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Plant-pathogen interactions during infection process of asparagus with *Fusarium* spp.

Research Article

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Abstract: Background: *Asparagus officinalis* L. is often infected by fungi from the *Fusarium* genus which also contaminate the plant tissues with highly toxic secondary metabolites. To elucidate the plant-pathogen interactions between asparagus and *Fusarium oxysporum* or *F. proliferatum*, a fungal mycotoxins profile was assessed together with an impact of the infection on all forms of salicylic acid content. Methodology: Fungal isolates were identified by their morphological features, species-specific PCR and transcription elongation factor 1a (TEF-1a) sequencing. Mycotoxins were assessed by high-performance liquid chromatography (HPLC). The salicylic acid and its derivatives content was analyzed by the HPLC method combined with fluorometric detection. The levels of free radicals were measured by electron paramagnetic resonance (EPR). Results: After infection both *Fusarium* pathogens formed fumonisin B_1 and moniliformin. Infection altered salicylic acid biosynthesis and conjugation rates both in the roots and stems when compared with non-inoculated plants. Samples with higher free radical concentrations in stems showed higher concentrations of all forms of salicylic acid. Conclusions: We postulate that infection by both *Fusarium* pathogens produces mycotoxins, which may be transported to the upper part of plant. Pathogen attack initiated a plant defense reaction involving increased salicylic acid levels and resulting in increase in free radical levels.

Keywords: *Asparagus • EPR • Fusarium oxysporum • Fusarium proliferatum • HPLC • Molecular identification • Mycotoxins • Salicylic acid*

Abbreviations:

APx - ascorbate peroxidase;
CAT - catalase;
EPR - electron paramagnetic resonance;
Foa - *F. oxysporum* f. sp. *asparagi;*
FR - free radicals;
FBs - fumonisins;
FB_1 - fumonisin B_1;
HR - hypersensitive reaction;
IR - induced resistance;
MeSA - methyl-salicylates;
MON - moniliformin;
PDA - potato dextrose agar;
PAL - phenylalanine ammonia lyase;
POD - peroxidase;
PR - proteins;
ROS - reactive oxygen species;
SA - salicylic acid;
SAG - salicylic acid glucoside esters;
SOD - superoxide dismutase;
SAR - systemic acquired resistance;
TSA - total content of free and glucoside bound salicylic acid.

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1. Introduction

Fungi of the genus *Fusarium* are severe pathogens of *Asparagus officinalis* L. – an important vegetable worldwide [1,2]. Infection of asparagus plants by particular *Fusarium* species depends on cultivar susceptibility, environmental conditions, agronomic practices and other factors [3-5]. *Fusarium proliferatum* (Matsushima) Nirenberg and *F. oxysporum* (Schlechtend) are the most common pathogens of asparagus causing crown and root rot. Considering their diverse ability to form mycotoxins, variability in pathogenicity and other characteristics, a correct identification of *Fusarium* species is very important [6-8].

Both *Fusarium* species are able to generate mycotoxins (moniliformin - MON and fumonisins - FBs), which pose potential health hazards to humans and animals [9-11]. The most abundant fumonisin produced in nature is fumonisin B_1 (FB_1), a suspected risk factor for esophageal and liver cancers, neural tube defects, and cardiovascular problems [12-15] in populations where food contains contaminated maize. On the basis of these reports the International Agency for Research on Cancer classified FB_1 as a probable carcinogen in humans (class 2B carcinogen) [16]. Tolerable concentration levels of fumonisins (FB_1 and FB_2) in food, feed and their components are regulated in several countries [17]. MON exhibits cytotoxic and cardiotoxic activities, causes developmental disorders and may also induce the development of Keshan's disease, which attacks mainly the cardiac muscle leading to the circulatory failure, cardiac rhythm disorders and cardiac contractility problems, as well as clots inside cardiac chambers causing embolism [18].

Interactions between plants and their pathogens comprise a rapidly developing field in plant science. The plant response to infection is determined by the genetic background of the host as well as the pathogen [19,20]. Plants have evolved integrated defense mechanisms against fungi, such as hypersensitive reaction (HR), leading to the rapid host-cell collapse at the infection site, and systemic acquired resistance (SAR). Salicylic acid (*o*-hydroxobenzoic acid) (SA) is a key molecule involved in the immune response in plants; however, the exact role of this compound remains the subject of an ongoing debate [21]. Biosynthesis of SA accompanies oxidative stress after a pathogen attack, which has been confirmed in many plant species [22-24]. In plant tissues SA exists as free SA and conjugated SA forms, including methyl-salicylates (MeSA), glucoside esters (SAG) and amino acid conjugates of the compound. The major form, however, is its 2-O-*β*-D-glucoside [25]. SA influences many physiological processes regulating seed germination, growth of the root system and leaves, chlorophyll biosynthesis, as well as flowering and thermogenesis [22,26-29]. Moreover, SA at higher concentrations (50-100 mmol L^{-1}) may trigger the local cell death programme [30,31]. During the hypersensitive response, SA alters hydrogen peroxide metabolism in infected plants by intensifying superoxide dismutase (SOD) activity and inactivating catalase (CAT) and ascorbate peroxidase (APx), thus leading to the accumulation of hydrogen peroxide, which oxidizes cell constituents, reduces the efficiency of photosynthesis and destroys cell membranes, leading to cell death [32,33]. On the other hand, SA also plays an important role in the signal transduction chain releasing defense reactions within SAR, *i.e.* biosynthesis of pathogenesis related proteins (PR) [19]. The role of SA in the development of induced resistance (IR) is confirmed by its local and remote accumulation in plant tissues in response to biotic and abiotic stressors [34,35].

During disease development, the level of free radicals (FR) was determined in order to correlate it with macroscopic observations of asparagus spears, the concentration of SA and concentration of mycotoxins (MON, FBs) in plant tissue. Free radicals are generated in areas surrounding infection sites as an early response to pathogen attack [37]. All the above components (FR, SA, mycotoxins and fusariosis symptoms) probably participate in the complex pathogenesis process [36-38].

Recently, electron paramagnetic resonance (EPR) was applied in multidisciplinary studies for direct detection of free radicals and paramagnetic species such as Fe, Mn and Cu [39-41]. EPR technique involves the interaction of electromagnetic radiation with the magnetic moments of electrons. When a sample with unpaired electrons is placed in the external magnetic field the electron spin will align parallel or antiparallel in the direction of the magnetic field, which corresponds to the energy state. The energy difference between these two states is proportional to the intensity of the applied magnetic field. If electromagnetic radiation corresponding to the energy difference is applied to the sample, resonance transition is possible between the lower and the upper energy states and we can observe an EPR line [40,42].

The present study was undertaken to elucidate plant-pathogen interactions between asparagus and its fungal pathogens. The principal aim was to evaluate the impact of infection by *F. proliferatum* and *F. oxysporum* on the concentrations of SA and mycotoxins (MON, FB_1) formed in asparagus tissues. Moreover, the relationships between mycotoxin levels and SA content (free and glucoside forms) were investigated. EPR spectroscopy was additionally used to investigate changes in free radicals level after plant inoculation.

2. Experimental Procedures

2.1 Plant material, fungal cultures and inoculation test

In a greenhouse experiment 1-year-old plants of asparagus cv. Andreas were used. Plants growing in pots (each with a capacity of 5 dm^3) with steamed soil were grown from seeds disinfected with a 5% suspension of Benlate fungicide in acetone. Isolates of *F. proliferatum* originated from asparagus spears cv. Eposs grown in Swidowiec (100 km west of Poznań, Poland), while *F. oxysporum* strains were isolated from spears collected at the Poznań farmers' market. Inoculums of 5 mm disks of potato dextrose agar (PDA) medium overgrown by one of the four single spore isolates, *i.e. F. proliferatum* (06-76sb and 06-94s) and *F. oxysporum* (07-25wz and 07-32s), were placed under incised skin of storage roots.

Storage roots of seven different seedlings were inoculated by each of the tested isolates separately, while six non-inoculated asparagus seedlings were used as the control. Asparagus plants were incubated in a greenhouse at 22ºC. The occurrence of *F. proliferatum* and *F. oxysporum*, as well as the level of salicylic acid and mycotoxins were determined in roots and stems of seedlings. After two weeks (1st term) from inoculation three random plants and after four weeks (2nd term) another three plants were used for the assessment (including the control).

2.2 Evaluation of *F. proliferatum* and *F. oxysporum* occurrence in asparagus seedlings

Each asparagus seedling was divided into two parts (storage root and stem base) and from each part five plant sections were put on PDA medium supplemented with streptomycin at 100 µg ml^{-1} to test the presence of *F. proliferatum* and *F. oxysporum*. Plant parts of 1 cm in length were disinfected with 1% sodium hypochlorite. Five sections (2 mm in diameter) were cut from each part (storage root and stem base) and transferred onto separate Petri dish with the medium. Fungal colonies grown from the sections were transferred onto standard media and identified according to the methods described by Booth [43], Gerlach and Nirenberg [44], Kwasna *et al*. [45], and Barnett and Hunter [46].

2.3 Molecular identification of *Fusarium* spp. isolates

Mycelia from 9-day-old single spore cultures of *F. proliferatum* and *F. oxysporum* grown on liquid medium (5 g L^{-1} of glucose, 1 g L^{-1} of yeast extract) were collected by vacuum filtration using a Büchner funnel. DNA was extracted and purified using a DNeasy Mini Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer's recommendations. Species-specific PCRs were used to verify mycological identification of species. In the polymerase chain reaction a forward primer: 5'-TGCATCAGACCACTCAAATCCT-3' and a reverse primer: 5'-TGTCAGTAACTCGACGTTGTTGTT-3' were used to detect *F. proliferatum* and a forward primer: 5'-CAGCAAAGCATCAGACCACTATAACTC-3' and a reverse primer: 5'-CTTGTCAGTAACTGGACGTTGGTACT-3' were used for *F. oxysporum* (Sigma-Genosys, Pampisford, UK). The species-specific primers were designed based on a partial sequence of the calmodulin gene [43,44]. The amplification reactions were carried out using a *Taq* PCR Core Kit (QIAGEN, Inc., Hilden, Germany). The reaction mixture was described earlier [49]. Amplification was carried out in a Biometra *Tpersonal* 48 thermocycler (Whatman Biometra, Goettingen, Germany) using the following programme: initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 40 s, primer annealing at 60°C for 40 s and extension at 72°C for 1 min. The amplification was ended with an additional extension at 72°C for 3 min. The PCR products were separated by electrophoresis in 1.5% agarose gel with 1´TBE buffer (89 mmol L^{-1} Tris-borate and 2 mmol L^{-1} EDTA, pH 8.0) and visualised under UV light following ethidium bromide staining. A Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas GMBH, St. Leon-Rot, Germany) was used as a molecular size standard.

Additionally sequence analysis of translocation elongation factor 1-a (TEF) was applied to confirm the morphological identification of all fungal isolates as described earlier [49]. Both molecular analyses confirmed identification of *Fusarium* isolates used for inoculation of asparagus.

2.4 Chemicals

Fumonisin B_1, moniliformin and SA standards were purchased with a standard grade certificate from Sigma-Aldrich (Steinheim, Germany). Sodium dihydrophosphate, potassium hydroxide, acetic acid, *n*-hexane and *o*-phosphoric acid were purchased from POCh (Gliwice, Poland). Organic solvents (HPLC grade), disodium tetraborate, *ortho*-phthalaldehyde, 2-mercaptoethanol and *t*-butyl-ammonium hydroxide, sodium acetate and all the other chemicals were also purchased from Sigma-Aldrich (Steinheim, Germany). Water for the HPLC mobile phase was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.5 Chemical analysis

Fumonisin B_1 was extracted from fresh plant tissue. Samples (5 g) of storage roots and stem bases,

remaining after the previous collection of 1 cm long parts for the detection of fungi, were homogenized for 3 min in 10 ml of methanol:water (3:1, v/v) and filtered through Whatman no. 4 filter paper according to the method described by Sydenham *et al*. [50]. The extract was adjusted to pH 5.8-6.3 using 0.1 mol L^{-1} KOH. A SAX cartridge was attached to the SPE manifold unit (Supelco, Bellefonte, PA, USA) and conditioned at a flow rate of 2 ml min^{-1} successively with 5 ml of methanol, followed by 5 ml of methanol:water (3:1, v/v). Next, an aliquot (10 ml) of the filtered extract was applied at a flow rate of 2 ml min^{-1}, then washed with 8 ml methanol:water (3:1, v/v), immediately followed by 3 ml of methanol. Fumonisin was eluted from the column with 10 ml of 1% acetic acid in methanol. The eluate was evaporated to dryness at 40°C under a stream of nitrogen. Dry residue was stored at -20°C until HPLC analysis.

The OPA (*ortho*-phthalaldehyde) reagent was prepared as follows: 20 mg per 0.5 ml methanol were diluted with 2.5 ml of 0.1 mmol L^{-1} disodium tetraborate, then mixed with 25 µl of 2-mercaptoethanol. The fumonisin B_1 standards (5 µl) or extracts (20 µl) were derivatized with 20 and 80 µl of the OPA reagent, respectively. After 3 min the reaction mixture (10 µl) was injected onto an HPLC column.

A Waters 2695 apparatus (Waters Company, Milford, MA, USA) equipped with a C-18 Nova Pak column (4 mm, 3.9x150 mm) and a Waters 2475 fluorescence detector (λ_{Ex}=335 nm; λ_{Em}=440 nm) were used to quantify the metabolite. Methanol:sodium dihydrophosphate (0.1 M in water) solution (77:23, v/v) - adjusted to pH 3.35 with *o*-phosphoric acid, after filtration through a 0.45 µm Waters HV membrane - was used as the mobile phase at a flow rate of 0.6 ml min^{-1}.

The detection limit for FB_1 was 0.1 ng g^{-1} FW. Positive results (on the basis of retention times) were confirmed by HPLC analysis and compared with the relevant calibration curve (r=0.9967). Recovery for FB_1 was 89% (measured in triplicates by extracting the mycotoxin from blank samples spiked with 0.1–10 ng g^{-1} of the compound). The relative standard deviation was below 8%.

Moniliformin was extracted from plant material with acetonitrile:methanol:water (16:3:1, v/v/v) using 5 ml of solvent per 1 g of sample. Extracts were defatted with *n*-hexane (3x50 ml) and then concentrated. The extract was purified on a Florisil column according to the method described by Goliński *et al*. [51].

MON was quantified by HPLC using a Waters 501 apparatus (Waters Company, Milford, MA, USA) with a C-18 Nova Pak column (4 mm, 3.9x300 mm) and a Waters 486 UV detector (λ=229 nm). MON was eluted from the column at the flow rate of 0.6 ml min^{-1} with acetonitrile:water (15:85, v/v) buffered with 10 ml of 0.1 mol L^{-1} K_2HPO_4 in 40% *t*-butyl-ammonium hydroxide in 1 L of solvent [52]. Retention time of MON was 11.5 min with the compound detection limit of 10 ng g^{-1} FW. Positive results (on the basis of retention times) were confirmed by HPLC analysis and by comparison with the relevant calibration curve (r=0.9990). Recovery for MON was 90%, (measured in triplicates by extracting mycotoxins from blank samples spiked with 10–100 ng g^{-1} of the compound). The relative standard deviation was below 7%.

SA in the free form as well as that conjugated as a glucoside (SAG) were determined according to the methodology recommended by Yalpani *et al*. [24]. Plant material was ground in liquid nitrogen to a fine powder and approximately 0.5 g was taken for analyses. SA was extracted twice with 3 ml of methanol, and after centrifugation, the supernatant was divided into two aliquot parts and the solvent was evaporated to dryness under a stream of nitrogen. A 5% solution of trichloroacetic acid (2.5 ml) was added to one part and then SA was extracted three times with 2.5 ml of the organic mixture of ethyl acetate:cyclopentane:isopropanol (100:99:1, v/v/v). In order to determine the total content of free and glucoside bound salicylic acid (TSA), 40 units of *β*-glucosidase in 0.5 ml of sodium acetate/acetic acid buffer (0.1 mol L^{-1}, pH 5.2) were added to the second part of the dry extract and incubated for 90 minutes at 37°C. The reaction was terminated by the addition of 2 ml of 5% trichloroacetic acid and then salicylic acid was extracted as described above. After solvent evaporation the dry residue was dissolved in 1 ml of the mobile phase (0.2 mol L^{-1} potassium acetate/acetic acid buffer, pH 5.0; with an addition of 0.5 mM EDTA) and analysed by HPLC combined with fluorometric detection using a Waters Company chromatograph (Milford, MA, USA) composed of a 2699 Separation Module Alliance and a 2475 Multi-λ Fluorescence Detector. A Spherisorb ODS2 Waters Company column (3 µm, 4.6x10 mm) with a flow rate of 1.5 ml min^{-1} was used for chromatographic separation. Detection parameters were as follows: λ_{Ex}=295 nm and λ_{Em}=405 nm. Retention time of SA was 6.0 min with a total analysis time of 12 min and detection limit of 10 ng g^{-1} FW. The content of SA released from its glucoside was calculated as the difference between assays with and without glucoside enzymatic degradation (SAG=TSA–SA). Furthermore, the percentage of SA in TSA content was calculated and labeled as $SA_{\%}$. The recoveries of the salicylic acid standard added to samples were 89 and 86% for SA and TSA, respectively (measured in triplicates by SA extraction from blank samples spiked with 10–1000 ng g^{-1} FW). The relative standard deviation was below 7%.

2.6 Electron paramagnetic resonance of asparagus plants

The EPR measurements were performed with a Bruker EPR EMX-10 X-band (9.4 GHz) spectrometer with magnetic field second modulation frequency of 100 kHz. The samples were stored and EPR spectra were recorded at a temperature of 77 K. The first derivative spectra were recorded using a magnetic field scan range width of 10 mT and 600 mT and amplitudes of the second modulation were 0.3 mT up to 1 mT. The values of the microwave power were adjusted to obtain non-saturating and non-broadening conditions for the spectral components.

The standard weak pitch sample with a concentration of free radicals of 2×10^{13} spins was used to determine the concentration of free radicals in samples. Concentration of free radicals (FR) was calculated from integrated intensity of free radical signals with g factor g=2.0035 and it was about 10^{15} spins in the samples.

2.7 Statistical analysis

Two-way analysis of variance of data obtained in two harvest terms was carried out to determine the effects of *Fusarium* isolates, plant parts and the *Fusarium* isolates x parts interaction on the occurrence of *F. proliferatum* and *F. oxysporum* as well as the variability in concentrations of FB_1, MON and salicylic acid content (SA, SAG, TSA, $SA_{\%}$). Least significant differences were calculated for each trait. The association between pathogen occurrence and FB_1, MON and salicylic acid contents was estimated using analysis of regression. Data were analyzed using the statistical package GenStat v. 7.1. [53].

3. Results

3.1 Evaluation of *F. proliferatum* and F. *oxysporum* occurrence in asparagus seedlings

In the greenhouse experiment disease symptoms caused by isolates of *F. oxysporum* and *F. proliferatum* were investigated. Brown lesions were observed on all asparagus roots in two weeks after inoculation with *F. oxysporum* or *F. proliferatum*, while on stems no symptoms were observed. However, 33% stems were infected with *F. proliferatum* two weeks after inoculation, whereas inoculation with *F. oxysporum* did not induce stem infection even after four weeks. Analysis of variance showed significant differences between pathogenic isolates in terms of most evaluated traits, except for MON and SAG contents two weeks after inoculation, and those of MON and TSA after four weeks (Table 1). Observed differences between almost all the examined traits were significantly related to the analyzed plant part.

3.2 Evaluation of mycotoxin biosynthesis

It is well known that the activity of toxigenic fungi results in mycotoxin biosynthesis immediately after inoculation. Two weeks after inoculation the concentration of FB_1 was higher in stems than in roots of asparagus for each analyzed isolate, with the highest concentration obtained from the *F. proliferatum* isolate (06-94s) at 29.9 ng g^{-1} (Table 2). Four weeks after inoculation the concentration of FB_1 was increased in stems only from the *F. oxysporum* isolate 07-25wz, while in all other

Terms	2-week period				4-week period			
Source of variation	Isolate	Part	Isolate x Part	Residual	Isolate	Part	Isolate x Part	Residual
Degrees of freedom	4	1	4	20	4	1	4	20
F. p.	0.70**	0.3	0.03	0.11	0.63***	0.83***	0.33***	0.04
F. o.	0.38**	1.2***	0.45**	0.07	0.33***	0.83***	0.33***	0.04
FB_1	421.01***	2034.3***	289.22***	199.01	275.14***	92.09	33.21	29.28
MON	11811.24	38363.4*	11811.49	5621.36	632148.11	20149333.11***	858920.05*	279512.18
SA	384.44***	31.7	67.51	47.42	841.61*	6607.16***	770.32*	243.24
SAG	1779.09	43544.1***	1357.47	736.18	910.18*	11981.38***	696.53	297.05
TSA	3633.11*	45924.3***	1573.28	927.38	2532.45	36381.31***	2331.18	1001.37
$SA_{\%}$	325.46*	33648.2***	243.22	109.24	891.61***	136.08	508.62**	77.93

Table 1. Mean squares from two-way analysis of variance (ANOVA) for investigated traits in both terms of observations.

** - significant at 0.05; ** - significant at 0.01; *** - significant at 0.001*
F. p. – F. proliferatum occurrence, F. o. – F. oxysporum occurrence, FB1 – fumonisin B1 content, MON – moniliformin content, SA – free salicylic acid content, SAG – salicylic acid in form of glucoside content, TSA – total salicylic acid content, SA% – percentage of free in total salicylic acid content

Isolate	FB_1				MON			
	2-week period		4-week period		2-week period		4-week period	
	root	stem	root	stem	root	stem	root	stem
07-25wz	0.5	5.1	0.5	0.7	0.0	0.0	141.1	2418.3
07-32s	0.6	18.9	4.2	0.6	0.0	0.0	0.0	2555.2
06-76sb	5.5	35.1	22	10.6	93.2	0.0	529.4	1385.0
06-94s	0.0	29.9	2.7	0.1	214.1	0.0	368.3	1881.1
Control	0.0	0.0	0.5	0.4	50.8	0.0	0.0	995.4
$LSD_{0.05}$	12.3		9.2		126.7		906.9	

Table 2. Concentration (ng g^{-1} FW) of fumonisin B1 (FB1) and moniliformin (MON) in different parts of inoculated asparagus (mean values; n=30).

cases higher concentrations of this toxin were recorded in roots. In the first term MON was found only in roots inoculated with *F. proliferatum* isolates (06-76sb and 06-94s), while the aboveground part (stem) did not contain the toxin at a detectable level. In the second term MON was detected in both analyzed parts of plants, with the concentration significantly higher in stems than in roots (Table 2).

3.3 Changes in salicylic acid content upon pathogen attack

Salicylic acid content was investigated in roots and stems of asparagus plants two and four weeks after root inoculation with *Fusarium* isolates, as well as the control (non-inoculated) plants.

Two factorial analysis of variance revealed a significant influence (at P=0.001) of *Fusarium* isolates on the content of free salicylic acid (SA) two weeks after inoculation, while asparagus organ (root, stem) showed significant differences (at P=0.001) in the contents of salicylic acid released from its glucoside (SAG), total salicylic acid (TSA) and the ratio between free and bound forms of the metabolite (Table 1). The highest TSA contents were observed after infection with both *F. proliferatum* isolates (06-76sb and 06-94s), *i.e.* 158.2 and 126.5 ng g^{-1} FW, respectively, and they were 2 and 2.5 higher than those observed for the control plants (Table 3). *F. oxysporum* attack caused a weaker (07-25wz) or no (07-32s) induction of salicylic acid biosynthesis both in roots and stems of asparagus plants. Nevertheless, inoculation caused a significant decrease in the ratio between SA and TSA ($SA_{\%}$) in stems for all the tested isolates – on average from 44.5 to ~21%.

Four weeks after inoculation, plant parts turned out to be a strongly differentiating factor for SA, SAG and TSA contents (at P=0.001). In turn, the *Fusarium* isolate influenced significantly $SA_{\%}$ (at P=0.001), SA and SAG (at P=0.05), while isolate × plant part mixed factors simultaneously influenced $SA_{\%}$ (at P=0.01) and SA (at P=0.05) (Table 1). The highest TSA content was observed for stems following *F. oxysporum* 07-25wz inoculation and it was detected at a concentration level of 156 ng g^{-1} FW (~170% of TSA content in stems of the control plants) with a simultaneous, significant increase of SA content from 35.3 up to 81 ng g^{-1} FW for the control and inoculated plants, respectively (Table 3). For the other isolates no significant increase in SA accumulation was observed; however, in case of *F. proliferatum* 06-94s a significant drop was observed for SAG and TSA contents in stems and roots *versus* the control plants (from 91.8 to 45.2 ng g^{-1} FW for TSA in stems). Four weeks after the inoculation with isolates 06-94s and 07-25w7 of *F. proliferatum* and *F. oxysporum*, respectively, a significant increase of $SA_{\%}$ in stems and a decrease in roots were observed for isolate 06-76sb of *F. proliferatum* (Table 3).

3.4 Free radical measurements

Examples of EPR spectra of paramagnetic substances in asparagus are shown in Figure 1. Figure 1a and 1b show spectra for roots, while Figure 1c and 1d show spectra for stems. In Figure 1a two lines with g=4.3 and g=2.5 are correlated to lines of iron ions Fe^{3+}. The small selected area of the EPR spectrum referred to as “FR RANGE” was amplified (see Figure 1b). This appears to be a common signal of free radicals generated in inoculated asparagus roots. In the whole range of the magnetic field for stems (Figure 1c) six lines of manganese Mn^{2+} ions and one line of free radicals were observed for stems. Concentrations of these paramagnetic centers were different for particular *Fusarium* isolates; however, no changes were observed in the structure of the EPR spectrum. The spectroscopic

Isolate	SA				SAG				TSA				$SA_{\%}$			
	2-week period		4-week period		2-week period		4-week period		2-week period		4-week period		2-week period		4-week period	
	root	stem	root	stem	root	stem	root	stem	root	stem	root	stem	root	stem	root	stem
07-25wz	17.1	8.1	13.0	81.0	1.1	70.3	8.3	74.8	18.2	78.4	21.3	156	94.4	11.1	60.9	52.8
07-32s	10.8	18.0	15.2	23.2	0.4	49.8	11.0	57.8	11.2	68.1	26.2	81.0	97.0	26.9	57.7	27.9
06-76sb	27.5	34.0	7.0	34.7	6.6	124.0	24.4	58.6	34.1	158.2	31.4	93.3	81.1	22.0	22.3	37.3
06-94s	24.8	29.0	9.3	29.9	1.3	97.7	7.8	15.3	26.0	126.5	17.1	45.2	94.9	22.7	54.0	66.4
Control	16.7	18.0	11.1	35.3	1.1	49.8	11.7	56.5	17.9	67.5	22.8	91.8	94.7	44.5	48.8	38.0
$LSD_{0.05}$	11.8		26.8		46.4		29.6		53.3		54.3		17.8		15.1	

Table 3. Content of salicylic acid and conjugated forms of salycylic acid in asparagus tissue (mean values; n=30).

SA – free salicylic acid [ng g^{-1} FW], SAG – salicylic acid in form of glucoside [ng g^{-1} FW], TSA – total salicylic acid [ng g^{-1} FW], SA% - percentage of free in total salicylic acid [%]

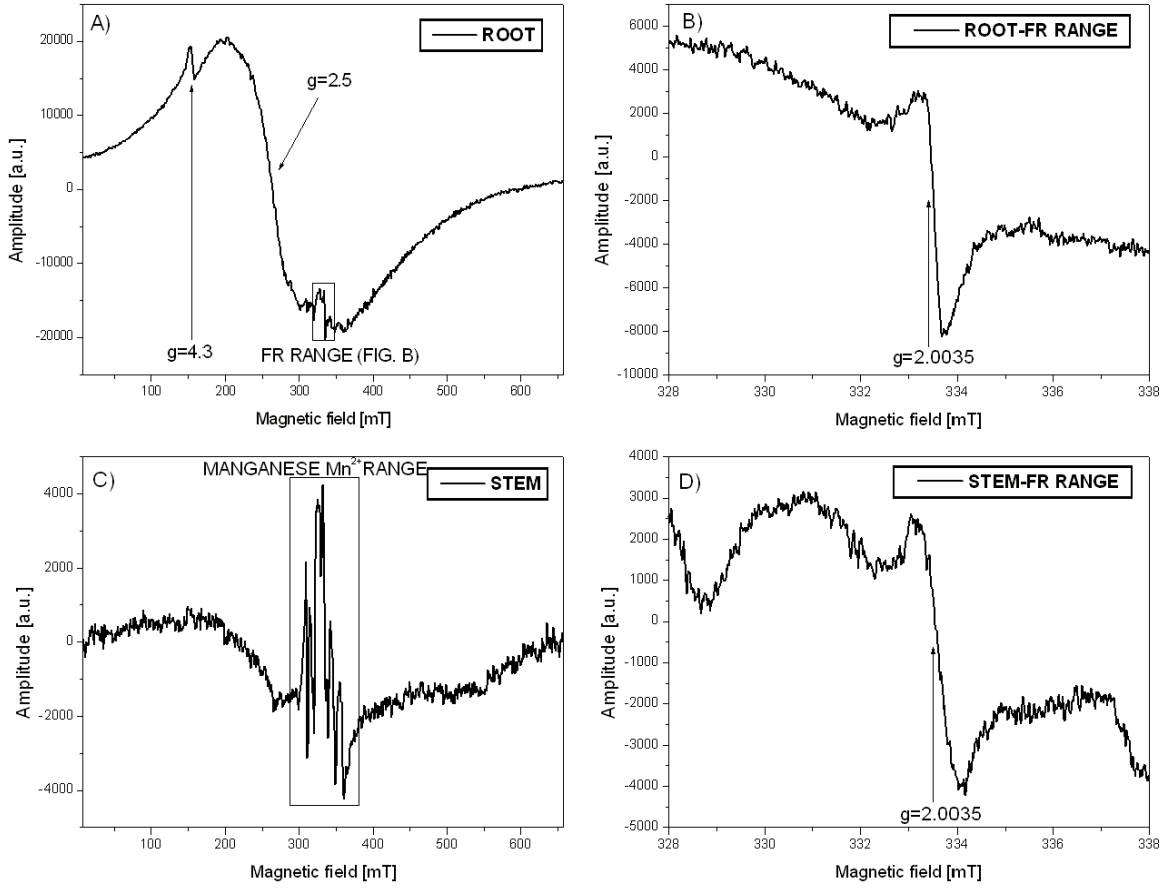

Figure 1. EPR spectra of asparagus inoculated with *Fusarium oxysporum* (isolate 07-32s) a, b) – root; c, d) – stem; b, d) – free radical range.

parameters for free radical lines were similar for both parts of plants with g-factor characteristic of particular paramagnetic centers (g=2.0035±0.0005) and line width (ΔB=0.8±0.03 mT) (Figure 1b and 1d).

4. Discussion

This study was focused on an interaction between asparagus plants and their fungal pathogens. Inoculation of asparagus roots with selected isolates of *F. proliferatum* (06-76sb and 06-94s) and *F. oxysporum* (07-25wz) changed SA biosynthesis and conjugation rates both in roots and stems when compared to non-inoculated plants. Isolate 07-32s of *F. oxysporum* was an exception here, with no significant increase of SA accumulation observed. The potential role for salicylic acid in IR of asparagus to *F. oxysporum* f. sp. *asparagi* (*Foa*) was described by He and Wolyn [54]. They reported that exogenous SA activated peroxidase (POD) and phenylalanine ammonia lyase (PAL), as well as lignifications upon *Foa* attack. In the other studies Mandal *et al.* [55] showed that the exogenous application of salicylic acid (200 µmol L^{-1}) through root feeding or foliar spray could induce resistance against *F. oxysporum* f. sp. *lycopersici (Fol)* in tomato. After 168 h, PAL and POD activities were about 5 times higher (compared to control plants) in case of salicylic acid absorption through the roots, and almost four times higher after treatments through foliar spray. The salicylic acid-treated tomato plants challenged with *Fol* exhibited significantly reduced vascular browning and leaf yellowing and wilting. To data, there is little information on changes in endogenous SA content in plants infected with *Fusarium* and the induction of intracellular resistance mechanisms.

In this experiment total SA content in asparagus tissue did not exceed 160 ng g^{-1} FW and was markedly lower when compared to values observed for plants treated with necrotrophic pathogens or abiotic stressors (up to 20 µg g^{-1} FW in tobacco leaves exposed to the ambient ozone, 75 µg g^{-1} FW in tobacco leaves inoculated with tobacco mosaic virus) [19,22,56-59]. This might indicate a relatively low ability of the investigated fungi to induce controlled HR cell death and SAR program in asparagus, regulated by this compound [28,60]. Two weeks after inoculation we observed an increase in the SA contents in roots as well as in stems of asparagus plants inoculated with *F. proliferatum* (both isolates) with a subsequent SAG increase in stems, especially for isolate 06-76sb. In *F. oxysporum* inoculated plants (isolate 07-25wz) an enhanced SA accumulation was observed only in roots (after two weeks) and SAG contents in roots and stems (after four weeks). The highest and comparable contents were observed for TSA in stems at two weeks (*F. proliferatum*), and four weeks (*F. oxysporum*) after inoculation and they were respectively 2 and 2.5 times higher than in the non-inoculated plants. As previously described, constitutive levels of SA can vary, not only between plant species, but also between cultivars of the same species [21] and organs of the same plant [61]. Differences in SA biosynthesis, conjugation and transport rates to the upper parts of plants may indicate differential abilities of *F. proliferatum* and *F. oxysporum* to infect asparagus plants and/or the diverse susceptibility of asparagus to the investigated fungi.

Two weeks after inoculation $SA_{\%}$ was about 90% of TSA in roots for all the isolates as well as the control plants. In contrast, we observed half or less $SA_{\%}$ than the level in the control (from 45% for the control to 11-27%) in stems of inoculated asparagus plants. This was probably due to phloem transport of the mobile free form of SA to upper parts of asparagus plants, and its conjugation in stems with glucose to O-*β*-D-glucoside for the purpose of nonspecific SAR, and possible biosynthesis of volatile methyl salicylate serving as a potential intra- and interplant signal transductor [58,62-64]. The observation that SAR spreads in the plant mainly in the apical direction and moves into grafted stems strongly suggests that signaling agents establishing SAR are translocated through the plant [65]. It was previously demonstrated that SA is accumulated As already indicated, SA is accumulated at sites in pathogen attack and then is transported *via* phloem to non-infected parts of the plant [21]. Edgar *et al.* [66] demonstrated that exogenous salicylic acid treatment prior to inoculation activated PR1 and BGL2 defense gene expression in leaves and provided an increased *F. oxysporum* systemic resistance, as evidenced by reduced foliar necrosis and plant death. In case of exogenous SA treatment of the foliar tissue did not activate defense gene expression in the roots of plants. This suggests that salicylate-dependent defenses may function in the foliar tissue to reduce the development of pathogen-induced wilting and necrosis. Moreover, Molodchenkova *et al.* [20] pointed to possible role of exogenous SA in the induction of trypsin and lectin inhibitors that are important in defense against *F. moniliforme* in maize sprouts. In this study a significant drop of $SA_{\%}$ in roots and an increase in stems were recorded after the next two weeks (especially for isolates 07-25wz and 06-94s). The significant increase in $SA_{\%}$ in asparagus stems four weeks after inoculation might be the effect of the observable increase in MON content.

Moniliformin exhibited a phytotoxic effect on jimsonweed (*Datura stramonium* L.) at concentrations of

50-800 µg mL^{-1} *via* foliar application, causing symptoms similar to those of the fungal isolates producing MON [67]. Fumonisin B_1 inhibits amylase production in germinating maize seeds and, as it was suggested by the authors, a high level of this toxin in maize seeds may have deleterious effects on seedling emergence [68]. According to Van Asch *et al*. [69], the concentrations of 102 µmol mL^{-1} MON and 13 µmol mL^{-1} FB_1 in the culture medium were required to cause over 50% reduction in growth of corn relative to the toxin-free control. In another study phytotoxicity and inhibitory effects of FB_1 and MON on chlorophyll biosynthesis were examined in an aquatic macrophyte *Lemna minor* [70]. FB_1 proved to be most active at 0.7 µg mL^{-1}, reducing the growth of *L. minor* fronds and their ability to synthesize chlorophyll by 53 and 59%, respectively, while MON exhibited the lowest phytotoxicity, with only a 16% suppression of the growth rate and a 54% reduction in chlorophyll at 66.7 µg mL^{-1}. Thus, we propose that the increase of SA and FR contents in stems may be a result of high MON accumulation, probably exceeding the phytotoxicity threshold.

The expression of genes responsible for mycotoxin biosynthesis is mainly regulated by temperature, pH, humidity and host genomic background [71]. The effect of external factors on mycotoxin biosynthesis is exerted at the transcription level through the activation of *fum1* gene of *F. verticillioides* under stress conditions.

Higher FR concentrations were observed in roots rather than in stems, which was probably due to the free radical generation by the wounded tissue in the inoculation site (roots). Oxygen-centered radicals have been studied to elucidate pathogenesis of several diseases and may play an important role in the biochemistry of the different stages of pathogen infection. The spectroscopic factor g=2.0035 suggests the accumulation of carbon-centered radicals with a nearby oxygen atom, resulting in a g-factor increase in comparision with typical pure carbon-centered radicals. In contrast to our results, typical oxygen-centered radicals have a higher g-factor (g>2.004) [72,73]. It is also interesting to compare FR concentration with salicylic acid content in inoculated with *Fusarium* asparagus plants. Samples with a higher FR concentration in stems (isolates 06-76sb and 06-94s) showed higher levels of all forms of salicylic acid (SA, SAG, TSA), which indicates that both factors are a measure of the stress level. In the second term reduced FR, SA and SA% concentrations followed by an increase of SAG content in roots were observed, which was probably due to FR formation in freshly wounded tissue.

High levels of Fe^{3+} ions in root samples were responsible for both EPR lines g=4.3 and g=2.5. In contrast, only Mn^{2+} ions responsible for specific 6 lines in the EPR spectrum in stems were observed, with no EPR lines from iron. The concentration of Fe^{3+} ions was related to the pathogen species, since a high Fe^{3+} concentration was observed in plants inoculated with *F. proliferatum* (584 787.9 for g=2.3-2.5). The level of free radicals in samples inoculated with F. proliferatum was higher (66.70x1015 spins/g) in comparison with *F. oxysporum* (45.45x1015 spins/g). This may indicate that *Fusarium* species have an effect on concentrations of both generated free radicals and complexes of Fe^{3+} ions in stems.

The above indicates an important role of *F. proliferatum* in pathogenesis, which is obviously related to ability to biosynthesis the mycotoxins and correlated with the conditions generating higher FR and SA levels.

To conclude, based on the described experiment we postulate that infection with both *Fusarium* pathogens generates mycotoxins, which may be transported to the upper (edible) parts of plants. Pathogen attack caused a plant defense reaction influencing the SAcontent and resulting in an increase in free radical levels. More extensive studies are necessary to explain and gain insight into the background of these biological processes.

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