Identification of causal agent of wilt of common sage (Salvia officinalis L.)

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Summary

Introduction: Common sage is cultivated in Europe and North America. It has strong antiviral, antibacterial and antifungal properties. This plant can be infected by different pathogenic fungi species, such as Alternaria alternata, Fusarium spp. (F. culmorum, F. equiseti, F. oxysporum), Phomopsis sclarea and Botrytis cinerea. Those species are the most frequently isolated fungi from sage stem base.

Objective: The aim of this study was to identify the causal agent of common sage wilt disease.

Methods: Studies were carried out in 2018–2020. 23 fungal isolates were identified based on their morphology and with use of PCR technique. Length and width of 100 conidia growing on SNA medium were measured after 7 days. Koch’s postulates were checked and the development of one fungus isolate (no. 13) was compared on seven media: the CMA, MEA, OA, PCA, SNA, PDA and Czapek medium. Sequences of the second largest subunit of RNA polymerase II (RPB2) were used to identify the pathogen.

Results: The fungus formed 3 kinds of spores: thin-walled, hyaline, slightly folded at the base, mostly 4-cell macroconidia, oblong, hyaline one- or two-cell microconidia and oval thick-walled chlamydospores. The Koch’s postulates were fulfilled. The fungus formed the most abundant aerial mycelium on the Czapek medium, and the least on the CMA medium. On the SNA medium, the mycelium grew into the medium and the aerial mycelium was not formed. The obtained RPB2 nucleotide sequence was 100% similar to the Fusarium oxysporum sequence deposited in GenBank (NCBI).

Conclusions: The results of this research can be used in further studies on the biological diversity of this species.

Key words: PCR, Fusarium oxysporum, morphology, RPB2

Słowa kluczowe: PCR, Fusarium oxysporum, morfologia, RPB2
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INTRODUCTION

Common sage (Salvia officinalis L.) is a plant from Lamiaceae family that originates from the Mediterranean basin [1]. Its natural habitat are sunny limestone slopes of up to 800 m AMSL [2]. Sage was cultivated in the medieval times inside monastic gardens, with species like hyssop (Hyssopus officinalis L.) and peppermint (Mentha x piperita L.) which symbolized mercy, forgiveness and cure [3]. Sage cultivation in Poland was initiated during Second World War [2]. Sage is appreciated in cosmetic industry because it contains essential oils which have a strong antiseptic, anti-inflammatory and antimicrobial effect [4]. It has been found that the addition of sage oil to feed reduces the proliferation of potentially harmful bacteria of coli group. Moreover, it has been shown that, due to its essential oil content, sage increases the production of fatty acids and bile in liver as well as pancreatic enzymes, such as pancreatic lipase and trypsin [5]. Sage oil is also widely used in food industry as an alternative to chemical preservatives [1]. Sage, like other spice plants, is a very good source of antioxidants in food, therefore it is used in the processing of meat. Both dried sage and sage extracts as well as essential oils inhibit microbial growth in meat products and reduce lipid oxidation processes in meat products, prolonging their shelf life [6]. Sage leaf extract may have a positive effect on patients with moderate to mild Alzheimer’s disease [7].

Sage plants growing in the wild are mainly resistant to diseases but as a result of environmental changes in the conditions of intensive cultivation, they become susceptible to infections by pathogens and damage inflicted by pests [8]. The main sage diseases in European countries are anthracnosis caused by Colletotrichum dematium (Pers.), grove and root rot caused by Rhizoctonia solani J.G. Kühn [6], whereas in the USA (California) mass death of sage seedlings infested with Fusarium oxysporum Schltdl was observed [9].

F. oxysporum is the cause of wilt of many plant species. It belongs to the genus Fusarium, family Nectriaceae, order Hypocreales, class Sordariomycetes and division Ascomycota [W1]. The fungus is common all over the world and it can sustain different climatic conditions as a saprotroph or pathogen of plants and humans. F. oxysporum can infect both mono- and dicotyledonous plants and its diversity within the genus is correlated with wide range of possible hosts. However, in spite of wide variety of host plants, the individual “variants” of the fungus show a high specificity for a particular plant species. These “variants” were designated as formae speciales (f. spp.). F. oxysporum has many special forms within the genus. Their number is constantly increasing. In 2018, 106 well-known special forms and 37 forms requiring further research were documented. The range of hosts of the special forms of F. oxysporum includes 45 families of plants, such as Asteraceae, Solanaceae, Cucurbitaceae and Fabaceae [10].

The symptoms caused by F. oxysporum are most often wilt and, less frequently, rot. The pathogen infects mainly by attacking the roots and colonizing the vascular bundles that darken or turn yellow (moving towards the upper parts of the plant). This type of symptom is known as Fusarium yellowing or Fusarium wilt. F. oxysporum also causes progressive and hypocotyl root rot but does not reach the vessels [11].

The teleomorph of F. oxysporum is unknown [12], therefore it is an anamorphic fungus [13].

Diseases caused by fungi are a huge and very common threat to pharmacopoeial raw materials [14]. Therefore, the aim of the present study was to identify and characterise one of the pathogens that threaten herbal crops [15]. F. oxysporum was also isolated from common sage stem base by Zimowska [9], however, no detailed studies of this species as a pathogen were performed.

MATERIAL AND METHODS

Isolation of fungus

The plant material consisted of 30 infected 4-week-old sage seedlings from the Department of Vegetable and Medicinal Plants, Warsaw University of Life Sciences. The pathogen was isolated from the base of the stem. Sections between healthy and diseased tissue were cut out with a sterile scalpel, disinfected for 2 minutes in 1% sodium hypochlorite and transferred with a sterile needle into Petri dishes (Ø 10 cm) with glucose-potato medium – PDA (Potato Dextrose Agar). The Petri dishes were incubated at 17°C for 5 days under fluorescent lamps (14 h day/10 h night). Pure fungal cultures were transferred to PDA slants, which were stored in a refrigerator (4°C).
Verification of Koch’s postulates

24 healthy 3-week-old sage seedlings were selected for the experiment. Plants were grown in multiplates filled with a sterile substrate (peat + perlite in a ratio of 3:1). Among 12 inoculated plants, the base of the shoot of 6 plants was damaged with a sterile scalpel. The inoculum consisted of the PDA medium disks (⌀5 mm), overgrown with the pathogen’s mycelium for 10-day-old cultures of the fungus. The discs were applied to the base of the shoot (1 disc/plant). The control variant consisted of 12 plants (6 without damage to the base of the shoot and 6 with damage) to which only the pure medium disks were applied. Both the control and inoculated variant were placed in plastic bags, wetted with distilled water and closed. Observations of the plants were carried out for 20 days before the onset of symptoms of the disease. For this study, isolates were selected on the basis of morphological features (presence of a particular type of spores, the appearance and measurements of conidia on the SNA medium, and colony morphology on the PDA medium), as described by Leslie and Summerell [16].

Then, the pathogen was reisolated from the inoculated plants.

Pathogen morphology

23 isolates were transferred onto the SNA medium. The length and width of 100 randomly selected conidia (macroconidia and microconidia) of all the isolates were measured after 7 days of growth of the cultures on a selective SNA medium (synthetic nutrient agar), according to Leslie and Summerell [16].

The slides were observed under the light microscope BX50 (Olympus). The photographic documentation of conidia was made with the use of the DP71 (Olympus) camera compatible with the microscope. Spores were measured using the Cell-F computer programme (Olympus).

Molecular identification

The DNA was extracted from the lyophilized mycelium of 23 pathogen isolates according to Tomczak et al. [17] with some modifications: 150 µl of a chloroform-octanol (24:1) mixture was used, samples were incubated at 65°C for 25 minutes and centrifuged at 12,000 rpm for 15 minutes (Eppendorf AG, Hamburg). DNA was stored at –80°C. The amplification of the RNA polymerase II gene fragments was performed with primers as follows: fRPB2-5F: 5’-GAYGAYMGWGATCAYTTYGG-3’, and fRPB2-5R: 5’-CRAARTGATCWCRT-CRTC-3’ [18]. The reaction was performed under the following conditions: pre-denaturation at 94°C for 5 minutes, 5 cycles of specific denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and elongation at 72°C for 2 minutes; 5 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 2 minutes; 30 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 54°C for 45 seconds, and elongation at 72°C for 2 minutes; final elongation at 72°C for 7 minutes and final cooling at 4°C, until the desired temperature was reached. The PCR reaction was performed on an Applied Biosystems Veriti 96 Well Thermal Cycler thermocycler.

The obtained PCR products were separated on a 1.3% agarose gel with addition of 1 µl of fluorescent dye ethidium bromide. The electrophoresis was performed in TBE buffer at 90 V for 35 minutes. The GeneRuler™ 1 kb DNA Ladder ready to use marker was used to determine the size of resulting PCR reaction products. A camera (Sygen) was used to read and archive the results.

Out of the 23 isolates, 6 distinct isolates were selected for the molecular analysis on the basis of the morphological features on the PDA and SNA media. The amplified DNA fragments of 6 fungal isolates were sequenced at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. The obtained nucleotide sequences were analysed using DNA Baser Sequence Assembler programme and compared with the sequences from GenBank using the BLASTn program.

Evolutionary analysis

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [19]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was (were) obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL)
approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1649)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 29 nucleotide sequences. There were a total of 1075 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [20].

Comparison of the morphology of cultures of fungal isolates on different media

Based on the morphology of isolates and their nucleotide sequences, a representative isolate (No. 13) was selected for comparison on 7 media: CMA (Corn Meal Agar), MEA (Malt Extract Agar), OA (Oatmeal Agar), PCA (Potato Carrot Agar), Czapek (Czapek-Dox Agar), SNA and PDA. The media were prepared according to Gams et al. [21].

Three Petri dishes of each medium were used per one isolate of the fungus. The inoculum was prepared from 10-day-old cultures of overgrown media discs (5 mm diameter) with mycelium of the pathogen.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

Isolation of the fungus and verification of Koch’s postulates

In 2018, a total of 23 isolates of the disease causal agent (FO1-FO23) were obtained from plants that showed darkening of the stem base and rot symptoms.

Koch’s postulates were fulfilled. Dark spots on the shoots were observed on all inoculated plants with an undamaged stem base (fig. 1A) and on plants with a damaged stem base (fig. 1B). The pathogen reisolates were the same as the isolates used to inoculate the plants.

Cultures of fungal isolates on the PDA medium produced a reddish pigment diffusing into the medium, which is a very characteristic feature of Fusarium fungi [22]. Therefore, the causal agent of sage plant wilt was initially classified into Fusarium. Fusarium culmorum (Wm.G. Sm.) Sacc., F. equiseti (Corda) Sacc. and F. oxysporum were also isolated by Zimowska [9] from the base of the sage shoot. In present study, only F. oxysporum was isolated from the base of the shoot of the plant, while in the work of Zimowska [9], except from the abovementioned pathogens, fungi of Alternaria, Phoma, Penicillium and Trichoderma genera were also isolated. Fusarium wilt of another sage species (Salvia sclarea L.) was also observed in plantations in Tajikistan [23].

Morphology and dimensions of conidia

After 5 days, the fungus produced 2 types of conidia on the SNA medium, macro- and microconidia. The macroconidia were slightly bent at the ends, thin-walled, hyaline, usually with 3 septa, sometimes 4 or 5, with average dimensions of 39.6 x 4 μm (fig. 2A). The microconidia were oblong, without septum, hyaline, 9 x 3 μm in size (fig. 2B). They were formed on unbranched monophialides with dimensions of 10.7 x 3 μm. Phialides formed directly on the mycelium hyphae (fig. 2C). Formation of chlamydospores along the hyphae was also observed (fig. 2D).

Both the morphology and size of the conidia were characteristic of the species F. oxysporum [16, 24]. The macroconidia observed by Booth [24] were 27–46 x 3-5 μm. As in the present study, they were pointed at both ends, thin-walled and had 3 septa. On the other hand, the microconidia were 5–12 x 2.2–3.5 μm. As in the present study, they were oval to slightly bent, without septa. The microconidia were formed on phialides in “wet heads”. The results of the present study were also compatible to those in Ohara and Takashi [25]. The pathogen also
produced chlamydospores, which were formed by *F. oxysporum* at the middle or at the end of the hyphae [22, 25, 26].

*F. oxysporum* is the main factor of wilt diseases of various plant species. For the first time, Gaetan and Madia [27] described sage wilt caused by *F. oxysporum* in Argentina in 2006. The disease was defined as Fusarium wilt. Kulik et al. [28] showed that *F. oxysporum* produces a large number of oval-shaped chlamydospores, which develop singly or in pairs. In the present study, spherical chlamydospores formed less abundantly, usually singly at the middle of the hyphae.

The individual special forms of the fungus show a high degree of specificity in relation to the host. In 1940, Snyder and Hansen (according to Puhalla [29]) distinguished various special forms of this pathogen on the basis of the species of the attacked host plants, e.g. *F. oxysporum* f. sp. *cubense*, isolated from banana plants (*Musa*) [30], and *F. oxysporum* f. sp. *ciceris*, isolated from chickpea plants (*Cicer arietinum*) [31].

**Identification of the fungus with the use of the PCR technique**

In the present study, 6 nucleotide sequences of *F. oxysporum* isolates were obtained. They were 1,034 bp in length and 100% similar one to another. A representative sequence has been deposited in GenBank under the accession number UGY87147. The nucleotide sequences obtained in this study were 100% similar to the sequence of *F. oxysporum* (MN457491) obtained from common pea (*Pisum sativum* L.) deposited in GenBank by Achari et al. [32].

The phylogenetic analysis shows that the isolate obtained from the common sage was classified
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Table 1.
GenBank accession numbers of the sequences of the Fusarium isolates used in the phylogenetic analysis and their respective references

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Figure 3.
Comparison of the gene sequence of the second largest subunit of RNA polymerase II - RPB2 of various species of fungi of the genus Fusarium. The tree was rooted to Fusarium poae (GenBank KU171706) Brown and Proctor [33]
into the same group as the other isolates belonging to the species *F. oxysporum* and representing the FOSC section (*F. oxysporum* species complex) (fig. 3, tab. 1). The tree with the highest log likelihood (−4337.00) is shown.

The *Fusarium* taxonomy and its phylogenetic basis are constantly updated. In the modern taxonomy of fungi, phylogenetic inference plays an important role in determining the identity of species which morphology is not a sufficient determinant of species affiliation [34]. Therefore, the gene sequence of the second largest subunit of RNA polymerase II, RPB2, is used in studies on the phylogeny of fungi, especially fungi of the genus *Fusarium*, and for their identification [35]. The RPB2 gene sequences are used for genetic analysis at the species level due to the ease of sequencing and comparing the obtained sequences with those deposited in GenBank. In addition, RPB2 sequences, when compared to ITS sequences which are also commonly used in the identification of fungi, are more specific for *Fusarium* species and reduce the risk of errors in species identification [36]. The results of the present study indicated that *F. oxysporum* was a pathogen of sage stem base. To the best of our knowledge, this is the first report of *F. oxysporum* as a pathogen of sage in Poland.

### Comparison of morphology of cultures on different media

The pathogen produced the most abundant aerial mycelium on the Czapek medium, and the least on the CMA medium. On the SNA medium, the mycelium grew into the medium and the aerial mycelium formed very poorly. The colonies on all the media were in different shades of white (fig. 4). According to data from the literature [16, 30, 31], some special forms of the fungus (*F. oxysporum* f. sp. *cubense* and f. sp. *ciceris*) formed white to purple colonies on the PDA medium. The fungi produced a pigment that diffused into the medium. The MEA medium was coloured intense red, the PDA medium was coloured deep red, the PCA medium was coloured bright pink, the OA medium was coloured violet and yellow, the Czapek medium was coloured yellowish and the SNA medium was coloured pale pink (only right beside the disk). The CMA medium did not change colour. According to the literature, most species belonging to the genus *Fusarium* grow rapidly on the PDA medium and colour the medium pink, beetroot or purple [16]. The fungus produced the most conidia and chlamydospores on the SNA medium [22], which was confirmed in the present study. There is no information in the literature about

### Table 1. (continued)

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CONCLUSIONS

*F. oxysporum* was identified as the causal agent of common sage wilt disease based on molecular studies and morphological features. The most important morphological features that enabled the pathogen's identification were: shape, size and type of conidia as well as fungal colony morphology on different media. This fungus was identified and characterised as a pathogen of sage stem base for the first time in Poland. The results of this research are the first step towards better recognition and comprehension of the biodiversity of *F. oxysporum* populations on common sage in terms of their host-specific pathogenicity and the occurrence of special forms and races. Moreover, the commonly known metabolic activity of the Fusarium genus emphasizes the urgent need to verify the contamination of raw material with known toxic secondary metabolites produced by *F. oxysporum*.

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