials and methods

Orobanche seeds were collected from sunflower fields in Edirne, in Trakya region of Turkey. They were identified as *Orobanche cernua* L in the Botany Laboratory of Biology Department in Trakya University by following the method of Davis et al. (1988). The sunflower cultivar was Orobanche-sensitive HA 89-B. The fungal isolate used in the experiment was collected from the infected Orobanche plants in the same fields (Edirne, 41°45′51″N, 26°59′11″E), and identified according to the method of Leslie et al. (2006) as *F. oxysporum* Schl Tdl. [Mycobanc no: MB#218372]. Infected Orobanche tissue with the fungus was surface-disinfected for 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 half-strength potato dextrose agar medium (½ PDA) and *Fusarium* selective medium (Nash and Snyder, 1962). After the incubation at 27.5 °C, the material was transferred to fresh PDA and grown for 7 days at 23 °C, which was followed by preparation of pure cultures (Herron et al., 2015). Fungal culture was preserved in deepfreeze conditions at −80 °C in Biology Department of Trakya University (Edirne, Turkey).

Pot experiments were performed according to the method of Müller-Stöver et al. (2002) partially modified by Aybeke et al. (2015). Two-thirds of a plastic pot (140 mm × 130 mm, No. 3) was filled with a 1:1 mixture of clay and sand. *Orobanche* seeds (50 mg per kg of soil; Müller-Stöver et al. 2002) were poured onto the soil and thoroughly mixed with a mini trowel, and then three sunflower seeds were sown in the pot. After 14 days, plants were thinned to one plant per pot (Aybeke et al. 2015).

Fungal inoculation experiments were performed by applying fungus directly onto the post-emergence Orobanche with a sterile forceps. 2–3 mm^2 of PDA agar fungal cultures at tip of the forceps were used for orobanche infection tests. *Orobanche* that were 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 between 15 °C and 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 orobanches after fungal inoculation was taken from the pot and immediately stored in a deep freezer at −86 °C for genetic and gene expression experiments.

2.1. Genetic studies (DNA extraction and RAPD procedures)

Genomic DNA isolations from *Orobanche* were performed with a DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The entire genomic DNA was diluted with nuclease-free water to a concentration of 25 ng/µl and used as template DNA for PCR reactions. Standard 50 µl PCR reactions were performed using 2 µl (50 ± 10 ng) of template DNA, 5 µl of 10× Taq buffer (Fermentas, EP0071), 1.25 U Taq DNA polymerase (Fermentas, EP0401), 6 µl of 2.5 mM $MgCl_2$ (Fermentas, R0971), 1 µl of 10× dNTPs (Fermentas, R0181), 1 µl of each primer and nuclease-free water (R0581, Fermentas). The primer sequences are given in Table 1.

The DNA was amplified using a thermal cycler PQLab gradient. 15 µl of PCR product with 3 µl of loading dye (R0611, Fermentas) was loaded onto a 2% agarose gel with ethidium bromide in 2× TAE (Tris 1.6 M, acetic acid 0.8 M, EDTA 40 mM) buffer. The molecular weight standard Gene Ruler 100 bp plus DNA (Fermentas, SM0321) was used according to the manufacturer's instructions. The DNA bands were visualised with a UV trans-illuminator (ViberLourmat, Quantum ST4), and the sizes of bands were calculated with the BIO-PROFIL, BIO-1D++ programme (Doganlar, 2012). A genomic template stability (GTS, 100%) test was performed as an indicator of disappearance/appearance of RAPD bands in comparison with the control group, indicating changes in RAPD profiles, as described in Pandey and Gupta (2015).

2.2. Gene expression studies

Expressions of antioxidant enzymes genes, Mn-superoxide dismutase (=SOD), Zn-superoxide dismutase (=SOD2, mitochondrial), catalase (=CAT), Glutamine synthetase (=GS), Heat shock protein gene (=HSP70) and apoptotic genes such as BCL2, BAX, Caspase (=CASP3) were carried out.

First, entire RNA was isolated from 100 mg samples of the control and experimental (fungus-infected) groups following the reagent kit manufacturer's instructions (PureLink® RNA Mini Kit, Life Technologies, USA) and using specific RNase-free DNase preparations. After extraction of total RNA, its concentrations were measured at 260 and 280 nm with a Qubit® Fluorometer (Life Technologies, USA) and the purity was assessed by a 260/280 ratio between 1.8 and 2.1. Integrity of the total RNA was tested by electrophoresis on 1% (w/v) agarose gel. Isolated RNA was dissolved in 50 µl of RNase-free H_2O and stored at −80 °C for subsequent analysis (Feng et al. 2009).

Synthesis of the first strand of cDNA was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) with a thermal cycler (Applied Biosystems® Veriti®) (step 1, 25 °C, 10 min; step 2, 37 °C, 120 min; step 3, 85 °C, 5 min). Finally, 20 µl of cDNA was obtained from each replicate. The cDNA was stored at −20 °C for subsequent steps of the analysis (Doganlar et al. 2015).

Quantitative real-time PCR (qRT-PCR) analyses were performed using the SYBR® Select Master Mix (Life Technologies, USA) on an ABI Step One Plus Real-Time PCR system (Table 1). Gene expressions were detected by fold change relative to the control group and rp49 mRNA was used as a normalisation standard (Doganlar et al. 2015). All tests were performed with three replicates. The comparative cycle-threshold (Ct) method (User Bulletin 2, Applied Biosystems, CA) was performed to analyse the expression levels of mRNAs per each control and experimental group. Relative fold changes of gene expression in the *Fusarium*-infected and control *Orobanche* groups were compared using analysis of variance (ANOVA) with Duncan's separation-of-means test using SPSS 18 software at a significance level of $P \leq 0.05$.

2.3. Statistical analysis

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

3. Results

While the control orobanche plants (Fungus-free) were intact, *Fusarium* infected ones (experimental groups) were quickly darkened and then completely died in an irreversible way (Fig. 1a–d). For example, for the AP5 primer, whereas band sizes ranged from 126.4 to 1889.3 bp in the control group, they ranged from 172.8 to 1113.8 in the experimental group. GTS (%), which was always 100 in the control group, decreased in the experimental group from 85 to 33.33 depending on the RAPD primer. The lowest rates of GTS were found in two primers, 1247 and OPC15 with 33.33%. Other GTS rates at rising levels