Biochemical Study of *Helianthus Annuus L.* Exudate, Related to *Orobanche Cumana* Wallr. Germination Inhibitors

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For biochemists and breeders, increasing Helianthus annuus L. (sunflower) resistance against the root holoparastic angiosperm Orobanche cumana Wallr., remains a challenge, considering that the damage caused by this parasite is still significant in many countries of Eurasia. Resistance factors, such as Orobanche seed germination inhibitors, have been studied in root exudates of different sunflower lines. Instrumental analyses of root exudates show the presence of germination inhibitor benzoic acid. Substances from the cinamic acid family, ferulic acid family, cumaric acid family known as Orobanche seed germination inhibitors, were not found. The germination inhibiting effect of benzoic acid through Orobanche seed germination tests, under standardized laboratory conditions, is already known.

Keywords: sunflower, Orobanche cumana, germination inhibitors, instrumental analysis, root exudates, benzoic acid

All known species of *Orobanche* are holoparasites with high host specificity. They produce a huge amount (up to 500.000) of small seeds (around 0.2 mm) for each plant [1]. The seeds only germinate in the presence of germination stimulants, specific substances exuded by the root of the host plant *Helianthus annuus* L.

Orobanche cumana Wallr., the weedy broomrape which threatens sunflower cultures, is countered by all practicable means, while broomrapes in the spontaneous flora are rare and endangered, even protected plants [2, 3]. The angiosperm species *Orobanche cumana*, as a root parasite, represents a major problem in cultivating the sunflower in Bulgaria, Romania, Russia, Turkey, Spain, Moldova, Serbia, Georgia [2, 4-6], China [7].

The top researches related to the biochemical aspects of the relationship between the host plant *Helianthus annuus* L. and the root holoparasit *Orobanche cumana* Wallr. are a big provocation for biochemists [8], especially in the context of climate change, in order to improve sunflower resistance against *Orobanche,* by studying the inhibitors of germination exuded by sunflower varieties, which could be used for the integrated control of *Orobanche cumana* [9].

Orobanche cumana shows some particularities against the other *Orobanche* species: on the one hand, related to its response to the natural germinating stimulant, it is specialized exclusively on sunflower root, but on the other hand, its aggressiveness is strong through its ability to rapidly develop new patotypes [10]. Compared to *O. cumana,* other *Orobanche* species like *O. ramosa* can parasitize plants from 21 botanical families [3].

For breeders, there is the advantage that the problematic of sunflower resistance against *Orobanche cumana* is more advanced, in comparison to other cultivated plants attacked by *Orobanche*, such as bean or tobacco [11]. Germination stimulants from sunflower, parthenolide, are specific for *O. cumana* and well known [11, 12], while other *Orobanche* species are stimulated by strigolactones [13-15].

Biological control with the insect *Phytomyza orobanchia* (in Russia) or with the help of fungi *Fusarium oxysporum f. sp. orthoceras* is not yet possible in the field [9, 5]. The use of herbicides is limited for economic and environmental reasons. Biochemists search for biochemical resistance factors and resistance mechanisms, for the causal relation between chemical compounds and resistance of root parasitic weeds from sunflower and other plants [12, 16-21]. Thus, the long-term control method against *Orobanche* remains the sunflower resistance breeding and the instrumental analyses of root exudates from *Helianthus annuus* is still a priority [4].

The objectives of the research are the instrumental analyses of root exudates from a sunflower resistant line, in comparison with Splendor, a susceptible sunflower variety, and also with a plant collection from the associated flora (leaved weeds), related to possible germination inhibitors.

Experimental part

Obtaining root exudates

Seeds were provided by the INCDA Fundulea. Sunflower plants grown in pots (figs. 1 and 2), are havely watered (Milli-Q quality), washed from the substrate and transferred to glass recipients with Milli-Q water. We want to use a small amount of water, but it has to cover the roots entirely. The glass recipients are covered with tin foil and placed on the SM 25 horizontal shaker (Edmund Bühler) for three hours [2]. Barber & Gunn (1974) found that mechanical stress enhances the secretion of substances from the roots of cereals [10]. The exudate is filtered at 60°C and concentrated to a volume of 3 mL by rotavapor.

The root exudate of the associated flora was prepared analogously, using a mixture of species that grow around the sunflower species (leaved weeds).

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Fig.1. *Helianthus annuus* and associated flora in greenhous

Fig. 2. Obtaining root exudates from the host plant *Helianthus annuus*

Art	Fraction I (mg) GC-MS	Fraction II (mg) HPLC
Associated flora	1.1	2.3
Helianthus annus var. Splendor	1.4	6.6
Helianthus annus resistant line	2.1	4.0
Blind sample	0.4	0.7

Table 1
SAMPLES FOR GC-MS AND HPLC

Concentration of root exudates

The solution with root is filtered and concentrated to 20 – 30 mL, using a Büchi Rotavapor R-124 with Buchi Waterbath B-480 at 50°C.

Initial separation of root exudates in a hydrophilic (fraction I) and a hydrophobic fraction (fraction II)

The initial separation of root exudates in a hydrophilic and a hydrophobic fraction is carried out with an Amberlite XAD 1180 column (after the collection of methods from the Department for Chemical Ecology and Ecosystem Research, Faculty of Life Sciences, University of Vienna [2]).

Chemicals: Amberlite XAD 1180, ultrapure water (Milli-Q quality), absolute ethanol.

Column preparation:10 g of Amberlite XAD 1180 get expanded overnight in 200 mL of ultrapure water (Milli-Q quality). This quantity is enough for four columns. We put 50 mL expanded Amberlite in each separation column [2, 22].

Fractionation

The aqueous sample (20-30 mL) is introduced into the column and eluted, first with 2x 75mL water (to obtain the fraction I, the hydrophilic phase), and then with 75mL absolute ethanol. The hydrophilic fraction I is concentrated by rotavapor to 25 mL. We took 1 mL of it to analyze in auto-sampler vial. The solution was dried in the SpeedVac overnight, weighed and sealed.

Derivatization of the hydrophilic fraction (fraction I) for gas chromatography in tandem with the mass spectrometry (GC-MS)

For GC-MS, the sample concentration should be about 3 mg/mL. Therefore it is necessary that sialylation will be made in micro vials. The concentrated extracts were dissolved in 200 µL chloroform-methanol-water mixture 1:1:1 (v), transferred into micro vials and dried again in the SpeedVac.

Then follows the oximation of the aldehyde groups, for example carbohydrates, for 17 h, with 30 µL solution 20 mg/L metoxiamin (CH3-O-NH2) in piridin, after then, for 1 h trimetilsialylation with 30 µL MSTFA, N-methyl-N-(trimetilsilil) trifluoroacetamid [23].

Gas chromatography in tandem with the mass spectrometry (GC-MS)

GC-MS analyses were made with Perkin Elmer Turbomass Autosystem XL, with a capillary column for separation of 20m x 0.18 mm, with a separation layer of 0.18µm.

Preparation of fraction II samples for high performance liquid chromatography (HPLC)

Column eluates with ethanol were evaporated in the rotavapor until dry (table 1). Furthermore they are dissolved in methanol (10 mg/mL), in which a drop of phosphoric acid was added. To dissolve, the bottles are placed for 15 min in an ultrasonic bath. 10 µL of content will be injected with an auto-sampler in the Dionex HPLC system.

Results and discussions

An important challenge for scientists is the rapid development of new aggressive races of *Orobanche cumana*, which overcome the resistance of sunflower lines in agriculture [4, 14].

In the complicated relationship between *Orobanche cumana* and host or non-host plants, the presence of substances with inhibiting effects on the germination of *O. cumana* in the root exudates is a useful screening tool for sunflower breeding [24].

To investigate the presence of substances with allelopathic effects, analyses were performed using high performance liquid chromatography, HPLC, with UV/VIS-Diodenarray-Detector and gas chromatography in tandem with the mass spectrometry GC-MS [7]. We analyzed root exudates of a resistant host sunflower line, of the susceptible sunflower Splendor line, and of the associated flora (leaved weeds).

HPLC chromatograms of root exudates are shown in figures 3-6. The Diodenarray-Detector recorded optical absorption spectrums of all present compounds and compared them with the spectrums from the database. If the identified spectrums correspond to at least 95% of that from the database, a proposal for identification is printed. The presence of germination inhibitors from the cinnamic acid family, ferulic acid family, cumaric acid family was checked, by comparing to the pure substance.

The benzoic acid was identified in *Helianthus annuus*, resistant variety, and in the associated flora, in considerable amounts, but it is absent in the Splendor variety, susceptible

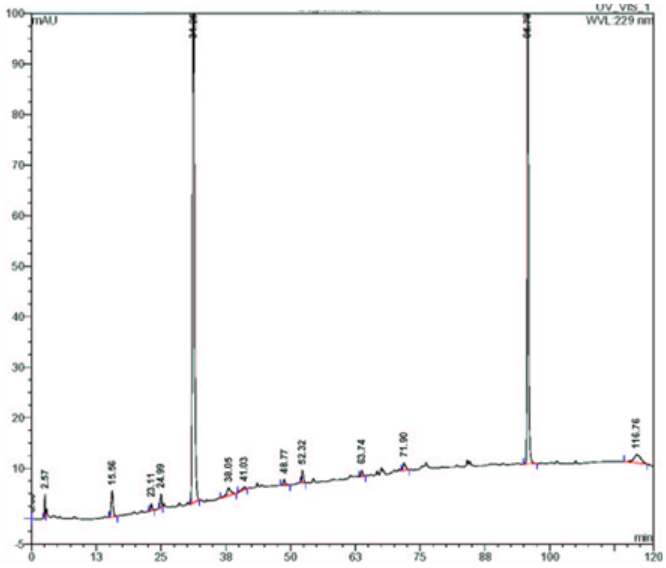

Fig. 3. HPLC analysis of root exudates from the associated flora

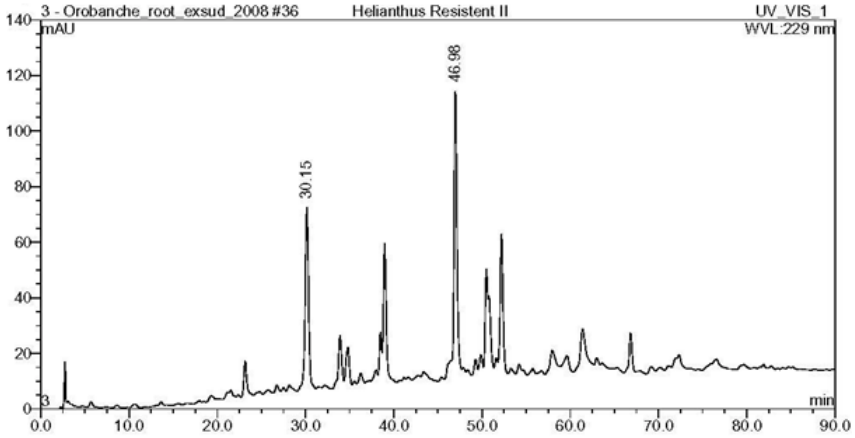

Fig. 4. HPLC separation by *Helianthus annuus,* resistant variety

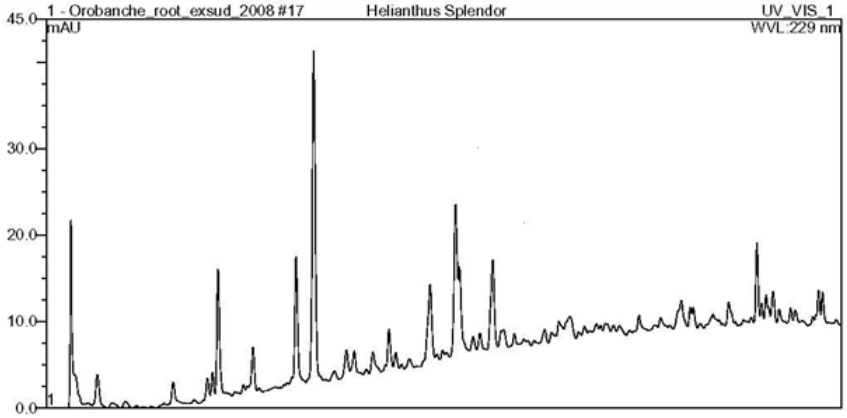

Fig. 5. HPLC separation by *Helianthus annuus,* Splendor variety

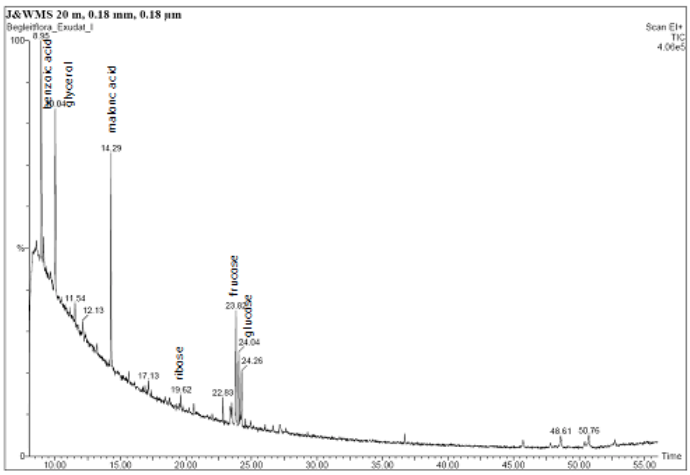

Fig. 6. GC-MS separation for root exudates of the associated flora

to *Orobanche*. Based on this analysis we could not establish the presence of substances from the cinnamic acid family, ferulic acid family, cumaric acid family, known as *Orobanche* seed germination inhibitors [2]. Inhibition of *Orobanche* seed germination by benzoic acid was already certified by standardized germination experiments [2, 3].

In the GC-MS analysis of root exudates from the associated flora, the hydrophilic fraction was separated (chromatogram in fig. 6). Chemical compounds have been identified on the basis of the mass spectra. We especially noticed the presence of carbohydrates and polyols, as well as that of organic acids, that could be a source of food for soil microorganisms [5].

Conclusions

The investigation of *Helianthus annuus* resistance factors against *Orobanche cumana,* parasitic angiosperm, which grows on the host roots and lives on their expense, means to make steps in understanding the close relationship between these partners [12].

To investigate the allelopathic effects between *Orobanche Cumana* with host and non-host plants, analyses were performed using high performance liquid chromatography, HPLC, with UV/VIS-Diodenarray-Detektor, and gas chromatography in tandem with mass spectrometry, GC-MS. In the analyzed host plant exudates from resistant sunflower varieties and also in that from the associated flora, benzoic acid was present in considerable amounts. Its identity was checked, both by separation HPLC retention time, as well as by the absorption spectrum. The presence of inhibiting substances for germination, like from the cinnamic acid family, ferulic acid family, cumaric acid family known as *Orobanche* seed germination inhibitors, could not be confirmed.

Benzoic acid was identified in *Helianthus annuus*, resistent variety, and in the associated flora, but it is not present in Splendor variety root exudates. The Splendor variety is susceptible to *Orobanche*. Therefore, benzoic acid can play an important role in the resistance of the sunflower against *Orobanche* attacks, as a step towards integrated control.

The host plant's and non-host plant's exuded germination inhibitors for *Orobanche* seeds could be efficient in agriculture. The research for germination inhibitors, along with the synthesis of germination stimulants [16, 25, 26] and with comparative genetic analyses of parasitism genes [27, 28], as parts of *Orobanche cumana* integrated control, will contribute to our understanding of these fascinating plants and need to be continued.

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Manuscript received: 24.03.2016