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Inhibitory effect of Greek oregano extracts, fractions and essential oil on economically important plant pathogens on soybean

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Abstract. *The inhibitory potential of Greek oregano extracts, fractions and essential oil on three economically important plant pathogens on soybean was examined. In vitro experiments were conducted to determine the effects of essential oil (EO), acetone exudate (AE), methanol extract (ME), and its polar (PF) and non-polar (NPF) fractions, originating from O. vulgare ssp. hirtum plants, on the mycelial growth of Phytophthora sojae, Pythium debarianum, and Alternaria alternata, which were isolated from diseased soybean plants. Seven variants: EO, AE, ME, PF, NPF at concentrations of 100 mg/ml, blank (with the solvent - DMSO) and control (without treatment) were performed with all isolates. The mycelial growth of P. sojae was inhibited to the greatest extent - over 95% reduction of growth under the impact of EO, ME, AE and NPF. In A. alternata variant there was also a high degree of growth inhibition - most pronounced in EO variant (97%) and least in ME variant (78%). The weakest inhibitory effect of the studied extracts was observed in P. debarianum – between 27% (NPF) and 36% (ME). In polar fraction treatments, growth enhancement was observed in P. sojae and A. alternata. Oregano essential oil, methanol extract, acetone exudate and non-polar fraction can be considered as promising candidate active compounds of potential biopesticide formulations for the control of soybean diseases.*

Keywords: carvacrol, agar-disk diffusion method, mycelial growth area, *Phytophthora sojae*

Introduction

Modern European agriculture relies mostly on the use of agrochemicals to ensure the economic sustainability of agricultural production. The negative effects of indiscriminate use of agrochemicals include environmental damage (contamination of water, air and soil resources), toxicity to nontarget organisms, toxicity to humans (mainly through the food chain), and development of pest resistance (Campos et al., 2019). In recent years it has been acknowledged that a variety of practices in the EU's agricultural sector are contributing to a wide-scale biodiversity loss, climate change, soil erosion and land degradation (Pe'er et al., 2020). The post-2020 common agricultural policy emphasizes environmental benefits, simplification, and stronger agricultural knowledge and innovation (EU SCAR, 2012). For this purpose, it is necessary to look for new directions for development, such as expanding of the organic production, production of high-quality food and products with high added value (Nikolova, 2017). The sustainable agriculture should be able to conserve soil quality by the use of biopesticides and biofertilizers, crop diversity and rotation. Biological control based on natural enemies, as well as the uses of natural products such as those derived from plants are considered effective and environmentally safe techniques for pest control. Botanical pesticides offer a good alternative to traditional chemicals for use in crop protection systems (Campos et al., 2019). Recently, the botanical pesticides have been gaining popularity and

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some plant products are being used globally as green pesticides. Higher plants essential oils and secondary metabolites such as phenols, flavonoids, quinones, tannins, alkaloids, saponins and sterols are promising for their different biological properties (Bhagat et al., 2014).

Oreganum vulgare ssp. hirtum is a species with a high essential oil yield. It is known as a culinary spice and also as a herb with medicinal properties. The plant is widespread throughout the Mediterranean region (Alekseeva et al., 2020). The essential oil of *O. vulgare* has demonstrated strong antifungal activity against *B. cinerea*, both *in vitro* and *in vivo* (Zhao et al., 2021) and inhibiting effect on a number of fungal pathogens of potato (Fusarium sp., *Alternaria alternata*, Neocosmospora sp. and *Botritis cinerea*) (Krumova et al., 2021).

Soybean is one of the five crops that dominate global agriculture, due to its high protein content and amino acid composition. 92% of soybean production in the world originates from the USA, Argentina, Brazil, China and India. In Europe, soybean plays a minor role and is cultivated mainly in the South and East (Karges et al., 2022). The history of soybean cultivation in Bulgaria is characterized by episodes of expansions in the past and decline after the country's accession to the European Union. The economically most important pathogen on soybean is *Phytophthora sojae*, causing annual losses worldwide of $1–2 billion. Along with *P. infestans*, this species has been developed as a model species for the study of oomycete plant pathogens (Tyler, 2007). *P. sojae* is the limiting factor for soybean production in regions with conditions favorable for the disease's development, such as warm temperatures and saturated soils (Dorrance and Martin, 2000). Cool and damp environmental conditions are most suitable for the other economically important oomycetous genus, *Pythium*, in soybean cultivation. Numerous Pythium spp. are known to be pathogenic to soybean. Moreover, the genus is recognized like one of the most important groups of seedling pathogens affecting soybean, causing both pre- and post-emergence damping-off (Zitnick-Anderson and Nelson Jr, 2015). *Pithium debarianum* and other Pythium spp. have been reported to cause stunting of soybean seedlings in USA (Strissel and Dunleavy, 1970).

Alternaria alternata is a cosmopolitan species with a wide host range. The species can cause diseases on many important crop plants or it can form asymptomatic symbiotic relationships as an endophyte (DeMers, 2022). Recently it has been reported for the first time in Turkey to cause necrotic spots on the upper surfaces of the lower leaves of soybean (Ustun et al., 2019).

The aim of our investigation was to evaluate the effect of essential oil and different types of plant extracts from the Greek oregano plant species, which is widespread in Bulgaria, on economically important oomycete and fungal pathogens in soybean crops.

Material and methods

Plant material and preparation of the extracts

Plant material

Aerial parts of *Origanum vulgare* ssp. *hirtum* were collected during the flowering stage from natural population at the Struma valley, Bulgaria. A Voucher specimen was deposited at the Herbarium, Institute of Biodiversity and Ecosystem Research (SOM), Bulgaria (CO1409).

Methanol extract and fractions

Crude methanol extract was prepared from air-dried, powdered aerial parts of the species macerated with methanol at room temperature for 24 h. After filtration, the organic phase was evaporated to dryness. Part of the extract was left for phytopathogenic activity analyses and another was continued to be processed to obtain fractions. The extract was dissolved in distilled water and chloroform in a ratio of 1:3 and processed in a separatory funnel several times to discoloration of the chloroform layer. The combined chloroform extracts were combined and evaporated to dryness to produce a non-polar fraction. The aqueous phase was also evaporated to dryness; it represents the polar fraction.

Acetone exudate

The exudate was prepared from air-dried, unground aerial parts by rinsing them with acetone for several minutes to dissolve the compounds accumulated on the surface of the plant tissue.

Essential oil

Essential oil was extracted on a Clevenger apparatus by water distillation from dry aerial parts of the plants.

Bioassay

The oomycetous *Phytophthora sojae* and *Pythium debarianum* isolates were obtained by baiting rhizosphere soil samples (Jung and Burgess, 2009) from diseased soybean plants grown in Soybean Experimental Station, Agricultural Academy in Pavlikeni. *Alternaria alternata* isolate was obtained from the necrotic spots on the leaves of soybean plants exhibiting symptoms of fungal disease from the same field location. The taxonomic identification of the isolates was performed using both classical methods and molecular techniques (Lyubenova et al., 2015). In order to assess the impact of the Greek oregano derived essential oil and extracts on the mycelial growth and development of plant pathogens we used the agar disk-diffusion method (Balouiri et al., 2016).

Small agar blocks (2x2 mm) were cultured in the center of 9 mm Petri dishes on the following nutrient media: for the oomycetous species we used carrot juice agar (CA) (16 g agar, 3 g $CaCO_3$, 100 mL HiPP Organic Pure Carrot Juice, and 900 mL distilled water) and *A. alternata* was cultivated on Potato-dextrose agar (PDA) (BD Difco™). The Petri dishes were incubated at 20°C for 12 hours for synchronizing the onset of growth. Five variants were performed with all isolates: pure essential oil, methanol plant extract (ME), ME polar fraction, ME non-polar fraction, and acetone exudate at a concentration of 100 mg/ml. The methanol extract was dissolved in methanol, and acetone exudate, ME polar fraction and ME nonpolar fractions in DMSO. Two drops with a volume of 15 µl of each extract were dripped into each plate at equal distances from its center. In the same way the essential oil was applied with a drop volume of 2 µl. Four replicates were made for each variant, one control without treatment and one with DMSO for each isolate. The Petri dishes were cultivated in a climatic chamber in darkness at 20°C. The results were documented after 3 days (*Pythium debarianum*) and after 10 days (*Phytophthora sojae* and *Alternaria alternata*). Pictures (CANON EOS 4000D) of all mycelial colonies were taken, and their mycelial growth areas were determined, using the image analysis program ImageJ (Schneider et al., 2012). The percentage of inhibition was calculated based on the obtained data (average mycelial growth area for each variant), using the equation:

$IMG = 100(C-T)\ C^{-1}$ where IMG is percentage of inhibition of the mycelial growth, C is the area of the fungal colony without treatment (control) and T is the area of the fungal colony with treatment (Zygadlo et al., 1994).

Results and discussion

The results for the *Pythium debarianum* variant were observed and recorded after three days, while for the *A. alternata* and *Phytophthora sojae* variants, results were observed and recorded after 10 days. This differentiation was necessary due to the considerably faster growth rate of *Pythium* isolate compared to the other two isolates. Both control variants without treatment and with DSMO had similar growth rate (Figures 1, 2 and 3). The oregano essential oil and extracts inhibited mycelial growth of all of the tested isolates to a different extent.

The essential oil of *O. vulgare* subsp. *hirtum* showed absolute inhibitory effect to *Phytophthora sojae* and *Alternaria alternata*, expressed in 97.83% IMG (Figure 1) and 97.72% IMG (Figure 2), respectively. Similarly, the non-polar fraction strongly inhibited the mycelial growth – 96.74% IMG of *P. sojae* (Figure 1) and 87.91% IMG of *A. alternata* (Figure 2). The methanol extract and acetone exudate occupied middle positions with regard to their inhibitory effect on both *Phytophthora sojae* and *Alternaria alternata* (Figure 1 and Figure 2).

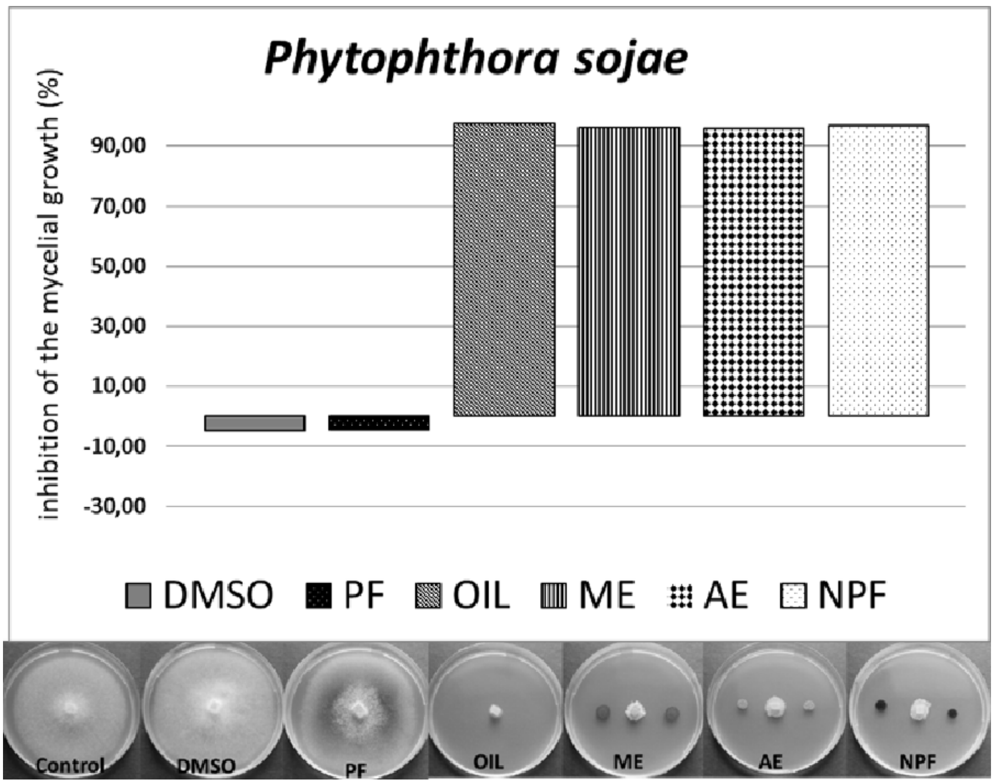

Figure 1. Impact of the *O. vulgare* subsp. *hirtum* oil, acetone exudate, methanol extract, and both polar and non-polar fractions on the growth of *Phytophthora sojae* under *in vitro* conditions after 10 days at 20°C

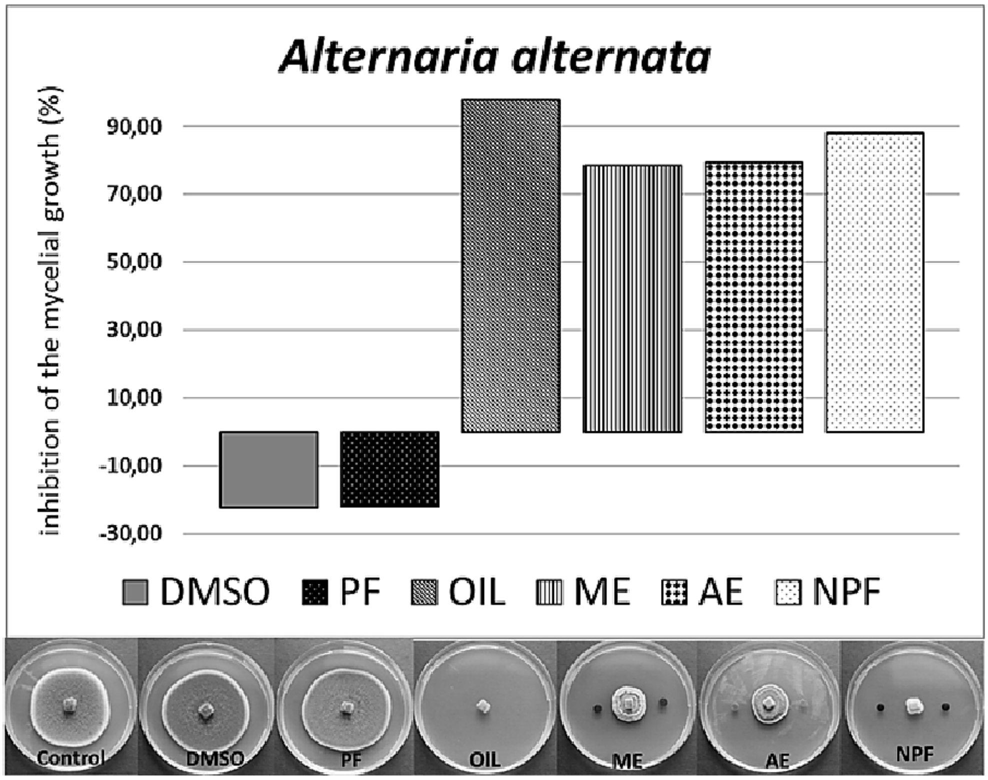

Figure 2. Impact of the *O. vulgare* subsp. *hirtum* oil, acetone exudate, methanol extract, and both polar and non-polar fractions on the growth of *Alternaria alternata* under *in vitro* conditions after 10 days at 20°C

In the *Pythium debarianum* variant, inhibition of mycelial growth was recorded following the same trend as in the *P. soje* and *A. alternata* variant, but to a lesser extent – the greatest inhibiting effect was recorded in the methanol extract variant (36.35% IMG) and between 27.88 IMG in polar fraction to 28.54% in acetone exudate variants (Figure 3). The non-polar fraction facilitated the mycelial growth of all isolates (Figures 1, 2 and 3).

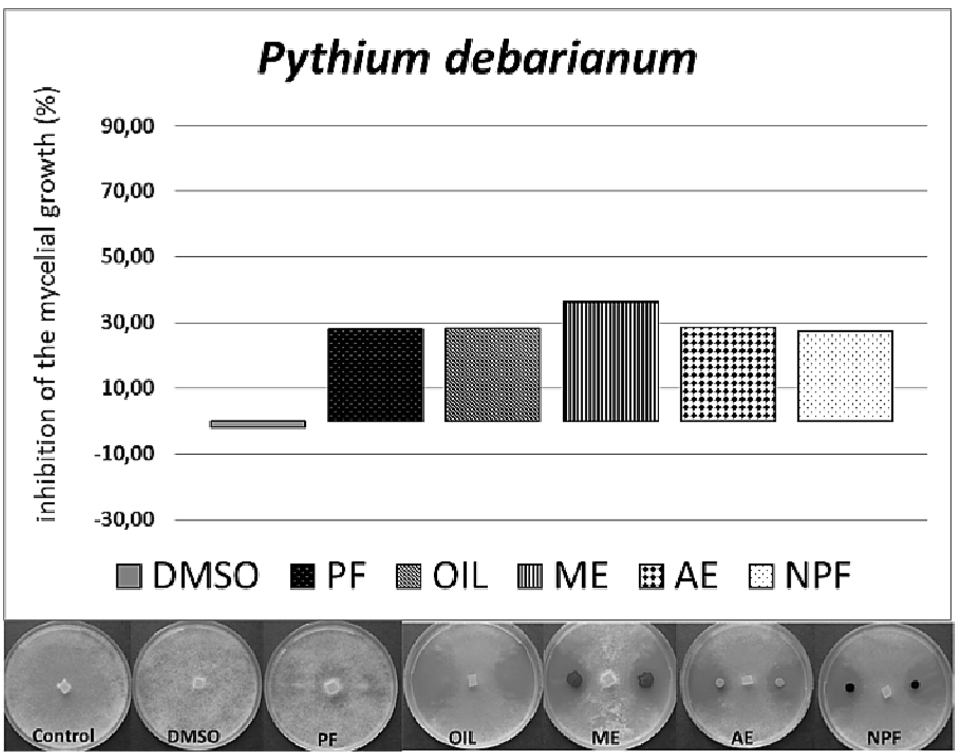

Figure 3. Impact of the *O. vulgare* subsp. *hirtum* oil, acetone exudate, methanol extract, and both polar and non-polar fractions on the growth of *Pythium debarianum* under *in vitro* conditions after 3 days at 20°C

In our previous study (unpublished) we found that carvacrol was the main compound in oregano essential oil (55.48%), acetone exudate (48.15%) and the non-polar fraction (50.34%). In methanolic extract carvacrol was found in smaller amount (13.24%) and completely absent in the polar fraction. The inhibitory effect of carvacrol is previously described in the literature (Noma and Asakawa, 2010; Zhang at al., 2019). The results from our study confirm the central role of carvacrol for inhibition of the mycelial growth of tested plant pathogens. Supporting this is the lack of inhibitory effect in the non-polar fraction, where carvacrol is absent. In the current study we also found a reverse correlation between the mycelial growth rates of the oomycete and fungal isolates and their susceptibility to the inhibitory effect of the studied essential oil and extracts. This could be explained by the longer exposure of the slow-growing isolates compared to the rapidly developing ones.

Conclusion

Greek oregano essential oil, methanol extract and its non-polar fraction as well as acetone exudate were able to strongly hamper the mycelial growth of the economically important soybean plant pathogens in *in vitro* conditions. This effect is primarily due to the high carvacrol content in the tested fractions. *Oregano vulgare* subsp. *hirtum* extracts, fractions and essential oil are promising candidates for use as active compounds in future biopesticide formulations. They show potential for inclusion in diverse ecological approach-based biocontrol strategies.

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