AUTHOR SUMMARY

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(4.3 Biological sciences – Molecular biology)

Influence of biotic and abiotic stresses on the parasitism of stem holoparasitic plants of genus *Cuscuta* L. (Convolvulaceae)

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General characteristics of the data

The dissertation is written in 277 pages (190 pages of main text and 87 pages of appendices). 10 author figures and 3 author tables are used in the literature review. The results are illustrated with 76 figures and 13 main tables and 337 references are cited. The presented results were published in 20 scientific publications, with 351 citations found in Scopus.

Summary and Relevance of the scientific questions

Members of the genus *Cuscuta* include about 200 species of stem holoparasitic plants in the family Convolvulaceae, which have a significant impact on natural and agricultural plant communities. In general, members of the genus *Cuscuta* are regarded as generalists - they infect a wide variety of plant species and a single plant can parasitize several different hosts simultaneously. The agricultural importance of several *Cuscuta* species is significant, causing severe crop losses worldwide.

Despite the enormous agricultural and ecological importance of parasitic plants of the genus *Cuscuta*, there are still huge uncertainties regarding the distribution, taxonomic status and molecular mechanisms of parasitism. Apart from this, the impact of abiotic and biotic stressors, although well-studied in plants in general, has hardly been addressed in these plants. Parasitic plants are not uncommon in challenging environments - with increased salinity or water defficiency, contaminated or at unfavourable temperatures. Suboptimal conditions can alter their host preference as well as their susceptibility to parasitism. On the other hand, additional stress from *Cuscuta* infection can have a significant impact on the ability of hosts to adapt to other stresses. In order to better understand their potential to impact modern agriculture in times of climate change, a more systematic approach needs to be applied, which requires the development of appropriate models of stress-resistant and stress-sensitive host-parasitic plant pairs.

This dissertation aims to advance knowledge of the distribution of these parasites in the Republic of Bulgaria, their host range, and genetic diversity through classical and molecular approaches. The results of scientific and experimental work carried out in the period 2017-2022 by the Molecular Biology of the Plant Cell group at the Department of Biochemistry of the Faculty of Biology at Sofia University in

collaboration with colleagues of the Departments of Botany, Biophysics, and Ecology and Environmental Protection of the Faculty, as well as in international collaboration with colleagues from China, Serbia, Austria and Italy are summarized. The dissertant, Assoc. Prof. Dr. Lyuben Zagorchev is the lead scientist in these studies, with a major role in planning and carrying out these studies, in the analysis of the results and in the preparation of the scientific publications.

The influence of an abiotic stress factor, salinity, on all stages of parasitism, both on the parasite and its hosts, was investigated. A wide range of biochemical and molecular biological approaches were used. The impact of parasitism on soil microbial communities was assessed by metagenomic analysis. Among the biotic stressors, gall-forming epiparasitic insects of the genus *Smicronyx*, as well as herbivorous insects of the gnawing and sucking type, were investigated. The hypothesis that members of the genus *Cuscuta* are potential reservoirs and vectors of plant viruses was also tested.

The obtained results have a significant contribution to the elucidation of fundamental scientific problems concerning plant-parasite interactions under suboptimal environmental parameters, which also have applied importance as a basis for predicting the effects of abiotic stress in the light of climate change on the distribution, agricultural and ecological impact of these widespread parasitic plants.

1. Introduction

Parasitic flowering plants comprise approximately 4000 species distributed in more than 20 families, representing about 1.5% of all known vascular plant species (Westwood et al. 2010), with most representatives in the families Orobanchaceae and Loranthaceae. These highly specialized organisms are characterized by partial or complete loss of photosynthetic ability and depend on their hosts for organic compounds, mineral elements, and water (Bungard 2004). Parasitic plants are classified into two main categories. Hemiparasites contain chlorophyll and are capable of photosynthesis. They obtain water and mineral elements from the host. Holoparasites are non-photosynthetic and depend entirely on the host (Hegenauer et al. 2017; Nickrent and Musselman 2004). Parasitic plants make contact with host plants and absorb nutrients through a specialized organ called the haustorium, a well-defined structural and physiological link to the host.

The genus *Cuscuta* includes about 200 species of stem holoparasitic plants in the family Convolvulaceae (Braukmann et al. 2013), which have a significant impact on natural and agricultural plant communities. In the Bulgarian flora, the genus is represented by 9 species, of which *Cuscuta campestris* is a North American species that is introduced and invasive to Bulgaria. The agricultural importance of several species of the genus *Cuscuta* is significant, causing severe crop losses worldwide (Parker 2012). The agricultural importance of *Cuscuta* in Bulgaria is not well documented. The life cycle of these parasites goes through several well-defined stages - germination, localization and coiling around a suitable host, haustorium formation and subsequent development. The ecological strategy of parasitic plants involves deep physical and possibly physiological dormancy (Jayasuriya et al. 2008), which ensures long-lasting seed presence in the soil and germination over tens of years. Tomatoes (*Solanum lycopersicum* L.) have been shown to be resistant (or incompatible) hosts for *Cuscuta* spp. (Amini et al. 2017; Sahm et al. 1995). The molecular basis of this resistance is largely unknown.

Nearly all *Cuscuta* species retain some ability to photosynthesize (McNeal et al. 2007a). Within the genus, photosynthetic capacity has been lost to varying degrees. Unlike other members of the genus, *C. campestris* retains a significant proportion of genes in the plastome (Braukmann et al. 2013). The critical step in the development of these plants is the localization, attachment, and formation of a haustorium on a susceptible host. Haustorium formation is initiated by the formation of an adhesive disc, also known as a prehaustorium. The next stage is the penetration phase, which is governed by a complex of enzymes that

modify the cell wall structure of the host cells. The cells at the tip of the haustorium are designated as 'search hyphae' whose function is to establish contact with the host's conducting elements. Upon reaching such 'search hyphae', they surround the conducting elements and form a connection, which can be with the phloem alone or with the xylem and phloem (Kaiser et al. 2015). The most basic and direct impact of the parasite on the host is undoubtedly related to the direct extraction of nutrients, both organic compounds and mineral elements, and water. A reduction in the photosynthetic activity of the host during infection with *Cuscuta* has also been reported (Saric-Krsmanovic et al. 2018).

Parasitic plants can be affected by abiotic stressors in a similar way as their hosts, e.g. by limitations in seed germination and young plant development due to drought and/or salinity, or indirectly, i.e. due to host-related limitations. The latter is particularly true for holoparasites, most of which have no or limited contact with the soil and do not photosynthesize. They are highly dependent on host availability and abiotic stressors potentially reduce this availability. "However, host 'quality' may not depend solely on the availability of biomass. When exposed to abiotic stressors, such as drought and salinity, hosts may accumulate higher concentrations of osmoprotectants, which are potentially resource-rich substrates for parasites, which may even improve host quality compared to hosts that are not stressed (Frost et al. 2003). Additionally, abiotic stress may affect the ability of the host to provide an adequate defensive response against infection, or conversely, this response may be stronger.

2. Aim and tasks

The main goal of the dissertation is to characterize the species diversity of the genus *Cuscuta* in Bulgaria and to study the influence of biotic and abiotic factors on parasite-host interactions.

The goal was chosen to test several main hypotheses.

Hypothesis 1. The introduced species *Cuscuta campestris* has a higher invasive and parasitic potential in comparison to the native species.

To test this hypothesis, the following scientific tasks were set:
1.1.   Characterization of the species and genetic diversity of the representatives of the genus in Bulgaria.
1.2.   Characterization of the host range in relation to species and genetic diversity.

Hypothesis 2. Parasite-host interaction is influenced by abiotic and biotic stressors.
To test this hypothesis, the following scientific tasks were set:
2.1.   To investigate the influence of biotic stressors - host species on parasitism in *Cuscuta* spp.
2.2.   To investigate the influence of abiotic factors - salinity on different stages of parasitism in *Cuscuta* spp.

Hypothesis 3. Parasites of the genus *Cuscuta* are subject to the influence of biotic factors that significantly alter their metabolism.
To test this hypothesis, the following scientific tasks were set:
3.1.   To investigate the influence of biotic factors - plant pathogens on parasitism of *Cuscuta* spp.
3.2.   Study of *Cuscuta* spp. as hosts and vectors of plant pathogens.

3. Material and methods

3.1 Plant material

Seed and vegetative material of *Cuscuta campestris*, *C. europaea*, *C. approximata* and *C. epithymum* were collected in the period 2017 - 2022 on the territory of the Republic of Bulgaria. Seeds of the remaining species were provided free of charge by colleagues from China, Serbia and the United Kingdom.

3.2 Methodological approaches

Molecular taxonomy and phylogenetic analysis - the taxonomic status of the samples was confirmed by specific PCR amplification, sequencing and BLAST of partial fragments of the small ribosomal subunit genes ITS1, 5.8S and ITS2, as well as a partial fragment of the large ribosomal subunit gene. Genetic diversity was examined by Random Amplified Polymorphic DNA (RAPD) analysis.

Metagenomic analysis - metagenomic analysis was performed to investigate the diversity of soil microbial communities - by PCR amplification of 16S (for bacteria) and ITS (for fungi) total DNA and NGS sequencing on an Illumina platform.

Transcriptome analysis - differentially expressed genes (DEGs) were identified by NGS sequencing on Illumina platform of iRNA libraries, then DEGs were annotated by GO and KEGG algorithms.

Enzyme activities - Enzyme activities were assayed either by spectrophotometric methods or by zymogram methods after separation on semi-denaturing polyacrylamide gel electrophoresis.

Blue Native/SDS PAGE - a combination of blue native and denaturing electrophoreses were used to separate thylakoid complexes after dissolving in digitonin.

Proteomic analysis - gel-based proteomic analysis was performed by a combination of isoelectric focusing/denaturing electrophoresis, software image analysis and subsequent MALDI TOF/TOF MS/MS identification of differentially presented proteins.

Antibody detection - immunoblot (Western blot) analysis with specific antibodies was applied after SDS PAGE separation and semi-dry transfer onto nitrocellulose membrane or after tissue printing onto nitrocellulose membrane. Specific antigens were detected by DAS-ELISA method.

Metabolomic analysis - Samples were analyzed using an ultra-performance liquid chromatography (UPLC) system and tandem mass spectrometry.

Photosynthetic measurements - the relative chlorophyll content was measured with a CCM-200 apparatus or spectrophotometrically. Photosynthetic parameters were measured with a Li-6400 apparatus. The prompt fluorescence (PF) of Chl a was measured with an MPEA fluorometer.

4. Results

4.1 Distribution, host range and genetic diversity of Cuscuta spp. in Bulgaria

4.1.1 Distribution and host range of *Cuscuta* spp.

In the period 2017-2021 plant material was collected from a total of 54 localities of four of the common species in Bulgaria as follows: *Cuscuta approximata* – 6 pcs., *Cuscuta campestris* - 35 pcs., *C. epithymum* - 9 pcs. and C. europaea - 4 p. (additional Table s4.1.1.1, distribution by floristic regions Fig. 11). All findings were determined morphologically and confirmed by DNA analysis. All sequences have been uploaded to the NCBI GenBank database (Table s4.1.1.2). In the present study, the host range, parasitized by *Cuscuta approximata*, *C. campestris*, *C. epithymum, C. europaea* was characterized (Fig. 13). At this stage, a total of 114 host species have been identified for the species studied, assigned to 87 genera and 33 families, all from the division of angiosperms (Magnoliophyta). The greatest diversity of host species is found among the representatives of the legume family (Fabaceae) - 19 species, followed by the families Asteraceae - 16 species, Lamiaceae and Rosaceae, - 8 species each. Among the hosts, 5 invasive species (*Amaranthus albus, A. retroflexus, Datura stramonium, Erigeron canadensis, Xanthium orientale* subsp. *italicum*), but also endemic species - the Bulgarian endemic *Campanula trojanensis*, were recorded.
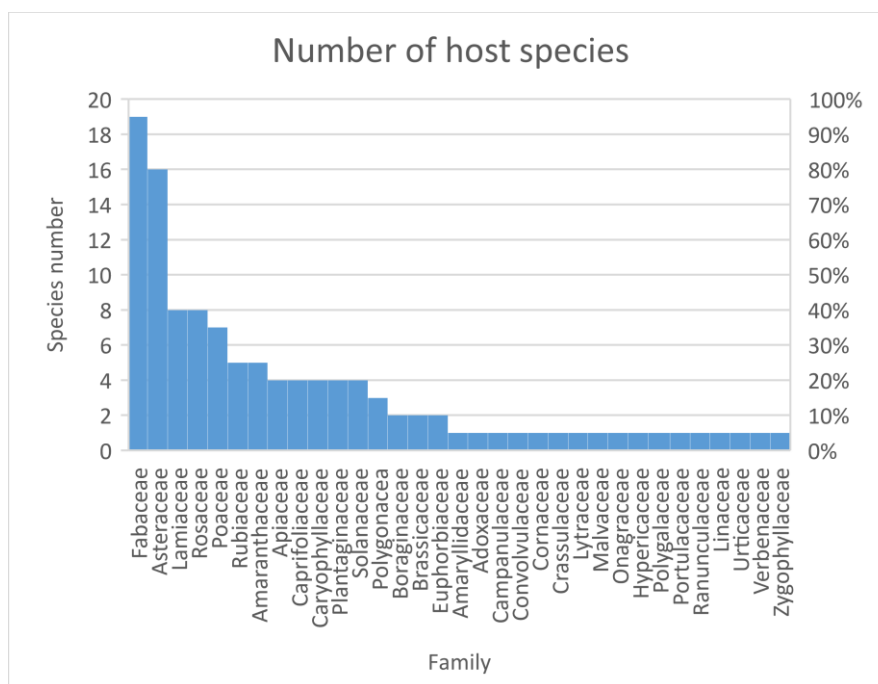

Figure 13. Distribution of identified host species by families, parasitized by *Cuscuta approximata*, *C. campestris*, *C. epithymum*, *C. europaea*

The host distribution of the four *Cuscuta* species by family is given in Figs. 14-17. In the case of *Cuscuta campestris*, the largest number of localities were surveyed (35 pcs.) and the largest number of hosts were found, 52 species from 19 families. In terms of natural distribution and niche overlap, it was found that there was some overlap between the four species in certain floristic regions (Fig. 11). The host overlap analysis is presented as an ecological network (Fig. 18). Although the most frequent hosts are clearly delineated and differ among the four *Cuscuta* species, there is a striking overlap in hosts at the family level, with representatives of Fabaceae, Lamiaceae and Apiaceae being preferred hosts by all four parasite species.

All species identified and collected from the order *Cuscuta* occur in the lowland, pre- and mid-montane vegetation belts. *Cuscuta europaea* is found at the highest altitude, at 1440 m in the Popovi meadows area in the Pirin Mountains. *Cuscuta epythimum* was also found at higher altitude in relation to the higher altitudinal range of its hosts. *Cuscuta campestris* also shows a distribution over a very wide altitudinal range, similar to *C. europaea*. In contrast, the distribution of *C. approximata* is within a relatively narrow range (Fig. 19).

4.1.3 Genetic diversity

Genetic diversity was primarily assessed by ITS sequences. A list of used sequences deposited in GenBank is given in Supplementary Table s4.1.1.2. A phylogenetic tree was constructed using the maximum likelihood method using the available sequences from the rDNA region (Fig. 20). A clear differentiation of *C. campestris* from the other three species was found, which also assemble in well-defined clusters. It is noteworthy that *C. europaea* is relatively similar to *C. approximata*.
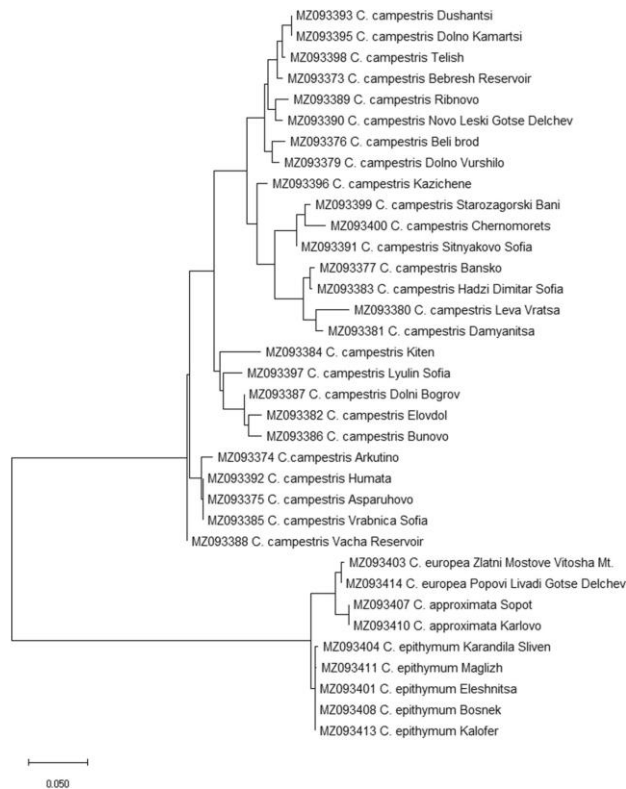
Figure 20. Phylogenetic tree of studied *Cuscuta* populations by maximum likelihood method based on sequenced ITS fragments.

RAPD analysis with 5 primer pairs was applied to detect interpopulation differences. The resulting fragments separated by agarose electrophoresis are attached as Supplementary Figures (s4.1.3.1-15). Table s4.1.3.1 appends the codes used for sample isolation. The results show that significant interspecies differences in RAPD profiles are observed (Fig. 21) and a combination of primers can be used for successful species identification. At the same time, a relatively uniform profile was observed for all primer pairs in *Cuscuta campestris*, whereas the other species showed significant polymorphism in RAPD amplification products. Based on the obtained profiles, a UPGMA phylogenetic tree of selected *Cuscuta campestris* populations was constructed (Fig. 22).
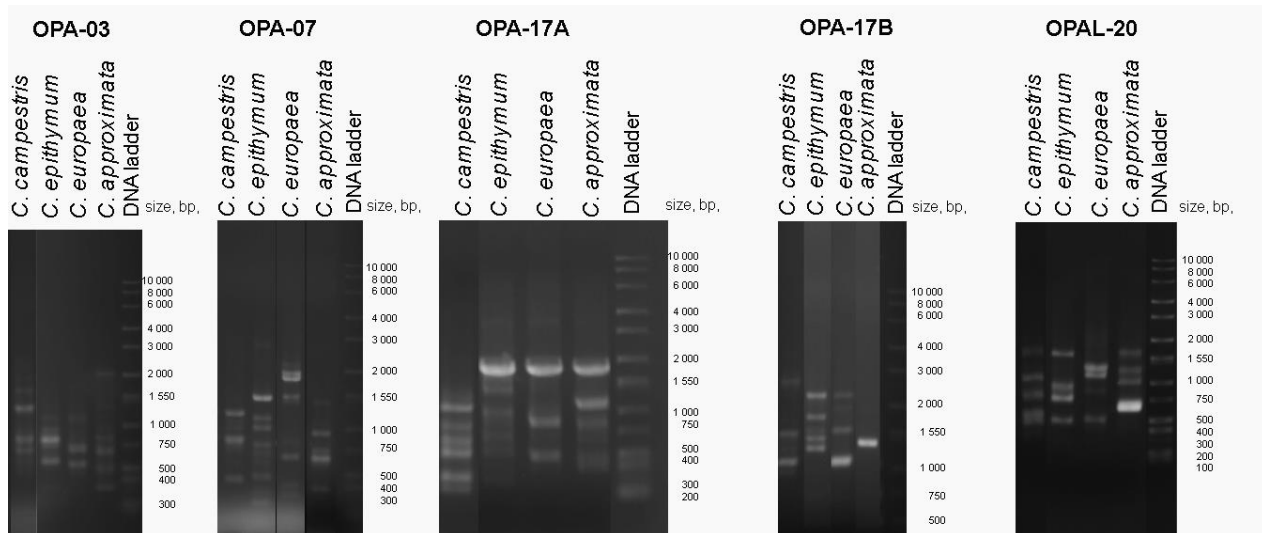
Figure 21. RAPD profiles of the four *Cuscuta* species with five primer pairs.

4.2 Influence of abiotic and biotic factors on germination and developmental stages before host infection

4.2.1 Influence of various factors on germination and subsequent development

The effect of seed age and genotype on germination was studied on seeds from 26 populations collected in the Republic of Serbia in the period 2005-2019. Data on individual populations, ITS sequences and phylogenetic tree are given in Supplementary Table s4.2.1.1. and Fig. 23. The results of the RAPD analysis are summarized in Table 4. Based on RAPD, a UPGMA tree was constructed (Fig. 24), according to which two clusters are clearly distinguishable.

Temperature has a significant effect on seed germination of different populations of *C. campestris*. The highest germination percentage in all populations, ranging from 64% (Cus62) to 9.3% (Cus59), was observed at 28ºC (Table 5). A correlation was found between germination and seed age, with a tendency for germination to decrease with increasing seed age. Testing of different bioclimatic variables using MaxEnt modelling (Ren et al. 2020) for the distribution of *Cuscuta chinensis* shows that it is temperature along with humidity have the greatest influence (Supplementary Figure s4.2.1.2).

The effect of different NaCl concentrations on germination rates was investigated on three *Cuscuta* species and three non-parasitic species of the family. Convolvulaceae. The reduction in germination differed among the species studied (Fig. 25). The statistical significance of the effect of plant species and salt concentration on the final germination rate (10th day) is shown in Table 6. Although the effect of salt concentration was significant at $p \leq 0.01$, the effects of plant species or the combination of species and salinity did not yield statistically significant differences.
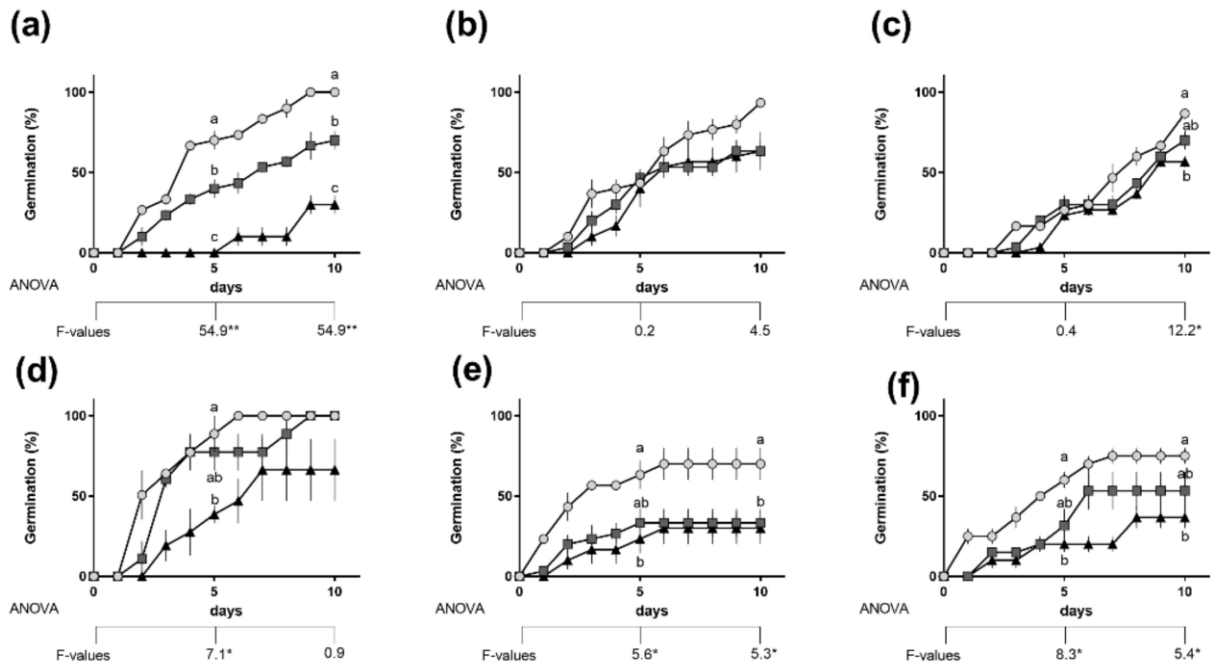
Figure 25. Germination of *Cuscuta campestris* (a), *Cuscuta chinensis* (b), *Cuscuta japonica* (c), *Ipomoea tricolor* (d), *Convolvulus arvensis* (e) and *Calystegia sepium* (f) at 0 (circles), 100 mM (squares) and 200 mM (triangles) NaCl. Different letters indicate statistical significance at p ≤ 0,05 (Tukey's post hoc test), one-way ANOVA (* p ≤ 0.05; ** p ≤ 0,01), n = 3. Levene homogeneity test: p > 0,05. Presented values are means ± SEM. (Published in Zagorchev L. et al. (2021) Plants, 10, 438)

The subsequent growth was followed up to the 7th day after germination and shows a similar inhibitory effect (**Fig. 26, 27**).
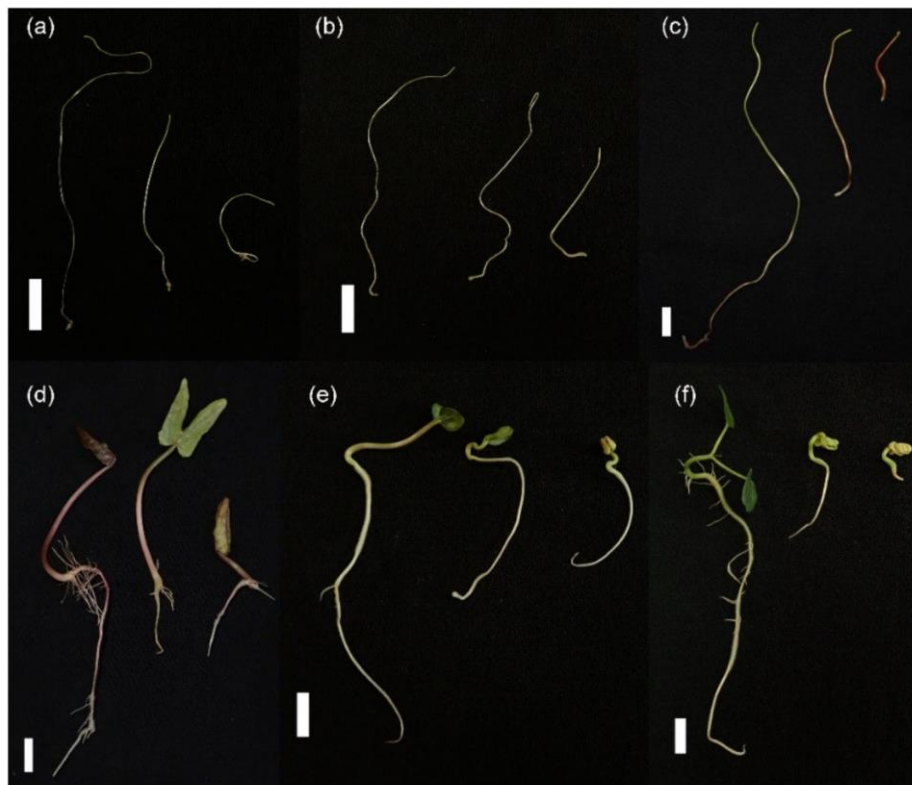
Figure 26. Seven days old *Cuscuta campestris* (**a**), *Cuscuta chinensis* (**b**), *Cuscuta japonica* (**c**), *Ipomoea tricolor* (**d**), *Convolvulus arvensis* (**e**) and *Calystegia sepium* (**f**) at 0, 100 mM and 200 mM NaCl (from left to right). Scalebar = 1 cm. (Published in Zagorchev L. et al. (2021) Plants, 10, 438)

Two major classes of hydrolytic enzymes, amylases and proteases, with possible roles in mobilizing seed reserves, were examined by zymogram analyses at the 24th and 48th hours of germination (Figs. 28 and 29). Growth and survival of germinated parasitic and nonparasitic plants were recorded. Pre-salting has some negative effects on the ability of *Cuscuta* to infect host plants and the periods of infection and secondary stem formation. Pre-emergence under saline conditions is reflected in a longer time to attach to the host (3-4 days delay) and a longer time to secondary stem formation (2-3 days delay). At the same time, about 15% of *C. campestris* germinated at 100 mM NaCl and almost 50% of those at 200 mM NaCl failed to successfully infect host plants. The effect at 100 mM NaCl was similar for *C. chinensis* and *C. japonica*, whereas at 200 mM NaCl both species failed to attach to the host. However, this appears to be the critical stage of parasite development, as further development, defined as the number of plants capable of forming a secondary stem, is not affected by salt pretreatment. A similar inhibition of growth rate was observed for all species at 100 and 200 mM NaCl (Fig. 30). While the reduction in non-parasitic species was in the range of 20-30%, the effect of germination under saline conditions was higher in *Cuscuta*, resulting in over 70% reduction in growth rate compared to controls (Fig. 30).
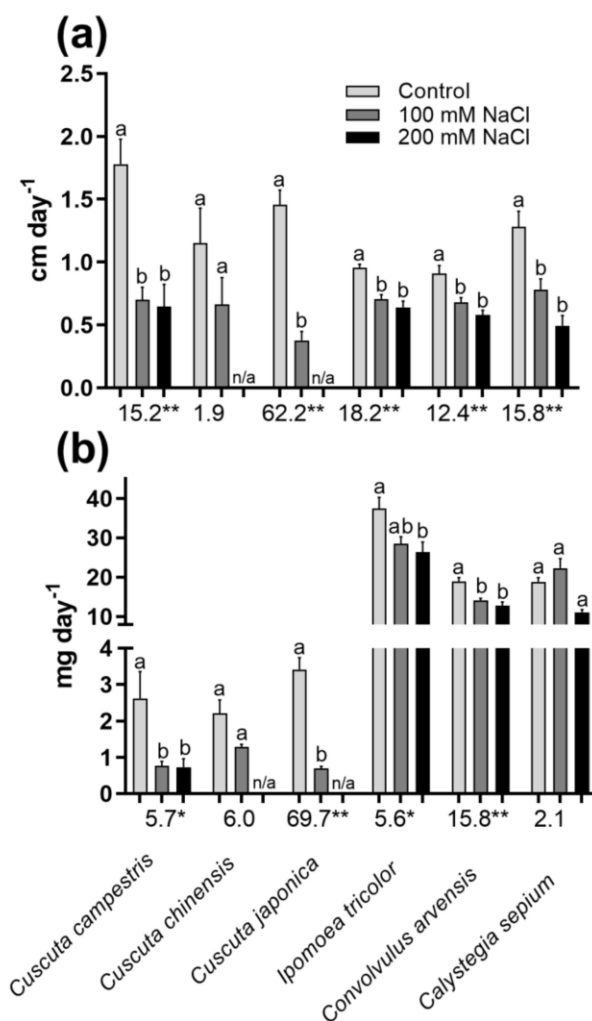
Figure 30. Growth rate in cm day^{-1} (a) and mg day^{-1} (b) of plants germinated at different NaCl concentrations on host *Arabidopsis thaliana* (*Cuscuta* spp.) or directly in soil (other species). Data represent mean values ± SEM. Different letters indicate statistical significance at p < 0.05, Tukey's post hoc test. One-way ANOVA: F-values are given below, * p < 0.05, ** p < 0.01. Levene's homogeneity test: p > 0.05. (Published in Zagorchev L. et al. (2021) Plants, 10, 438)

4.2.2 Influence of host and salinity on haustorium formation

The formation of haustoria is largely dependent on the type of host (Fig. 31). In *Lycopersicum esculentum* (tomato), a characteristic browning is observed in the haustorium area, probably caused by an active host deeeeee response, whereas in *Zea mays* (maize) haustoria do not penetrate the host parenchyma.
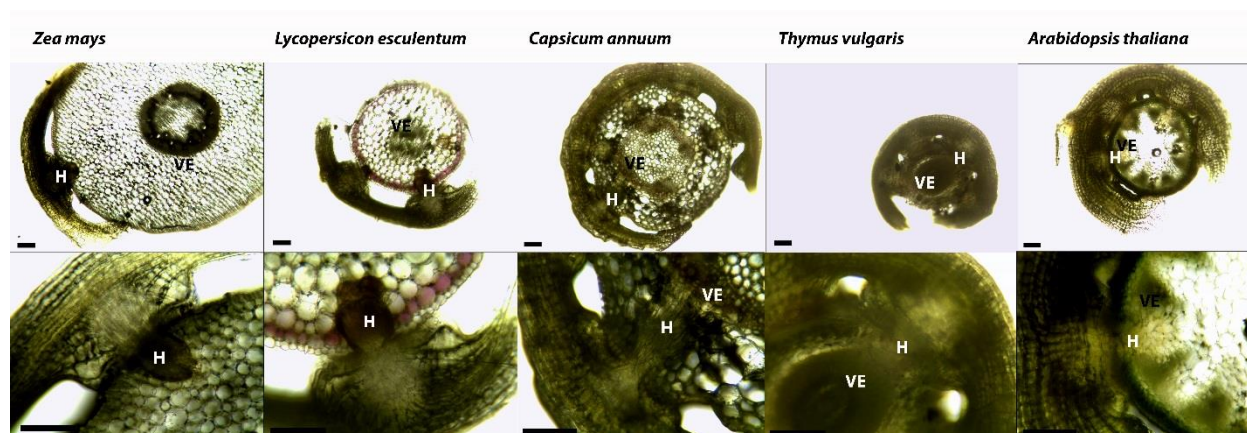

Figure 31. Haustoria formation in *Cuscuta campestris* on different host species. H – haustoria; VE – vascular elements. Scalebar = 200 µm.

Enzymes likely involved in haustorium formation were examined by zymogram analyses of cell wall proteins (Figs. 32-35). The analysis showed that the most pronounced isoforms associated with parasite-host interactions were observed in the macroscopically visible haustorium stage. Immunoblot analyses of carbohydrate epitopes from hydroxyproline-rich proteoglycans at the infection site of compatible (*Arabidopsis*), resistant (tomato), and incompatible (maize) hosts at 0 and 150 mM NaCl. Anti-arabinogalactan antibodies LM2, JIM8, and MAC207 and anti-extensin antibody JIM12 were used (Fig. 36). The results suggest that the three hosts have different arabinogalactan protein profiles, at least some of which are relevant to the salt stress response, to *Cuscuta campestris* infection, or to both.

4.3 Influence of abiotic and biotic factors on parasite development after successful infection

4.3.1 Impact of salinization depending on the host species

The critical stage in the development of *Cuscuta* spp. is the formation of the secondary stem, which determines whether or not the parasite will survive. The influence of a combination of biotic (host species) and abiotic (salinity) factors on parasitic plant development has been traced in hosts, cultivated plants, of different families. In the experiments conducted, none of the tomato cultivars proved to be suitable hosts for *C. campestris* in either control or salinity conditions. The dieback of the parasite was accompanied by a characteristic browning of the host stem, although this resistance was not absolute and successfully developed parasites were observed in 0.57% of infections (Fig. 37). Members of the family Cucurbitaceae also appear to be incompatible hosts in this particular case, although without an apparent protective response (Fig. 37).
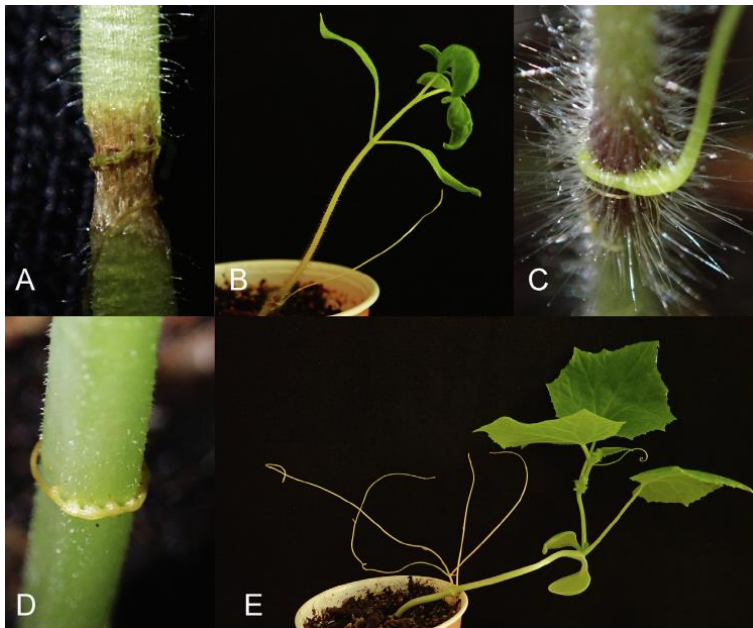
Figure 37. Growth of *Cuscuta campestris* on different hosts. A – incompatible interaction with *Solanum lycopersicum*; B – successful development on *Solanum lycopersicum*; C – overcoming of the defense reaction of *Solanum lycopersicum*; D – incompatible interaction with *Cucumis sativum*; successful development oon *Cucumis sativus*. (Published in Zagorchev L. et. al. (2022) Journal of the Saudi Society of Agricultural Sciences. 21 (5), 324-330)

The survival rate, depending on the host plant, tends to decrease with increasing salt concentration (Table 7). The time frame between haustorium formation and the appearance of secondary stems, or lag phase (Koch et al. 2004), varies substantially among plant families and within a plant. In most of the Fabaceae, the lag phase is no longer than 48 hours. In the Apiaceae, secondary stem emergence takes 6-7 days, whereas in the Solanaceae it is prolonged to 10±2 days. The growth rate of *C. campestris* strongly depends on the host plant, including under control conditions. At the plant family level, median growth rates are comparable when host plants are members of Fabaceae or Apiaceae and decrease with salinity (Fig. 38).
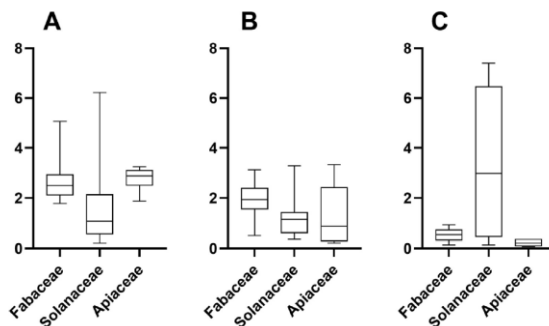
Figure 38. Median growth of *Cuscuta campestris* and distribution of data in dependence on the host family at control (A), 100 mM NaCl (B) and 200 mM NaCl (C) treatment. (Published in Zagorchev L. et. al. (2022) Journal of the Saudi Society of Agricultural Sciences. 21 (5), 324-330)

However, the data are not normally distributed (Fig. 38), but are mainly related to large differences in the growth rate of *C. campestris* depending on species and cultivar (Fig. 39).
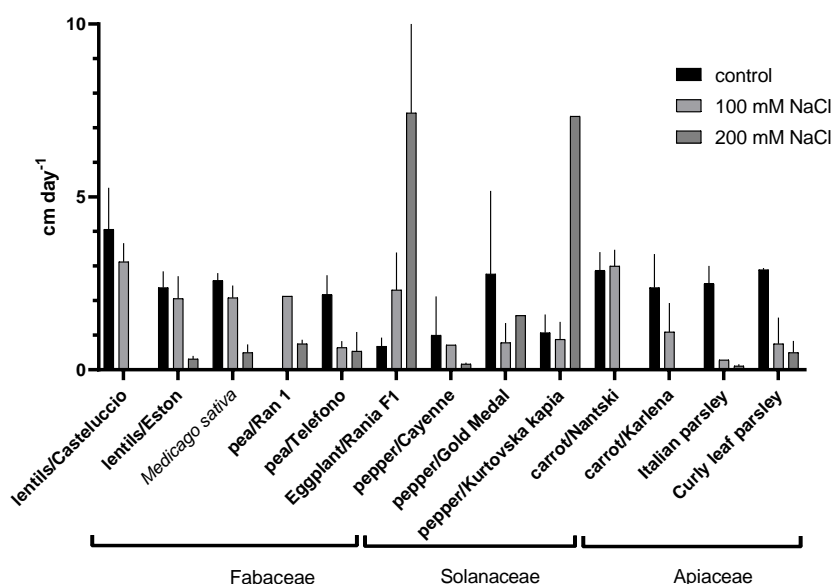
Figure 39. Mean growth in cm day^{-1} of *Cuscuta campestris* on different hosts under different salt concentrations. Mean values ± SEM. (Published in Zagorchev L. et. al. (2022) Journal of the Saudi Society of Agricultural Sciences. 21 (5), 324-330)

Statistical analysis shows that the host plant family is not particularly determinant of growth in *C. campestris*, but has a strong relationship with salinity (Table 8). For example, the effect of abiotic stress is significantly different among members of different families. Within individual families, the effect of elevated salinity significantly affected the growth of *C. campestris* with little dependence on host plant species (Table 8). However, within individual host plant species, cultivar also had a significant effect on parasite growth.

Table 9. Two-way ANOVA of *Cuscuta campestris* growth in cm day^{-1} on different hosts under different salt concentrations. (Published in Zagorchev L. et. al. (2022) Journal of the Saudi Society of Agricultural Sciences. 21 (5), 324-330)

Cases	Sum of Squares	df	Mean Square	F	p
Family	7.483	2	3.742	2.140	0.123
salinity	21.354	2	10.677	6.108	0.003
Family * salinity	79.977	4	19.994	11.437	< 0.001
On species level					
within Fabaceae					
species	3.356	2	1.678	3.773	0.033
salinity	30.877	2	15.439	34.722	< 0.001
species * salinity	4.006	4	1.002	2.253	0.085
within Solanaceae					
species	9.648	1	9.648	2.923	0.098
salinity	50.820	2	25.410	7.699	0.002
species * salinity	23.691	2	11.846	3.589	0.040
within Apiaceae					
species	3.909	1	3.909	7.640	0.012
salinity	13.265	1	13.265	25.925	< 0.001
salinity * species	2.896	1	2.896	5.661	0.027
On cultivar level					
within *Lens culinaris*					
cultivar	7.521	1	7.521	30.723	< 0.001
salinity	4.533	1	4.533	18.516	0.003
cultivar * salinity	0.574	1	0.574	2.346	0.164
within *Pisum sativum*					
cultivar	1.548	1	1.548	17.292	0.001
salinity	7.239	2	3.619	40.441	< 0.001
cultivar * salinity	1.752	2	0.876	9.786	0.003
within *Capsicum annuum*					
cultivar	19.577	2	9.788	9.403	0.002
salinity	14.020	2	7.010	6.734	0.007
cultivar * salinity	52.550	4	13.138	12.620	< 0.001
within *Daucus carota*					
cultivar	3.688	1	3.688	12.107	0.008
salinity	1.882	1	1.882	6.179	0.038
cultivar * salinity	2.644	1	2.644	8.679	0.019
within *Petroselinum crispum*					
variety	0.637	1	0.637	8.936	0.011
salinity	22.243	2	11.122	156.064	< 0.001
variety * salinity	0.055	2	0.027	0.383	0.690

The concentration of L-proline was also measured in *Cuscuta campestris* at different host and salt concentrations (Fig. 40). There was a general trend of increasing concentration in proportion to the applied salt stress, which, however, was not related to growth rates. In an experimental host system, *Arabidopsis thaliana* treated with 0 (NS - no salt), 50 (LS - low salt) and 150 (HS - high salt) mM NaCl, the antioxidant enzymes peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) as well as L-proline concentration were examined in the parasite *Cuscuta campestris*. The results are presented in Figure 41.
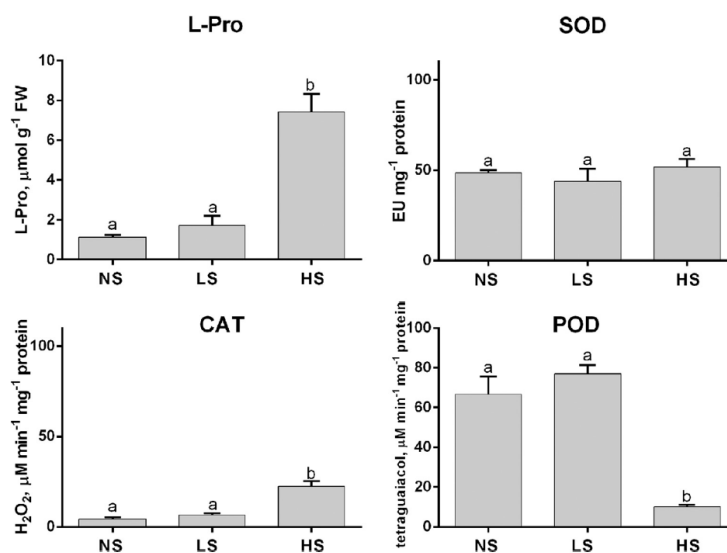
Figure 41. L-proline (L-Pro) concentration and activities of superoxide dismutases (SOD), catalases (CAT), and guaiacol peroxidases (POD) in *Cuscuta campestris* parasitizing *Arabidopsis* at increasing salt concentrations. Columns show mean +/- SEM values (n = 5). Different letters indicate significant differences (P < 0.05), Tukey's post-hoc test. (Published in Zagorchev L. et. al. (2018) Plant Physiology and Biochemistry. 132: 408-414.)

Although not in direct contact with the soil, exposure of the host plant to salinity also resulted in significant changes in L-Pro concentrations and CAT and POD activity in the parasite (Fig. 41). All changes induced by salt concentration, except SOD, were statistically significant according to one-way ANOVA (Table 10). Additionally, the accumulation of L-proline (Fig. 42) was examined in *Cuscuta campestris* and *Cuscuta europaea* on salt-tolerant (*Thellungiella salsuginea*) and salt-sensitive (*Arabidopsis thaliana*) hosts. A concentration of 300 mM NaCl was applied, which was sub-lethal for *Arabidopsis* and almost completely inhibited growth but did not significantly affect *Thellungiella* (Ghars et al. 2008).

4.3.2 Effect of salinization on the metabolome

The studies were conducted on the model plant pair *Arabidopsis thaliana* (glycophyte) and *Eutrema salsugineum* (halophyte = *Thellungiella salsuginea*) treated with 0, 100 and 200 mM NaCl and infected with *Cuscuta chinensis*. Targeted metabolomic analysis of the parasitic plant was performed by UPLC-MS/MS analysis. A total of 538 metabolites were detected which were distributed as follows: 1) flavonoids, 104; alkaloids, 29; lignans and coumarins, 13; phenolic acids, 100; lipids, 78; amino acids and their derivatives, 65; quinones, 3; organic acids, 104; nucleotides and their derivatives, 88; steroids, 1; others, 64. Differentially accumulated metabolites between treatments were relatively few in number (Table 11). Two main trends were observed, with relatively few differential metabolites between salt treatments in the same host, in the range of 10-20 in *Arabidopsis* (CA) and between 20 and 60 in *Thellungiella* (CT), but significantly higher numbers, between 160 and 180 between different hosts in the same salt treatment.

Table 11 Differential metabolites (increased or decreased) in *Cuscuta chinensis* on host *Arabidopsis* (CA) and *Thellungiella* (CT) treated with 0, 100 and 200 mM NaCl.

group name	All sig diff	down regulated	up regulated
CA0_vs_CA100	21	12	9
CA0_vs_CA200	19	9	10
CA100_vs_CA200	13	8	5
CA0_vs_CT0	181	93	88
CA100_vs_CT100	168	68	100
CA200_vs_CT200	166	73	93
CT0_vs_CT100	55	20	35
CT0_vs_CT200	57	26	31
CT100_vs_CT200	24	17	7

From the results obtained, it can be concluded that the influence of the host, even in closely related species on the metabolic profile of the parasitic plant is significant. However, the metabolic profile may also depend to a considerable extent on environmental conditions (Ye et al. 2002), which is particularly true for flavonoids in *C. chinensis* and is monitored by the influence of salinity on this profile. This trend is evident from PCA analysis (Fig. 43) and cluster analysis (Fig. 44).
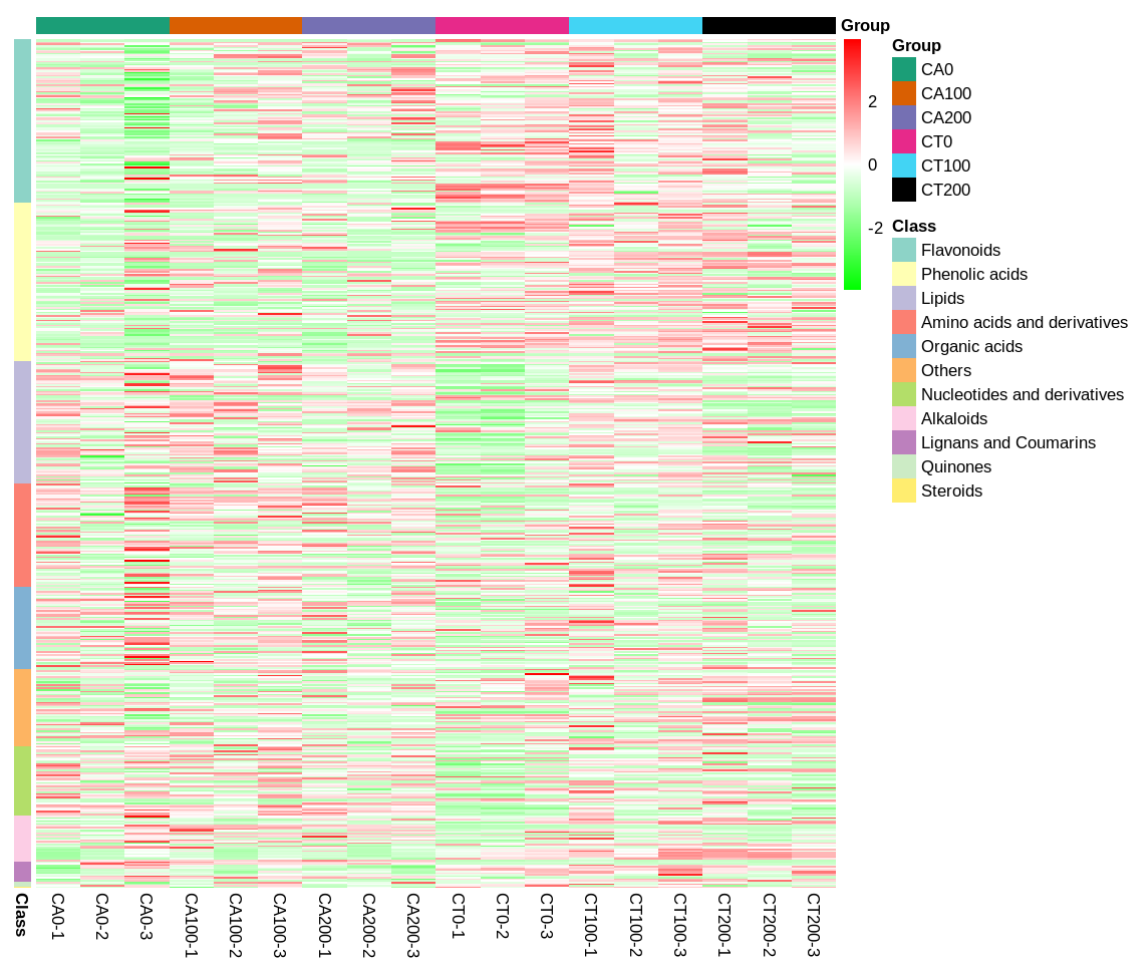
Figure 44. Heat map of cluster analysis of individual metabolite groups with distribution between samples in Cuscuta chinensis on Arabidopsis (CA) and Thellungiella (CT) hosts at 0, 100 and 200 mM NaCl.

Differential metabolites between individual samples are appended in Supplementary Tables s4.3.2.1-9. Comparative analysis between groups - number of differential metabolites between different salt concentrations in one host compared to number of differential metabolites between different salt concentrations in the other host are presented as Venn diagrams (Fig. 45).

4.4 Impact of Cuscuta parasitism on host metabolism and soil microcommunities

4.4.1 Influence of *Cuscuta* parasitism on the host proteome

Changes in the host proteome were examined in *Arabidopsis thaliana* infected with *Cuscuta australis*. The differential amount of proteins was assessed by two-dimensional polyacrylamide gel electrophoresis, separately in the stem and leaves of host plants (Fig. 46 a,b and Supplemental Figs. s4.4.1.1-4). A total of 21 protein spots in leaves and 24 protein spots in stem showed an increase or decrease ≥ 2.5-fold (Fig. 47).
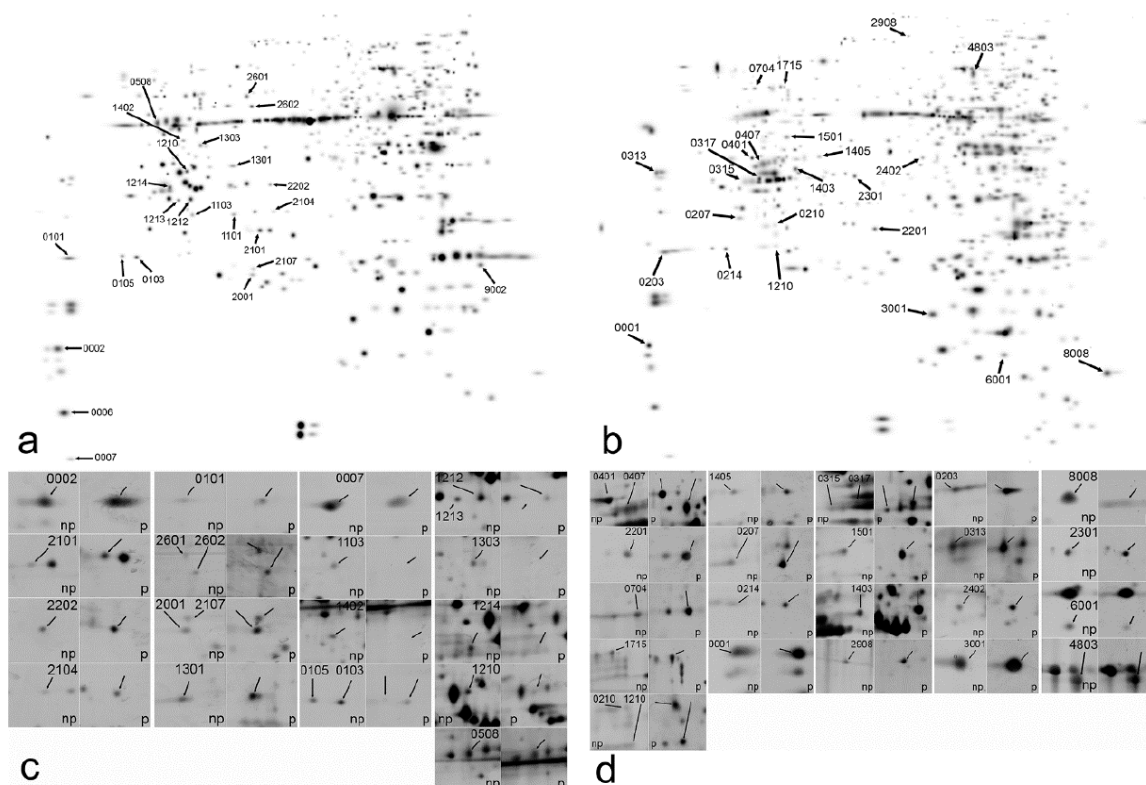
Figure 46. PDQuest master gel image of 2D-PAGEs of leaves (a) and stem (b) of *Arabidopsis* and the corresponding differentially regulated protein spots (c and d). *Arabidopsis* is either parasitized (p) or nonparasitized (np) by *Cuscuta australis.* Protein spot numbers correspond to those automatically assigned by PDQuest. (Published in Zagorchev L. et al. (2022) Plants. 11, 2904).

A total of five protein spots in the leaves and two in the stem were not identified. Of the protein spots identified (Tables s4.4.1.1 and s4.4.1.2), one protein was detected in a total of four reduced spots in leaves: glyceraldehyde-3-phosphate dehydrogenase (SSPs 1210, 1212, 1213, and 1303). One protein was detected in two protein spots with increased expression in the stem: Atmp 24.1 glutathione S transferase (SSP 2201 and 3001), but also two isoforms of aspartate aminotransferase were detected in two spots with increased expression (SSP 0315 and 1403). Chloroplast transketolase 1 was detected in spots up-regulated in both leaves and stem (SSP 2601 and 1715, respectively), and chloroplast phosphoribulokinase was detected in spots down-regulated in both leaves and stem (SSP 0508 and 8008, respectively). A member of the glycine-shearing T-protein family was detected in a down-regulated spot in leaves (SSP 0105) and an up-regulated spot in the stem (SSP 0407). According to the Gene Ontology (GO) biological process

annotation, proteins associated with different types of stress, including biotic (response to bacterial pathogens) and abiotic (salinity, cold, heavy metals), were evenly distributed between both up- and down-regulated spots (Tables s4.4.1.1 and s4.4.1.2). However, a significant fraction of these are related to either photosynthesis or carbohydrate metabolism.

4.4.2. Influence on host photosynthetic activity

Two types of experiments were conducted to establish this effect. In one experiment, the model plant *Arabidopsis thaliana* was subjected to parasitism by *Cuscuta australis*. After 72 h of infection, documented by haustorium formation macroscopically and microscopically (Fig. 48), a significant reduction in most photosynthetic parameters was observed in the host (Table 12). Notable was the nearly twofold reduction in chlorophyll content, as well as statistically significant reductions in Pn, gs, and Tr. Changes in Ci were not statistically significant but followed the trend of decrease.

Table 12 Effect of parasitism on host chlorophyll content and photosynthetic characteristics. Data are shown as mean ± standard error (n = 6). Different lowercase letters indicate statistically significant (Student's t-test) difference between parasitized and nonparasitized plants. (Published in Zagorchev L. et al. (2022) Plants. 11, 2904)

	Non-parasitized	Parasitized
Photosynthetic rate (P_N) /(μmol CO_2 m^{-2} s^{-1})	**3.61 ± 0.73a**	2.71 ± 0.33b
Stomatal conductance (g_S) /(μmol H_2O m^{-2} s^{-1})	**0.19 ± 0.05a**	0.12 ± 0.01b
Concentration of intercellular CO_2 (C_i) /(μmol CO_2 m^{-2} s^{-1})	305.66 ± 1.34a	301.58 ± 6.23a
Transpiration rate (T_s) /(μmol CO_2 m^{-2} s^{-1})	**3.30 ± 0.74a**	2.31 ± 0.097b
Relative chlorophyll content	**17.51 ± 2.06a**	8.79 ± 1.96b

In the second group of experiments, the incompatible host *Ipomoea tricolor* Cav. (Solanales, Convolvulaceae) was used as a host for *Cuscuta campestris*. Although successful infection and haustorium formation occurred with this host (Fig. 49), subsequent development of the parasitic plant was slow.

Differences in the shape of the chlorophyll fluorescence induction curves in the characteristic phases appear as specific peaks in the differential curves shown in Supplementary Figs. s4.2.2.1-4. The O, J, I, and P characteristic phases of Chl a fluorescence were used to calculate 17 parameters of the JIP assay (Fig. 50) and structural and functional changes in the photosynthetic apparatus. The JIP-test parameters provide a better visualization of the effects of C infection. campestris on the leaves of I. tricolor of different ages (L1, L2 and L3).

In all three leaf types, the parameters PSII performance index (PI$_{ABS}$) and performance index of both photosystems (PI$_{total}$) were very sensitive to infection with *C. campestris*. Some inactivation was observed in the oldest leaves L1 and L2, with PI$_{ABS}$ values decreasing compared to day 16. The responses of L3 to infection were reversed, i.e. values increased compared to the first measurement on day 18, indicating activation of PSII in the presence of the parasite. PI$_{total}$ showed some activation of PSI following infection, on days 28 and 32 for L1, on days 21-28 for L2 and on days 21 and 25 for L3.
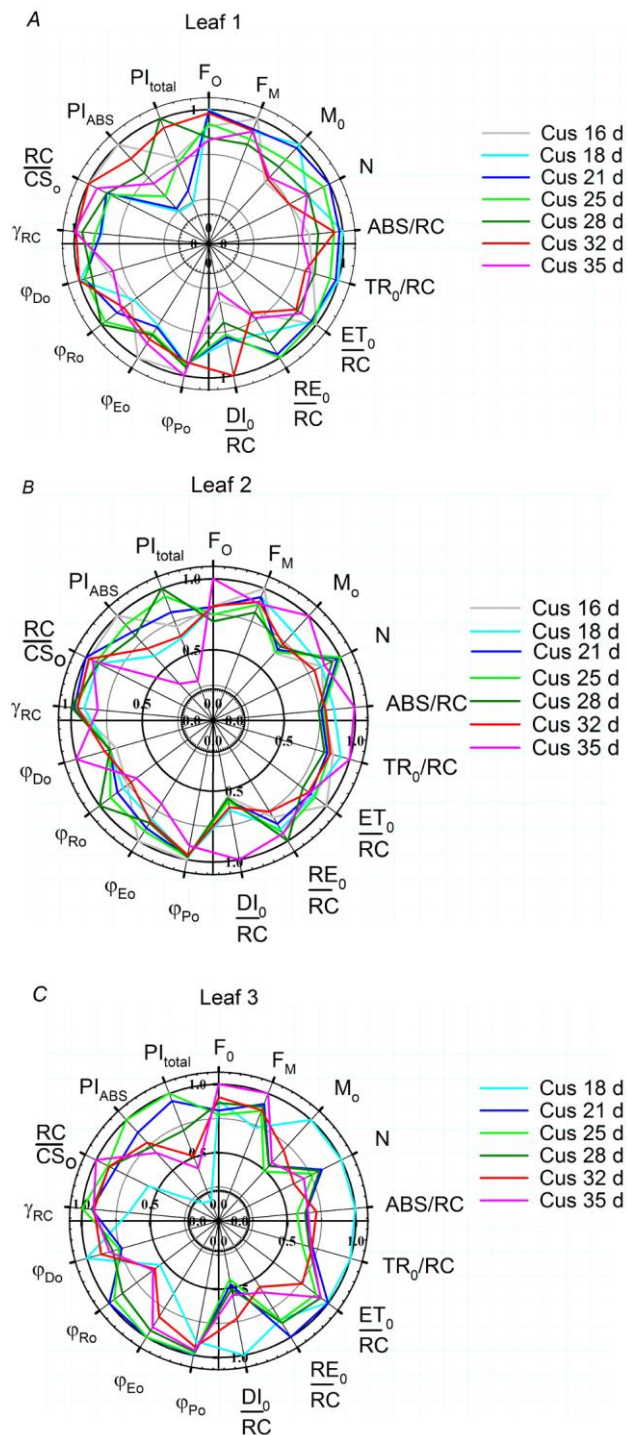
Figure 50. JIP assay parameters in leaves of different ages (L1, L2, and L3) calculated from the Chl a fast fluorescence induction curves as the ratio between the parameter value for *Ipomoea tricolor* infected with *Cuscuta campestris* and the same parameter value on the same day for control plants. The ratios thus obtained for a given parameter were normalized to the maximum value of that parameter. (Published in Zagorchev L. et. al. (2020) Photosynthetica. 58 (SI): 237-247.

4.4.3. Influence on the host transcriptome

Transcriptome analysis was initially performed on *Trifolium repens* plants infected with *Cuscuta australis*. Normalized unigene values were used for differential expression analysis with a threshold of log2(fold change) > 1 and p < 0.05 (Love et al. 2014). A total of 1 601 differentially expressed genes (DEGs) were identified between infected and control hosts, including 945 DEGs with increased expression and 656 DEGs with decreased expression. GO and KEGG enrichment analyses were performed to establish function (Figs. 51 and 52).

DEGs with increased expression from the biological process category were those for nitrogen metabolism (GO:0006807), cellular nitrogen metabolism (GO:0034641), and biosynthetic

processes (GO:0009058). Among the molecular functions, DEGs with increased expression are mainly related to the activity of structural molecules (GO:0005198), structural part of ribosome (GO:0003735) and oxidoreductase activity (GO:0016491). KEGG pathway analysis showed that the 105 DEGs with increased expression between infected and control hosts were significantly enriched for genes in the ribosome pathway (ko03010; Fig. 52A), whereas the 15 DEGs with decreased expression were significantly enriched for genes in the plant-pathogen interaction pathway (ko04626; Fig. 52B).
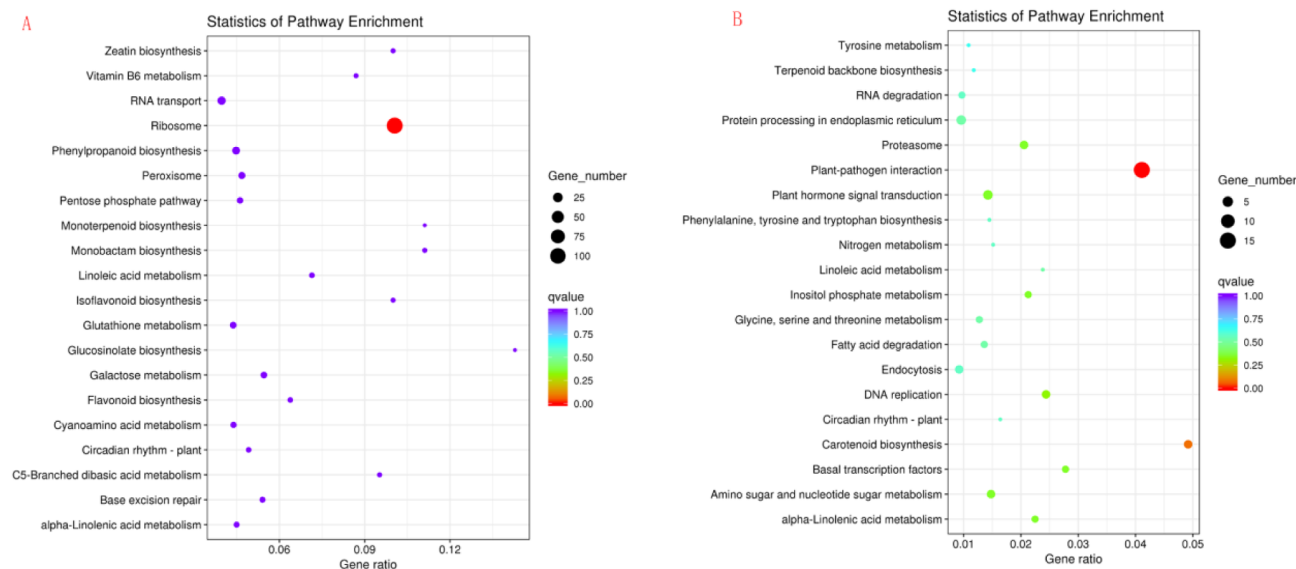
Figure 52. Enriched differentially expressed genes (DEGs) between infected (P) and uninfected (CK) hosts based on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. (A) DEGs with increased expression. (B) DEGs with decreased expression. (Published in Zhou L. et al. (2021) Scientia Horticulturae. 289. 110458)

Functional annotation of the genes indicated that 15 DEGs with reduced expression fell into a specific category of plant-pathogen interaction, including CML, CALM, CNGF, RPM1 (RPS3), EDS1, FLS2, and PR1 (Table 13). In addition, 1224 of the DEGs were annotated as transcription factors and correspond to different transcription factor families. The most highly represented transcription factor families are MYB, WRKY, and bHLH, with 89, 61, and 58 genes, respectively (Fig. 53).

Table 13. Differentially expressed genes (DEGs) enriched in plant-pathogen interaction pathways in response to *Cuscuta australis* parasitism. (Published in Zhou L. et al. (2021) Scientia Horticulturae. 289. 110458)

Gene ID	KO ID	KO Name	Gene Annotation
Cluster-16854.14882	K13448	CML	calcium-binding protein CML
Cluster-16854.18259	K02183	CALM	calmodulin
Cluster-16854.7818	K05391	CNGF	cyclic nucleotide gated channel
Cluster-34712.0	K05391	CNGF	cyclic nucleotide gated channel
Cluster-16854.9195	K05391	CNGF	cyclic nucleotide gated channel
Cluster-35121.0	K13457	RPM1, RPS3	disease resistance protein RPM1
Cluster-16854.8014	K13457	RPM1, RPS3	disease resistance protein RPM1
Cluster-16854.8015	K13457	RPM1, RPS3	disease resistance protein RPM1
Cluster-16854.8017	K13457	RPM1, RPS3	disease resistance protein RPM1
Cluster-16854.8021	K13457	RPM1, RPS3	disease resistance protein RPM1
Cluster-16854.7230	K18875	EDS1	enhanced disease susceptibility 1 protein
Cluster-16854.7576	K13420	FLS2	LRR receptor-like serine/threonine-protein kinase FLS2
Cluster-16854.7583	K13420	FLS2	LRR receptor-like serine/threonine-protein kinase FLS2
Cluster-16854.6611	K13420	FLS2	LRR receptor-like serine/threonine-protein kinase FLS2
Cluster-16854.12166	K13449	PR1	pathogenesis-related protein 1

Similar analysis was performed on *Arabidopsis*, infected with *Cuscuta chinensis* A total of 2 216 DEG were found, of which 1 411 up-regulated and 805 down-regulated (**Fig. 54**).

Figure 54. DEG in parasitized (AC0) vs. non-parasitized (A0) *Arabidopsis*.

GO (Fig. 55) and KEGG (Fig. 56) analysis show significantly different results from the previous case. The largest number of DEGs with increased expression between infected and control hosts were significantly enriched for genes involved in signal transduction of hormonal stimuli, chlorophyll biosynthesis, and the light phase of photosynthesis (Fig. 56A), whereas the major DEGs with decreased expression were significantly enriched for genes in the plant-pathogen interaction pathway (ko04626; Fig. 56B).
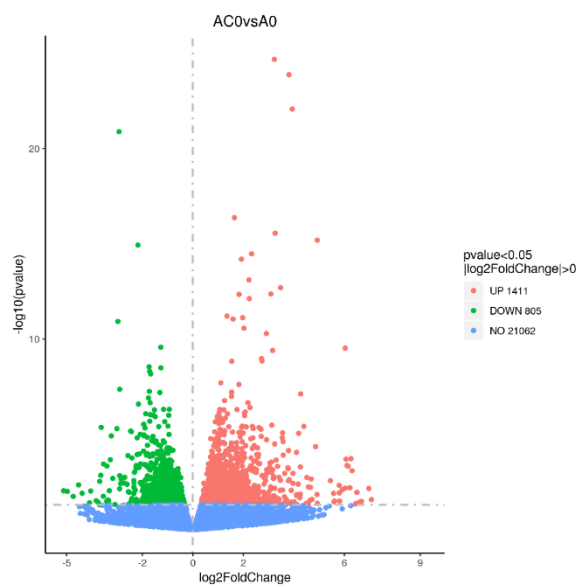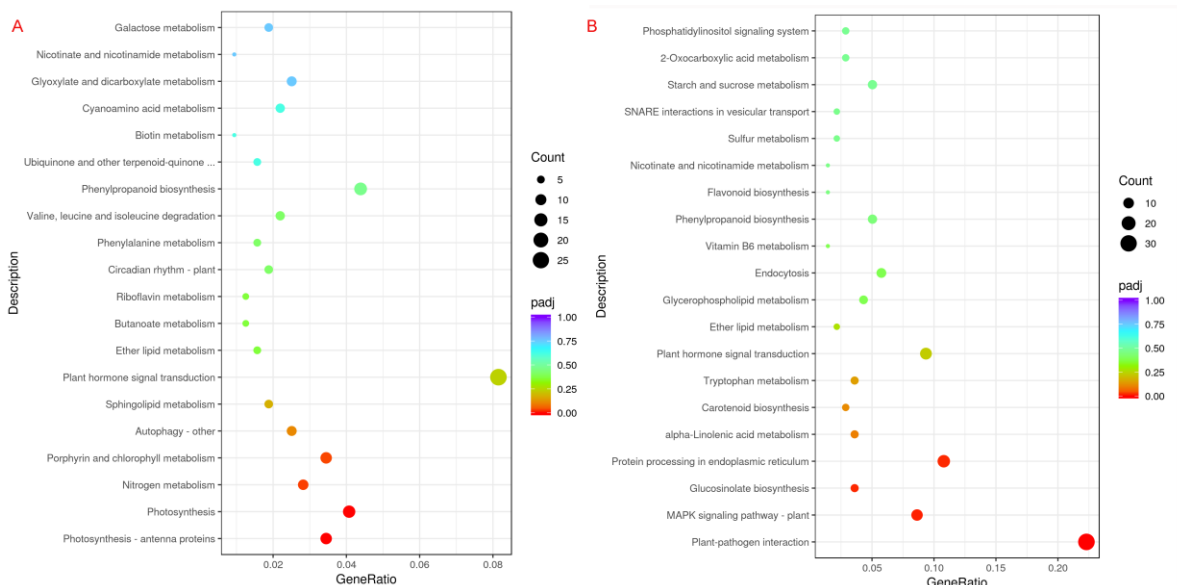
Figure 56. KEGG analysis of differentially expressed genes in infected (AC0) versus uninfected (A0) *Arabidopsis*.

4.4.4 Interfering with the response to abiotic stress

The impact of infection with *C. campestris* on the four studied biochemical parameters in the aboveground host tissues was dependent on the distance to infection (Fig. 57). L-Proline concentrations were measured separately in uninfected *Arabidopsis* (niA), uninfected leaves of infected *Arabidopsis* plants (iA), and infected leaves (iL), whereas SOD, CAT, and POD activity was further measured in the zone of haustorium formation, i.e. the site of infection (iS). There was a slight increase in L-Pro concentration in infected *Arabidopsis* (Fig. 57), which, however, was not significant (ANOVA, Table 14). In contrast to L-Pro, the activity of all three antioxidant enzymes increased significantly toward the site of infection (iS > iL > iA > niA). ANOVA analysis indicated that enzyme activities in relation to infection were generally more significant in iS than in iL and insignificant in uninfected leaves of infected *Arabidopsis* (Table 14).
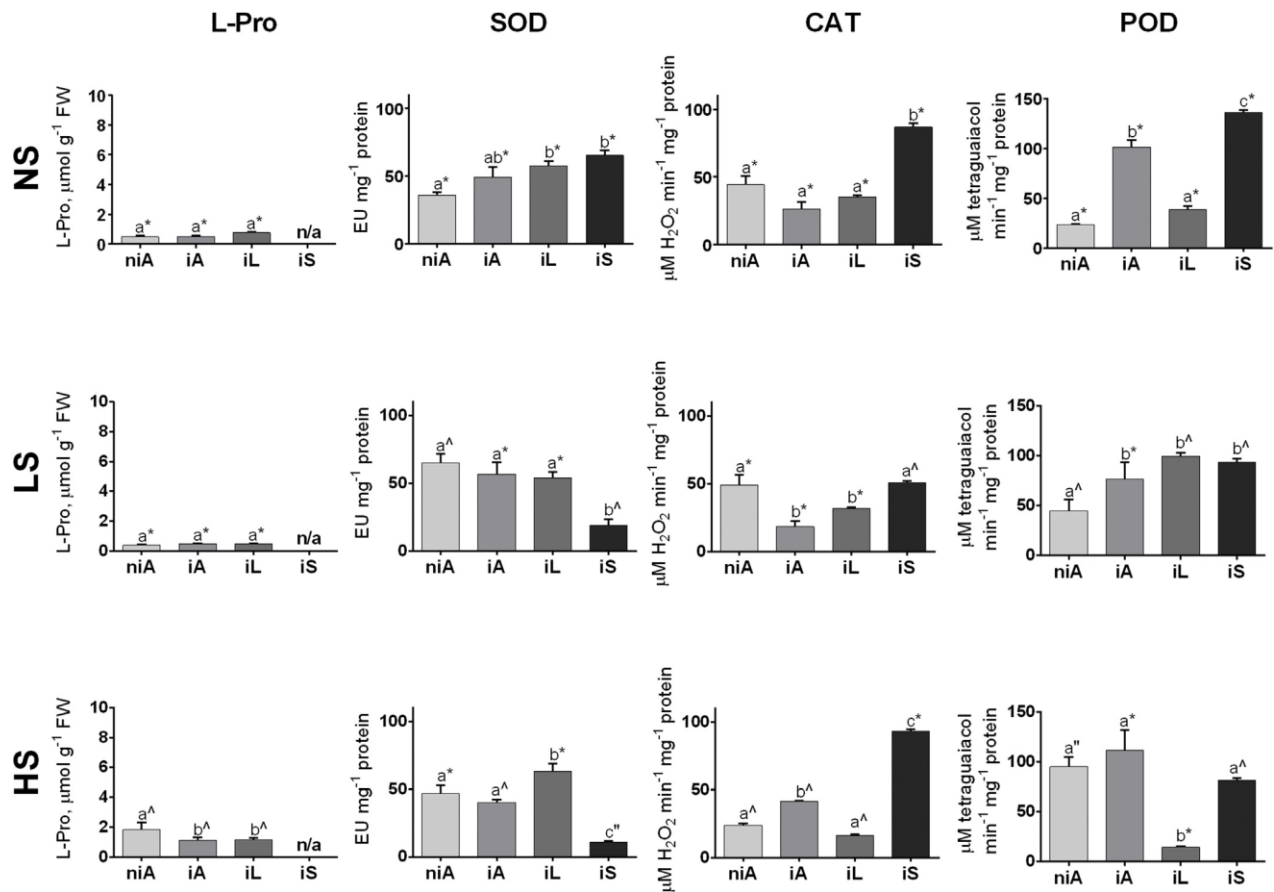
Figure 57. L-proline (L-Pro) concentration, superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (POD) activity in leaves of uninfected *Arabidopsis* plants (niA), uninfected leaves of infected *Arabidopsis* (iA), infected leaves (iL), and infection site (iS) in control (NS), low-salt (LS), and high-salt (HS) treatments. Columns represent means ± SEM (n = 5). Different letters indicate significant differences at P < 0.05 within panel, Tukey's post-hoc test. Different symbols indicate significant differences at P < 0.05 between different salt treatments of the same sample type, Tukey's post-hoc test. (Published in Zagorchev L. et. al. (2018) Plant Physiology and Biochemistry. 132: 408-414.)

The effect of parasitism of *C. campestris* on L-Pro accumulation and antioxidant enzyme activity in roots of infected *Arabidopsis* is presented in Figure 58. All four stress markers increased under NS in infected plants. This trend was maintained for CAT and SOD under salinity treatment (Fig. 58), whereas the concentration of L-Pro and the activity of POD in infected plants was lower compared with noninfected plants under both LS and HS treatments.

4.4.5. Impact on soil communities

4.4.5.1 Metagenomic analysis of soil communities

Effective operational taxonomic units OTUs, Chao 1 score and Shannon index of soil bacterial and fungal communities of *Alternanthera philoxeroides*, infected or not with *C. australis* are presented in Table 15. Parasitism did not significantly affect the relative abundance of major bacterial genera and classes in the rhizosphere soil of *A. philoxeroides* (Fig. 59). However, parasitism significantly increased the relative abundance of Acidobacteria Gp4 and Acidobacteria Gp9 at the order level in the rhizosphere soil. Parasitism significantly reduced the relative abundance of OTUs of the bacterial genera Rhizocola, Pseudoxanthomonas, and Craurococcus, while increasing the abundance of Rhodospirillales, Latescibacteria, and unidentified genera (Fig. 60).
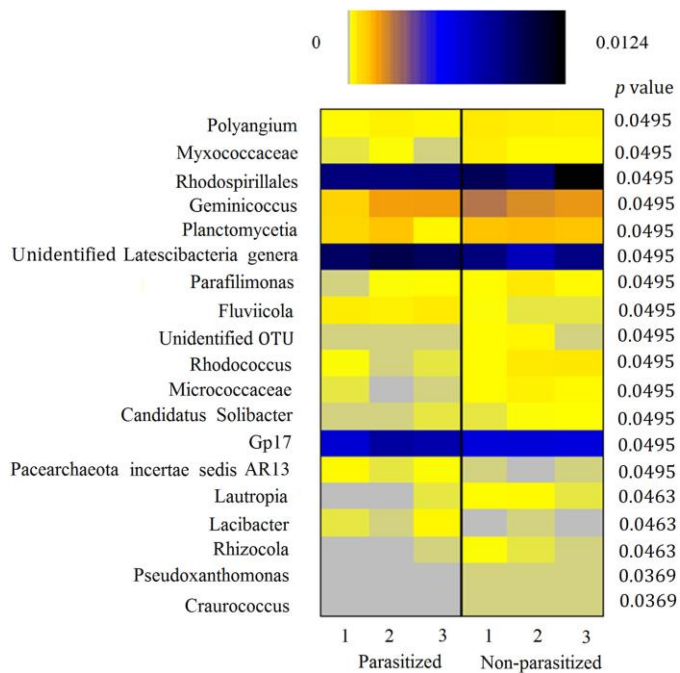
Figure 60. Heatmap showing the significant differences (P < 0.05) in the amount of bacterial taxa in *Alternanthera philoxeroides* rhizosphere soil between parasitized and unparasitized plants. The different colours indicate the relative amount of different OTUs (%). (Published in Yang B. et al. (2019) Applied Soil Ecology. 143: 1-9.)

Similarly, parasitism did not significantly affect the relative abundance of major fungal genera and classes in the rhizosphere soil of A. philoxeroides (Fig. 61), but significantly increased the relative abundance of Trichosphaeriales at the order level. Parasitism significantly reduced the abundance of the fungal genera Piriformospora, Orbiliaceae, Xylomyces and Devriesia, while increasing the abundance of Helotiales, Preussia and Davidiella (Fig. 62).
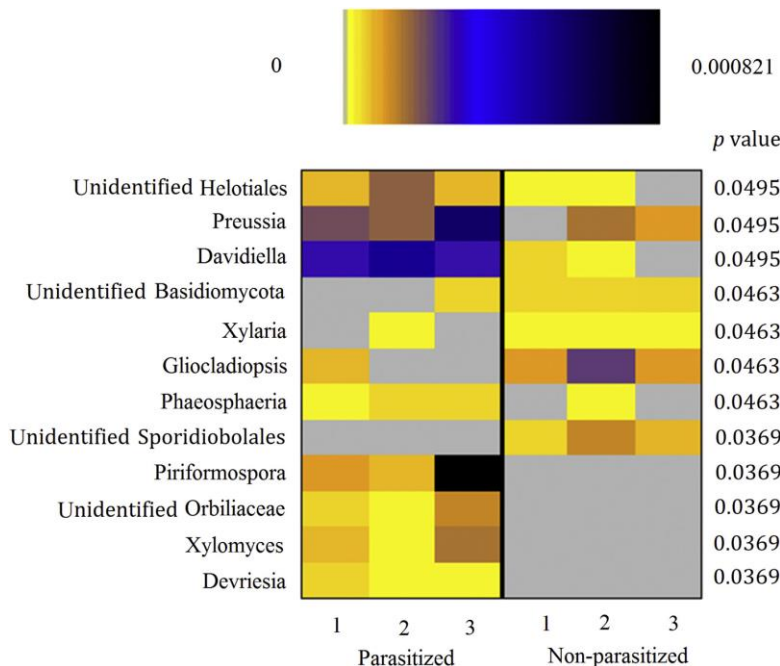
Figure 62. Heatmap showing the significant differences (P < 0.05) in the number of fungal taxa in the rhizosphere soil of *Alternanthera philoxeroides* between parasitized and unparasitized plants. Different colors indicate relative amount of different OTUs (%). (Published in Yang B. et al. (2019) Applied Soil Ecology. 143: 1-9.)

4.4.5.2 Soil enzymes

Four soil enzymes were investigated to assess the effect of the parasitic plant on soil properties - sulphorylases, glucosaminidases, acid phosphatases and beta-glucosidases in soil of *C. campestris* -infected *Medicago sativa* under laboratory conditions. The enzyme activities are presented in Figure 64.
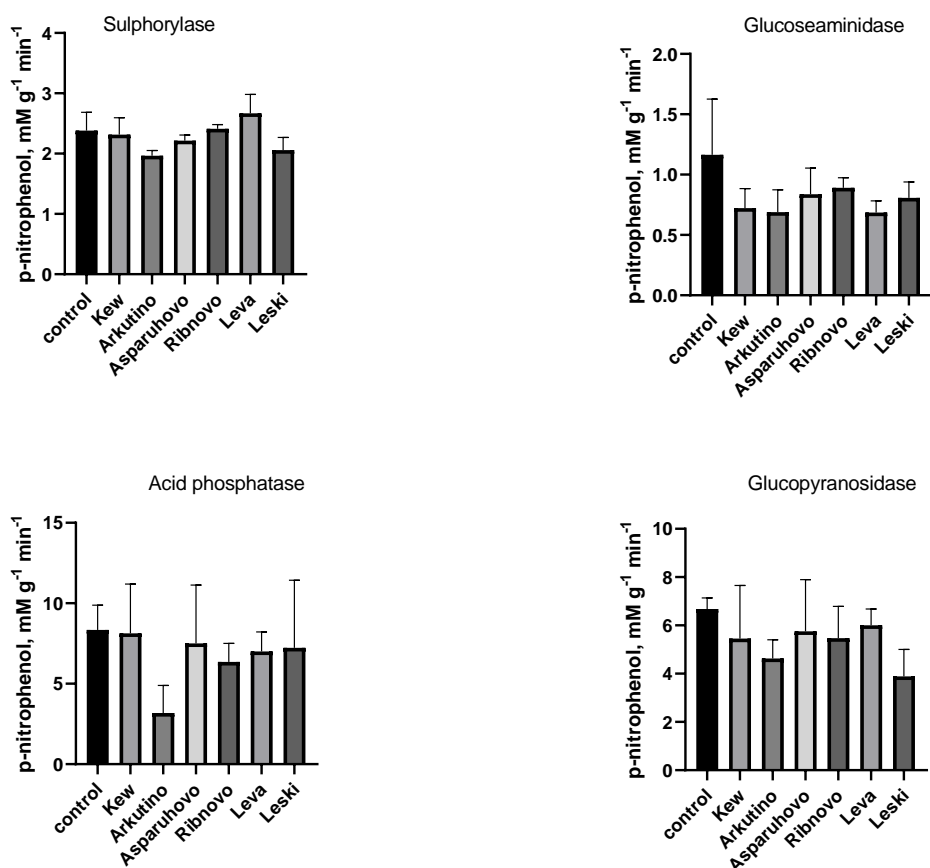
Figure 64. Soil enzyme activities in the rhizosphere of *Medicago sativa* infected with *Cuscuta campestris* from different populations.

There is a tendency towards a decrease in the activity of all four enzymes, which leads to a lower exchange of organic matter and minerals in the soil and consequently to a lower availability of phosphate, sulphate and nitrogen compounds, which is undoubtedly a negative effect. The observed differences between populations seem to be related to the fresh mass of the parasitic plant (Fig. 63). The correlation is inversely proportional - the lowest activities are observed when the parasite is least developed.

4.5 Interaction of the parasite-host pair with other biotic factors

4.5.1 Epiparasitic gall-forming insects of the genus Smicronyx

Smicronyx spp. larvae cause the formation of characteristic spherical galls (Zhekova et al. 2014). *Smicronyx* sp. galls are found in about 10% of *C. campestris* but are not found in other species of the genus nor on host plants. The average size of galls is 1.5-2.5 cm long and 0.5-0.8

cm wide, usually fusiform according to the morphotype summarized by Isaias et al. (Isaias et al. 2013) or irregularly shaped (Fig. 65 a) with a larva inside (Fig. 65 b).
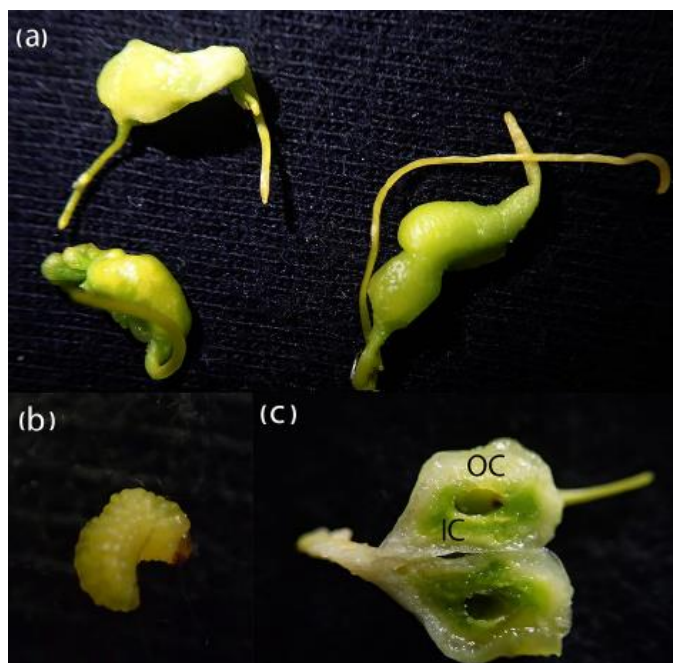
Figure 65. Galls of *Smicronyx* (a) and larva (b), found on *Cuscuta campestris*. The longitudinal section (c) reveals two cortices around the larval chamber, greenish inner cortex (IC) and yellowish outer cortex (OC). (Published in Zagorchev L. et al. (2018) Planta. 248 (3): 591-599)

4.5.1.1 Photosynthetic activity of galls

A transverse section of the galls reveals two distinct areas, a greenish inner cortex around the larva and a pale yellow outer cortex (Fig. 65). Chlorophyll concentration was significantly higher in the galls compared to the stem (Fig. 66).
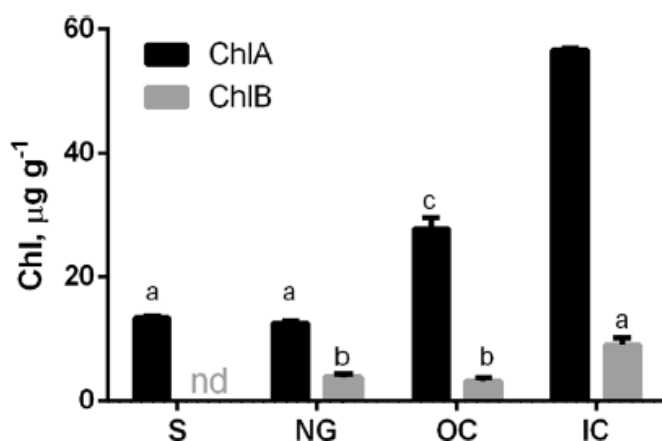
Figure 66. Chlorophyll a and chlorophyll b concentrations in uninfected stems of *Cuscuta campestris* (S), stems up to 5 mm from the galls (NG), outer cortex (OC) and inner cortex (IC) of the galls. Nd - not detected. Different letters show statistically significant differences at $P < 0.05$, Student's t-test. (Published in Zagorchev L. et al. (2018) Planta. 248 (3): 591-599)

The photosynthetic ability of the galls was investigated in comparison with a model plant, *Arabidopsis thaliana*, by OJIP assay. Induction curves showed significantly lower fluorescence of the galls, especially in the outer cortex (Fig. 67). The differential curves are presented in Fig. 68.
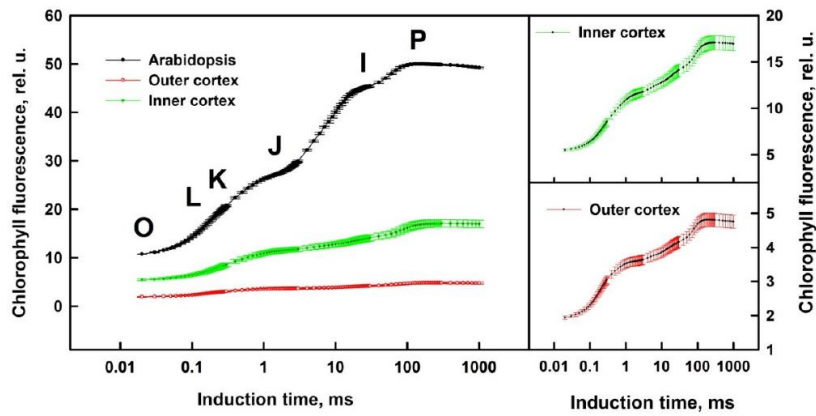
Figure 67. Induction curves recorded for inner and outer cortex of *Smicronix* galls and in leaves of reference photosynthetic plant *A. thaliana* (averaged from seven replicates). The fast fluorescence of both cortices of the galls was measured on approximately 2-mm-thick sections. ICs for the inner cortex are presented top right and the outer cortex bottom right. (Published in Zagorchev L. et al. (2021), Cells, 10, 1399)

The parameters of the JIP assay provide a better visualization of the differences between *Smicronix* and reference plant *A. thaliana* (Fig. 69).
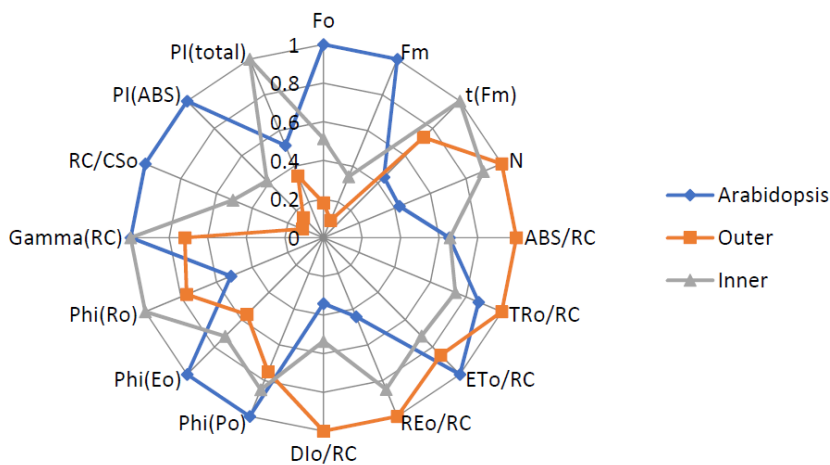
Figure 69. Spider-web plot of 16 JIP-test parameters calculated from induction curves of fast Chl A fluorescence in the outer and inner cortex of galls and in *Arabidopsis* leaves. (Published in Zagorchev L. et al. (2021), Cells, 10, 1399)

The lower values of minimum and maximum fluorescence (parameters F_o and F_m) in the inner and outer cortex compared with the leaves of *A. thaliana* are the result of the low density of photosynthetic structures (R_C/C_{So}). In contrast to PSII, PSI functions more efficiently in the galls (especially in the outer cortex) than in *A. thaliana*. Overall, energy transfer to RCs in galls significantly exceeds energy transfer in *A. thaliana*, i.e., PSA in a normal photosynthetic plant functions optimally but is not optimized in *Smicronyx* galls. Much of the excitation energy in galls is dissipated as heat by the reaction centers of PSII, especially in the outer cortex. To further elucidate the photosynthetic mechanism of *Smicronyx* galls, thylakoid complexes were digested with digitonin and separated by two-dimensional BN-PAGE electrophoresis (Fig. 70). Obviously, the number of complexes is lower in *C. campestris* than in *A. thaliana*. In *C. campestris* at least PSI and LHCII were detected, but most of the other complexes identified in *A. thaliana* are absent.
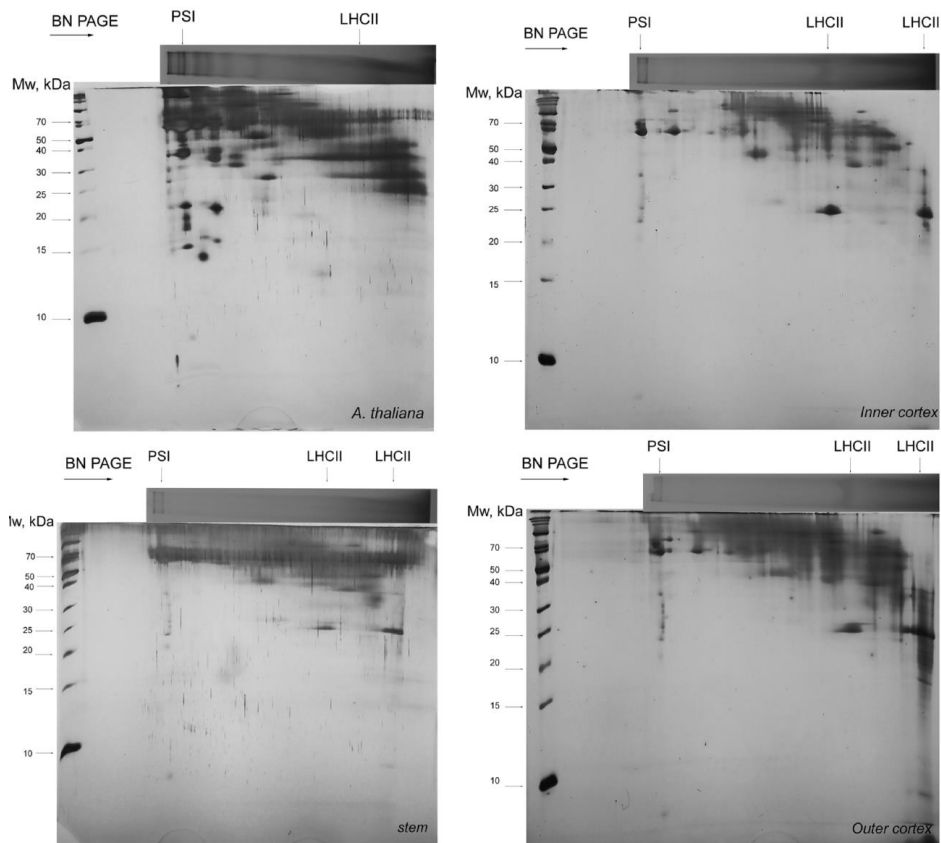
Figure 70. 2D BN/SDS PAGE separation of thylakoids in *Arabidopsis thaliana*, *Cuscuta campestris* stem and galls of *Smicronyx* (inner and outer cortices). Dissolution in 1% digitonin. (Published in Zagorchev L. et al. (2021), Cells, 10, 1399)

4.5.1.2 Zymograms of enzyme activities

The protease and amylase activity zymograms are presented in Figs. 71 and 72. The combined protease activity in the galls relative to the uninfected stems, based on the combined peak area of all isoforms, increased gradually with an almost tenfold increase in the outer cortex but slightly lower in the inner cortex (Fig. 71 d).

Isoform	MW, kDa	S	NG	OC	IC
PROT1	98	-	-	+	+
PROT2	68	-	-	+	+
PROT3	56	+	+	+	+
PROT4	49	+	+	+	+
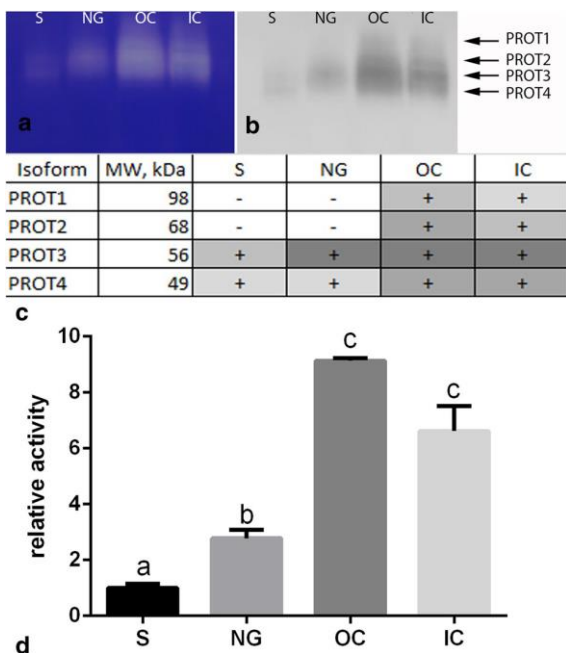
Figure 71. Protease (PROT) isoforms and relative activity in uninfected stems of Cuscuta campestris (S), near galea (NG), outer cortex (OC) and inner cortex (IC) of galls. a-original zymogram; b-software-processed c-molecular weight in kDa and relative activity of PROT isoforms. Different letters indicate significance at P < 0.05, Student's t-test (Published in Zagorchev L. et al. (2018) Planta. 248 (3): 591-599)

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Six isoforms of α-amylase were detected, of which four, AMYL1-4 with molecular weights ranging from 50 to 120 kDa, were present in all samples examined and with similar hydrolytic activity toward starch (Fig. 72 a-c). Two α-amylase isoforms with lower molecular weights, AMYL5 and AMYL6, were represented only in the inner and outer cortex (Fig. 72 a, b). AMYL5 (Mw 30 kDa) was also represented in the uninfected stems, but with markedly higher activity in the outer cortex (Fig. 72 c).

Isoform	MW, kDa	S	NG	OC	IC
AMYL1	120	+	+	+	+
AMYL2	80	+	+	+	+
AMYL3	65	+	+	+	+
AMYL4	50	+	+	+	+
AMYL5	30	+	-	+	+
AMYL6	25	-	-	+	+
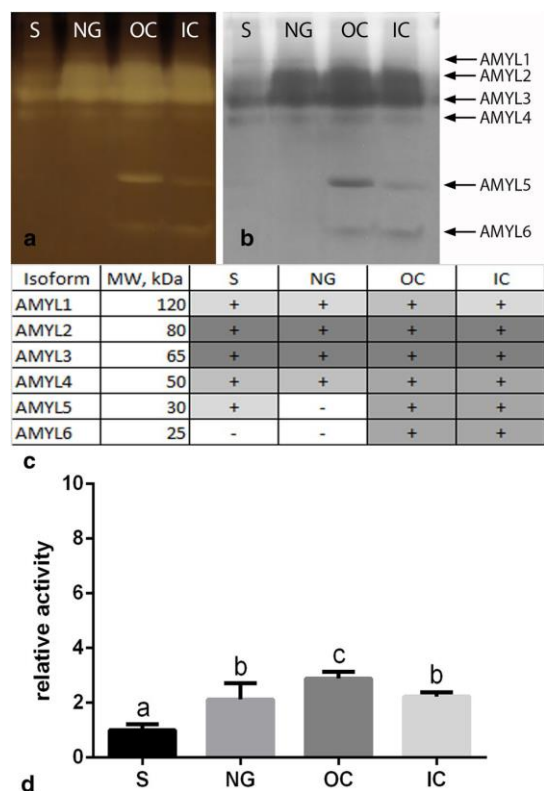
Figure 72. Amylase (AMYL) isoforms and relative activity in uninfected stems of Cuscuta campestris (S), near the galls (NG), outer cortex (OC) and inner cortex (IC) of the galls. a-original zymogram; b-software-processed c-molecular weight in kDa and relative activity of AMYL isoforms. Different letters indicate significance at P < 0.05, Student's t-test (Published in Zagorchev L. et al. (2018) Planta. 248(3):591-599)

The five antioxidant enzymes examined (Figs. 73-77) can be divided into two groups depending on whether activity increases or decreases in the galls. The enzymes with decreased activity in the galls are ascorbate peroxidase, catalase, and glutathione reductase. The relative combined activity of guaiacol peroxidases is highest in the outer cortex but decreases in the inner cortex of the galls. All four isoforms of superoxide dismutase increased in activity from the uninfected stalk to the inner cortex (Fig. 77). The combined activity of SOD increased gradually with a nearly tenfold increase in the inner cortex.
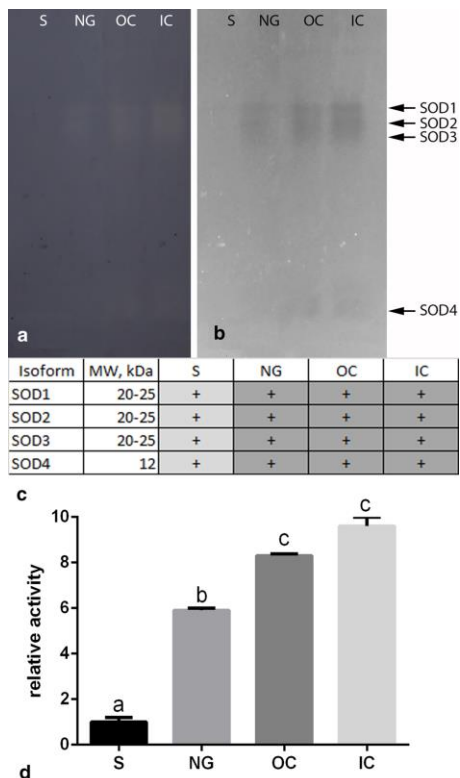
Figure 77. Superoxide dismutase (SOD) isoforms and relative activity in uninfected stems of *Cuscuta campestris* (S), near galls (NG), outer cortex (OC) and inner cortex (IC) of galls. a-original zymogram; b-software processed c-molecular weight in kDa and relative activity of SOD isoforms. Different letters indicate significance at P < 0.05, Student's t-test (Published in Zagorchev L. et al. (2018) Planta. 248(3):591-599)

Isoform	MW, kDa	S	NG	OC	IC
SOD1	20-25	+	+	+	+
SOD2	20-25	+	+	+	+
SOD3	20-25	+	+	+	+
SOD4	12	+	+	+	+

4.5.2 Feeding of gnawing and sucking herbivorous insects

Arabidopsis plants are infected with *C. campestris*, and then salinized with 0 or 150 mM NaCl for one week. Larvae of *Lycoriella ingenua* (Dufour, 1839) (Diptera: Sciaridae) or *Metcalfa pruinosa* were then transferred to them. In the case of the experiments with Sciarid flies, *Lycoriella ingenua* larvae (Fig. 78 a) caused visible damage to Arabidopsis leaves adjacent to the soil (Fig. 78 b, c). Salt treatments did not visibly affect larvae and herbivore activity did not visibly affect parasitic plant development. In the case of *M. pruinosa* the herbivorous insects feed on leaves or stems of *A. thaliana*. Interestingly, in the case of *Cuscuta*-infected plants, the nymphs of *M. pruinosa* feed preferentially on the parasitic plant, completely ignoring the host plant.
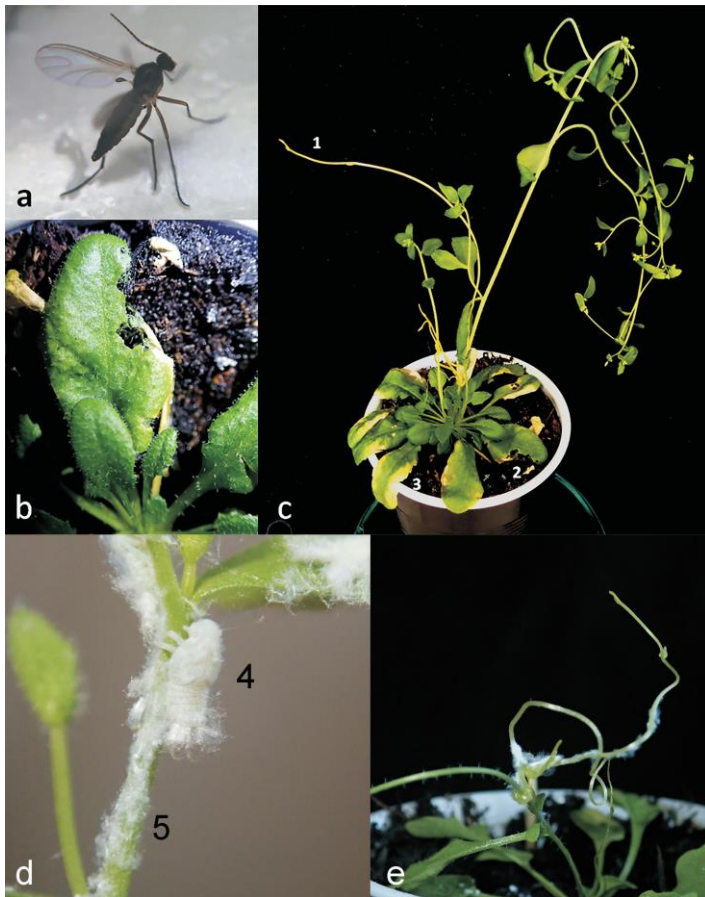
Figure 78. *Lycoriella ingenua* (a) feeding on *Arabidopsis* leaves (b) on triply stressed plants (c). 1, *Cuscuta campestris*; 2, *Lycoriella ingenua*-damaged leaves; 3, yellowing of leaves at 150 mM NaCl. *Metcalfa pruinosa* feeding on *Arabidopsis* (d) and *Cuscuta campestris* parasitizing *Arabidopsis* (e). 4 - nymph of *Metcalfa pruinosa*; 5 - white wax secreted by the insect. (Published in Zagorchev L. et al. (2022) Comptes rendus de l'Academie Bulgare des Sciences. 75 (6), 835-844)

Chitinases, one of the major classes of pathogenesis-related proteins in roots (Fig. 79) and leaves (Fig. 80) of *Arabidopsis*, were examined by zymogram analysis. In *Cuscuta campestris*, the number and intensity of chitinase isoforms increased upon treatment with herbivorous insects (Fig. 81).
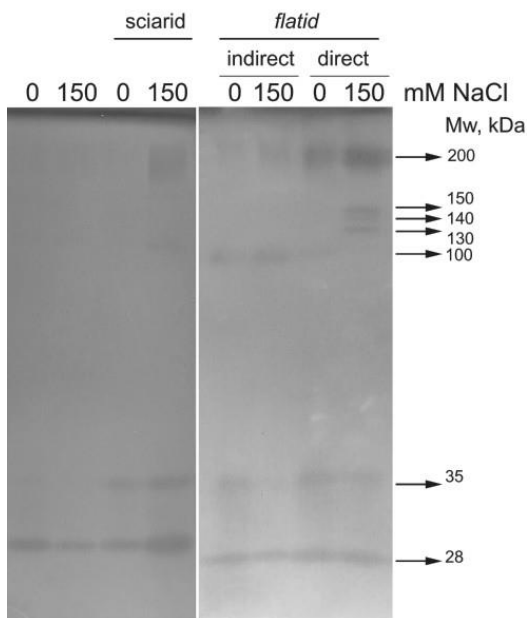
Figure 81. Chitinase profile of *Cuscuta campestris* subjected to different combinations of stress. 5-15% T gradient SDS PAGE with 0.1% glycol chitin in the gel matrix. sciarid = *Lycoriella ingenua*; flatid = *Metcalfa pruinosa*. (Published in Zagorchev L. et al. (2022) Comptes rendus de l'Academie Bulgare des Sciences. 75 (6), 835-844)

4.5.3 Role of *Cuscuta* spp. as vectors of plant viruses

A total of 36 populations of *Cuscuta* spp. collected during the summer (June-September) of 2020 were surveyed. The main *Cuscuta* species that was found was *C. campestris* - 27 populations in total. A total of two populations of *Cuscuta europaea*, four populations of *Cuscuta*

epithymum and two populations of *Cuscuta approximata* were identified. The locations of all populations are shown in Figure 82 and additional Table s4.5.3.1. Only two of the viruses examined, TYLCuV and CMV, were detected (Table s4.5.3.1). A single population of *C. epithymum* was found to be double positive for TYLCuV and CMV. All other populations belong to *C. campestris*. Eight (30%) were positive for CMV, 5 (19%) were positive for TYLCuV and 3 (11%) were positive for both viruses (Table s4.5.3.1).

Tissue printing of *Arabidopsis* infected with *C. campestris* under laboratory conditions (Fig. 83 a) shows characteristic granular staining of CMV virus particles in the stem of *C. campestris* (Fig. 83 b), *Arabidopsis* stem over *Cuscuta* infection (Fig. 83 c), and in a haustorium cross section (Fig. 83 d). The staining is concentrated in the conducting elements (Fig. 83 c). No staining was observed in control plants (Fig. 83 e). The results were further confirmed by DAS-ELISA and Western blot analyses.
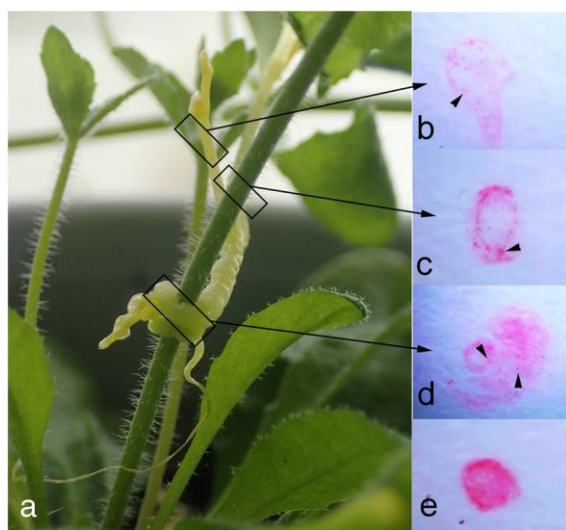
Figure 83. CMV immunolocalization on tissue prints of a *C. campestris* – infected *Arabidopsis* (a). Arrows indicate localization of immunostaining on stem sections of *C. campestris* (b), stem of infected *Arabidopsis* (c), site of infection (d), and control *Arabidopsis* plants (e). (Published in Teofanova D. et al. (2022) Phytoparasitica. 50 (3), 555-566)

5. Discussion

5.1 Distribution, host range and influence on the host

The present results (Figs. 13-17, Table s4.1.1.2) clearly demonstrate that all members of the genus *Cuscuta* are host generalists (Koch et al. 2004). In agreement with similar studies in a relatively nearby country, Hungary (Barath and Csiky 2012), the results also show that *Cuscuta campestris* has the widest host range and is distributed in relatively more diverse ecological conditions (Figs. 11, 19). In contrast to the studies cited above, however, the present results show little overlap in the host range (Fig. 18). Although it occurs primarily in human-impacted areas, such as gardens and along roadsides, where common hosts include widespread weeds such as *Polygonum aviculare, Convolvulus arvensis*, both reported as preferred host species (Baráth 2021), and *Portulaca oleraceae*, was also found to parasitise Balkan endemic plants such as *Achillea clypeolata*, and in sensitive habitats such as sand dunes.

Cuscuta campestris, in addition to parasitizing indigenous plants and anthropophytes, is found in the greatest number of habitats, most of which are constructed or colonized by ruderals and weeds. It parasitises in many places along roadsides, silos, abandoned fields, pigsties, in farmyards and vegetable gardens, etc., areas closely associated with various anthropogenic activities, but mostly land fertilisation, manure storage and spreading. An interesting fact is the involvement of C. campestris in some coastal marine habitats such as Black Sea sand dunes. It has also been observed in the margins of some saltwater lakes and longhouses along the Black

Sea coast. In these habitats the environmental conditions are extreme (high solar radiation, salinization of the substrate, etc.).

With respect to cultivated plants, neither maize as a monocot nor tomatoes have been found to be suitable primary hosts for *C. campestris* (Fig. 31). This finding supports previous reports in the scientific literature (Dawson et al. 1994; Krause et al. 2018; Ntoukakis and Gimenez-Ibanez 2016; Singh and Singh 1997). However, the literature shows a lot of inaccuracies in the nomenclature used regarding host classification. In an attempt to systematize these discrepancies, we propose an algorithm for estimating host quality (Fig. 85).
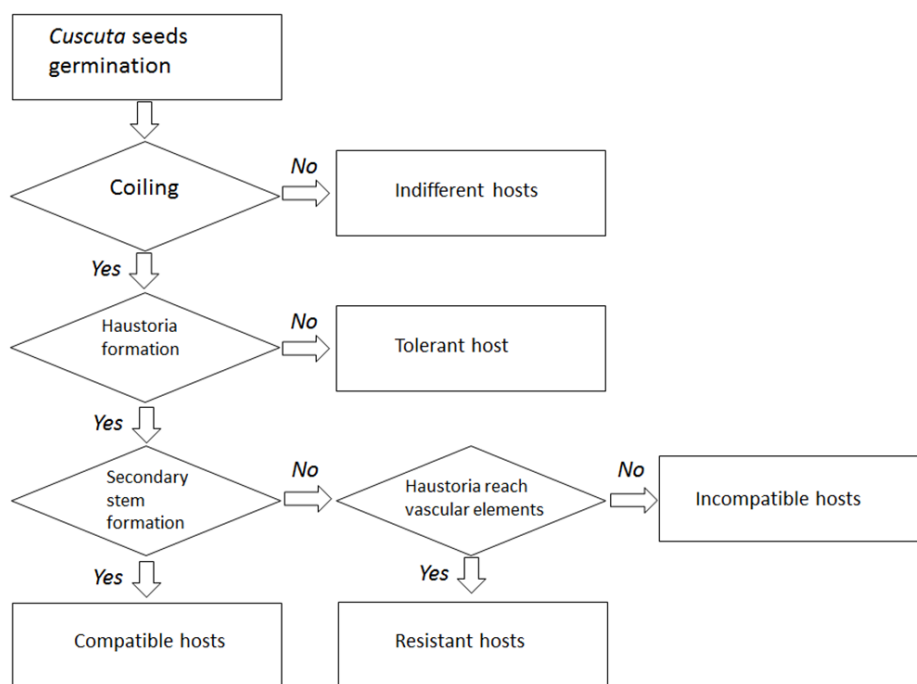
Figure 85. An algorithm for categorizing different hosts with respect to their susceptibility to parasitism by *Cuscuta* spp.

Host species resistance is usually determined by the induction of an injury-like response leading to the death of certain host and parasite cells (Kaiser et al. 2015; Runyon et al. 2010), which can occur either at the stem surface or later during haustorium penetration. This reaction appears to be characteristic of tomato, as observed here (Figs. 31, 37). In contrast to resistant hosts, incompatible hosts (i.e., *Zea mays*) do not exhibit an active visual response. Compatible hosts, which can also be scored as susceptible, may further differ in their ability to support the growth of *C. campestris*. The host plants selected in this study, representatives of the families Cucurbitaceae, Fabaceae, Solanaceae and Apiaceae, are common hosts for *Cuscuta* spp. (Lanini and Kogan 2005; Marambe et al. 2002) and are among the important cultivated plants in Europe and particularly in Bulgaria. While representatives of Fabaceae and Apiaceae are suitable hosts (Table 7, Figs. 38, 39), those of Solanaceae show different success for the parasite. All host plants tested from the Fabaceae family provided very rapid attachment and a short lag phase for the parasite, probably due to the presumably higher nitrogen content of this family and the high nitrogen requirement of *Cuscuta* members (Marambe et al. 2002).

In terms of genetic diversity, the results (Figs. 20-22) are not significantly different from those published for members of the genus in the literature (Fahad et al. 2011; Tajdoost et al. 2013; Kazemitabar et al. 2014). The observation that RAPD markers are suitable for species identification (Lin and Lin 2003), but not as successful for characterizing intraspecific diversity of

Cuscuta spp. is confirmed. No correlation between genotype and host range, geographic distribution, or seed germination was found (Figs. 20, 22-24, Table 5, s4.1.1.1).

A significant result was the establishment, primarily of *Cuscuta campestris*, as a reservoir and vector of plant viruses. The four viruses tested were selected on the basis of their agricultural importance (Scholthof et al. 2011). To date, there is no evidence of these viruses being found in cuckoo yarns, although these parasitic plants are known hosts and vectors of other viruses such as Potato virus Y (Birschwilks et al. 2006), Tobacco rattle virus (Dikova 2006) and Grapevine leafroll-associated virus-7 (Mikona and Jelkmann 2010). TYLCV and CMV have been found among populations of *C. campestris* (Fig. 82, Table s4.5.3.1). The occurrence of CMV and TYLCV in C. campestris is an alarming phenomenon, suggesting that this species may represent a reservoir and vector, in addition to their usual insect vectors, for the spread of these viruses among both natural and agricultural habitats. Although parasite-host transmission was not investigated in the present experiment, parasite-host transmission has been demonstrated at least for CMV (Figs. 83-84). This is in agreement with previous findings for other viruses (Mikona and Jelkmann 2010; Birschwilks et al. 2006) and further demonstrates the potential of parasitic plants as viral vectors.

The impact of Cuscuta spp. parasitism on hosts is significant and this has been demonstrated by examining photosynthesis and the host transcriptome. In the present study, a total of 1601 DEGs were identified between P (parasitized) and CK (controls) of T. repens with and without parasitism by C. australis, respectively (Figs. 51-54, Table 13). Functional annotation revealed that 15 DEGs were enriched within the plant-pathogen interaction pathway (Fig. 51-54, Table 13). Similar results were obtained for Arabidopsis upon infection with Cuscuta chinensis (Figs. 54-56). In Arabidopsis, the expression levels of PR1, PR2, and PR5 are up-regulated in response to drought stress (Liu et al. 2013). Overexpression of AtRPS2 and AtRPM1 confers broad-spectrum resistance to fungal pathogens, bacterial pathogens, and pests (Li et al. 2019). However, in this study, Cluster-16854.12166 (PR1), Cluster-35121.0 (RPM1, RPS3), Cluster-16854.8014 (RPM1, RPS3), Cluster-16854.8015 (RPM1, RPS3), Cluster-16854.8017 (RPM1, RPS3), and Cluster-16854.8021 (RPM1, RPS3) had decreased expression in T. repens during parasitism of C. australis. This suggests that parasitized plants actively suppress defense mechanisms to prevent active defense responses.

Species of the genus *Cuscuta* also exhibit a distinct effect on both light (Fig. 50, Figs. s4.2.2.1-4) and light-independent (Table 12) host photosynthetic responses, even when this host does not provide optimal parasite development (Fig. 49). The results for light-independent photosynthetic responses are in agreement with the results of Shen (Shen et al. 2007), who found that changes in the photosynthetic process in leaves of *Mikania micrantha* infected with *C. campestris* were dependent on the age of the leaves. Similar to our results, the presence of the parasite *C. reflexa* caused stimulation of net photosynthesis in the host and caused a delay in leaf senescence in *Ricinus communis* (Jeschke and Hilpert 1997) and in *Coleus blumei* (Jeschke et al. 1997). Transcriptome analyses in *Arabidopsis* show that there is significantly increased expression of genes associated with the light phase of photosynthesis (Fig. 55). At the same time, there was a significant suppression of light-independent responses (Table 12).

The decrease in CO_2 assimilation can be further explained by down-regulation of at least the large subunit of RuBisCO (Table s4.4.1.2), but also by down-regulation of chloroplast glyceraldehyde-3-phosphate dehydrogenase and phosphoribulokinase (Table s4.4.1.2), which are involved in ribulose-1,5-bisphosphate regeneration. Accordingly, their downregulation leads to a decrease in CO_2 assimilation, also referred to as "Calvin-Benson cycle retardation" (Price et al. 1995; Elena López-Calcagno et al. 2017). Furthermore, many of the differentially regulated proteins in the present study are associated with modulating carbohydrate or amino acid

metabolism and, according to the GO annotation on biological processes, are related to the response to different types of stress.

The host response to *Cuscuta* infection is generally thought to trigger JA- and SA-related defense pathways (Runyon et al. 2010), and increases in the expression of multiple genes and proteins associated with the response to pathogens have been reported. Borsics and Lados (Borsics and Lados 2002) reported up-regulation of PPRG2 of the homologous to pathogenesis protein family (PR-10) as a result of *Cuscuta* parasitism. In *Mikania micrantha* infected by *C. campestris*, up-regulation of a chitinase homologous gene, Mmchi1, has been reported (Li et al. 2010a). Transcriptome analyses largely contradict these data (Figs. 52 and 56).

Using next-generation sequencing, we found that parasitism alters the composition and diversity of the microbial environment in the rhizosphere of invasive *Alternanthera philoxeroides* infected by *C. australis* (Figs. 59-62). This confirms previous findings that short-term parasitism by *C. campestris* significantly altered indices of functional diversity of soil microbial communities using the BIOLOG assay (Li et al. 2014). Similarly for *C. australis*, Bardgett et al. (Bardgett et al. 2006) found significant changes in subsoil properties in a natural grassland ecosystem infested by the root hemiparasite *Rhinanthus minor*. The indirect or systemic effect of parasitism by *C. campestris* on host roots is also well expressed for a number of enzymes (Figs. 58, 79). This effect can be explained by the disruption of xylem/phloem transport caused by the parasitism mechanism of *C. campestris* (Birschwilks et al. 2006), which directly affects communication between the site of infection and the root. The significant underground effect of *Cuscuta* spp. parasitism has been previously confirmed, resulting in a deficiency of assimilates (Jeschke and Hilpert 1997). Ultimately, all of these mechanisms lead to significant changes in the activities of key soil enzymes (Fig. 64), which in turn leads to impaired soil organic matter turnover (Acosta-Martinez et al. 2018).

5.2 Impact of abiotic stress on parasite-host interactions

Abiotic stress, studied mainly in as salinity, affects every stage of *Cuscuta* spp. development, from seed germination, through haustorium formation, to successful parasitism and development. A comparative analysis of seed germination of parasitic and nonparasitic members of Convolvulaceae under salinity conditions indicated slower germination in parasitic plants, further delayed by salinity (Fig. 25), which is consistent with existing literature. The strategy of nonparasitic climbing plants involves rapid initial growth and climbing on other plants to outcompete surrounding species (Shen et al. 2016). The relatively large seeds of these species germinate as quickly as possible (Fig. 25), mobilizing starch by activating amylases (Fig. 28). In contrast, even when physical dormancy was artificially disturbed (by scarification), germination in *Cuscuta* spp. was significantly slower. The ecological strategy of parasitic species involves deep physical and possibly physiological dormancy (Jayasuriya et al. 2008), which ensures long seed retention in the soil and continuous germination over several years. Slower germination would allow emergence when potential hosts have already established, which is critical due to low photosynthetic capacity (McNeal et al. 2007b) and impaired ability to assimilate minerals from the soil due to gene loss (Vogel et al. 2018), which does not guarantee survival in the absence of a host. Therefore, initial growth in *Cuscuta* is directed towards immediate attachment to a potential host.

Clearly, parasitic *Cuscuta* are much more sensitive to salinity than other non-parasitic members of the family. Convolvulaceae. Not only the germination rate (Fig. 25) and growth in the absence of a host (Figs. 26 and 27) were negatively affected, but also the further ability to infect potential hosts and the growth rate after establishment (Fig. 30). At the level of haustorium formation, certain differences in enzyme profiles were observed at the site of contact in both the parasitic plant and the hosts (Figs. 32-35), which differences were dictated by both host species

and salinity. The involvement of proteases (Bleischwitz et al. 2010) and pectinmethyl esterases by the parasite (Yokoyama et al. 2020; Srivastava et al. 1994) is well known and confirmed in the present experiment. With respect to AGPs (Fig. 36), at least in *Arabidopsis* and *Solanum lycopersicum*, a differential increase in LM2 and JIM8 recognized fractions was observed as a result of infection, consistent with previous results indicating an active involvement of host AGPs in the process of haustorium formation (Albert et al. 2006). This was initially associated with adhesive disc formation, but was subsequently also associated with searching hyphae (Hozumi et al. 2017). Albeit in a different experimental subject, but AGPs have been found to be significantly affected by salt stress and are critical for cell morphogenesis (Fig. 86).

Salinization has a significant impact on the development of the parasite and after successful infection. Increased salt concentration exhibits a negative and dose-dependent effect on parasite growth (Frost et al. 2003a). In some host plants, this effect is already significant at 100 mM NaCl and completely devastating at 200 mM NaCl, whereas in others the lower salt concentration does not significantly alter parasite growth (Fig. 39). However, increased salt concentration can also increase host susceptibility (Frost et al. 2003a), as shown in eggplant and in several pepper cultivars (Fig. 39). Obviously, survival and parasite growth rate are not directly related in all cases. While in *Petroselinum crispum* the survival of the parasite increased with salinity and the growth rate decreased, the opposite was observed in *Capsicum annuum* cv. Kurtovska kappia (Fig. 39, Table 7). Survival is strongly dependent on haustorium formation, which in turn can be suppressed by cell wall lignification (Jhu et al. 2020) caused by increased salt concentration. Secondly, parasitic plant growth can be influenced by several factors. Negative effects would be expected from reduced host biomass availability and accumulation of toxic ions, especially Na^+ (Wallace et al. 1978), while Cl^- ions are reported not to penetrate haustoria to any significant extent (Förste et al. 2020a). The salinity parasite also showed a general increase in L-proline concentration (Fig. 40), which does not seem to be directly related to growth rates.

Most of the effects of abiotic stress on *Cuscuta* spp. are induced by indirect, host-mediated effects. However, the parasite is also capable of its own metabolic stress response (Srivastava et al. 2004; Vurro et al. 2011). A significant increase in L-Pro concentration was observed in response to salinity (Fig. 41), much more pronounced than in the host plant (Fig. 57). The relative insensitivity of SOD activity (Fig. 41) may be explained by the nonphotosynthetic nature of the parasite. Given that the photosynthetic mechanism is the main source of superoxide radical overproduction at salt concentration (Pintó-Marijuan and Munné-Bosch 2014), it can be expected that in *Cuscuta* this does not pose a significant challenge. In contrast to SOD, the opposite decrease in POD activity and increase in CAT activity (Fig. 41) suggests the importance of H2O2 production and the possible transfer of this reactive oxygen species from the host to the parasite.

Regarding the metabolic analysis of *Cuscuta chinensis* parasitizing glycophytic and halophytic hosts under salt treatment, the main expectation is to determine whether the response to salt stress of the parasite depends on the salt tolerance of the host. Contrary to expectations, no significant differences were found in typical stress metabolites such as proline or glycine betaine (Tables s4.3.2.1-9). The only significantly affected metabolites that could be attributed as stress metabolites were from the flavonoid group. There are a number of reports of their involvement in the salt stress response (Chutipaijit et al. 2009; Sarker and Oba 2018). However, from the results obtained, it appears that the flavonoid profile in *Cuscuta* changes mainly under the influence of host species and not so much under the influence of salt stress (Fig. 44).

The effect of parasitism of *C. campestris* on the response to host abiotic stress is either direct or indirect. The direct effect consists of metabolic changes in and near the site of, for example, in the iS (infection site), where haustoria form, and in the iL (infected leaves), which is a

direct effect of parasitism. The indirect effect affects host tissues distant from the site of infection, and can be defined as systemic - a whole plant reaction. Not surprisingly, all biochemical markers examined were affected mostly at the site of infection and in infected leaves (Fig. 57), and the interaction between infection and salinity was also more pronounced there (Table 14). The significant antioxidant response provided by higher CAT, SOD, and POD activity suggests parasitism-induced oxidative stress (Demirbas et al. 2013; Saric-Krsmanovic et al. 2018).

5.3 Interaction with hosts under biotic stress conditions

There is relatively little literature on pathogens or epiparasites on members of the genus *Cuscuta*. One such example is the gall-forming weevil beetles of the genus *Smicronyx*. The most obvious phenotypic difference between the galls and the uninfected stem borer of *C. campestris* is the accumulation of chlorophyll, especially in the greenish inner cortex of the galls (Fig. 65). The cross section of the galls is very similar to previous publications (Zhekova et al. 2014; Anikin et al. 2017), showing a larval chamber surrounded by a chlorophyll-rich inner cortex and a paler outer cortex (Fig. 65). The elevated chlorophyll concentration (Fig. 66) in the inner cortex confirms the possibility that *Smicronyx* galls are photosynthetically active (Anikin et al. 2017), a significant difference in gall functionality compared to *Cuscuta* tissues.

Anikin et al. (Anikin et al. 2017) investigated some parameters of PSII fluorescence in *Smicronyx smreczynskii* galls on *C. campestris* and found an increased amount of active PCs of PSII and increased photosynthetic efficiency in galls compared to stems of *C. campestris*. In the present study, similar results were obtained and extended with the capabilities of the JIP assay (Figs. 67-68). The normal Chl a/b ratio in green plants is about 3. LHCII consists of three parts: a core antenna containing only Chl a and most closely associated with the reaction center; minor Chl a/b-binding proteins that occupy an intermediate position between the core antenna and the peripheral antenna; and the outermost Chl a/b-binding proteins, LHCII. These three antenna groups account for 14, 15, and 67% of the total chlorophyll in PSII, respectively (Ke 2001). LHCII is the major component of the thylakoid membrane and the main light-harvesting antenna of PSII, with a Chl a/b ratio close to 1 (8:7 molecules). In addition to light harvesting, LHCII plays a role in membrane alignment, which has consequences in regulating the energy distribution between the two photosystems. The normal Chl a/b ratio in PSI is 5-6. Our results from electrophoretic separation of thylakoid complexes show much less PSI and LHCII in the galls compared with *A. thaliana* (Fig. 70). The lack of LHCII results in less efficient energy transfer in PSII in the galls than in *A. thaliana*. Other authors have also observed low efficiency of PSII (Van der Kooij et al. 2000;). Sherman (Sherman et al. 1999) found that chloroplasts of *C. pentagona* contain a number of proteins necessary for successful CO_2 fixation, and the proteins in thylakoids are organized similarly to other higher plants, except for the large percentage of thylakoids organized in grana. *C. campestris* under certain circumstances is able to properly organize its photosynthetic apparatus and carry out significant photosynthesis.

The activity of hydrolytic enzymes further confirms the functional distinction between the inner and outer cortex, as well as in comparison with uninfected stems. Both proteases (Fig. 71) and α-amylases (Fig. 72) showed increased activity, and several isoforms were detected only in galls, consistent with previous findings that insect-induced plant galls are metabolically active, starch- and protein-rich structures (Giron et al. 2016). Increased metabolic and photosynthetic activity lead to a significant increase in SOD activity, especially in the inner cortex of galls (Fig. 77). This is essential for the removal of superoxide radicals generated during photosynthesis, even under optimal conditions, and for the maintenance of redox homeostasis (Foyer and Noctor 2011). The increased activity of these enzymes is expected due to the putative oxidative stress in the inner cortex of mature galls (Santos Isaias et al. 2014).

The study of the impact of feeding on herbivorous insects was conducted with two species differing in their feeding mode, the gnawing larvae of scyarid flies and the sucking larvae of the cicada *Metcalfa pruinosa*. In addition, plants were also subjected to salt stress to test the relationship between abiotic and biotic stressors. While the lack of direct feeding of scyarid larvae with C. campestris is expected due to their soil habitat, the preferred feeding of cicadas on the parasitic plant (Fig. 78) is considered unusual behavior. Relatively early studies reported that, in the case of aphids, some species tend to feed on *Cuscuta* even if the host plant is not a natural food for aphids and is therefore avoided (Harvey 1966).

Salinity alone did not induce visible changes in chitinases in either *Arabidopsis* roots or leaves (Figs. 79, 80), although induction of pathogenesis-related (PR) proteins, including chitinases, in response to abiotic stresses has been reported (Grover 2012). However, salinity modulates the chitinase profile in response to insect herbivory in roots as well as in the parasitic plant. Interactions between biotic and abiotic stressors are well documented and known to elicit similar responses, and it is known that salinity can increase the defense response against herbivorous insects (Renault et al. 2016). A previous study also reported impaired defenses to herbivorous insects on host plants infected by *Cuscuta* (Runyon et al. 2008). With respect to chitinases, this was not confirmed in our study. The lower intensity of chitinolytic bands in leaves in the *Cuscuta* + flatid treatment must be due to the lack of direct damage by cicadas on Arabidopsis. In contrast, the response of *C. campestris* to herbivorous insects in terms of the number and intensity of chitinolytic bands (Fig. 81) was more pronounced than that of the host plant. These results are consistent with reported data on sensing by the host and signal transduction elicited by herbivorous insects by *Cuscuta* (Zhuang et al. 2018).

6. Conclusions

Based on the results obtained, the following more important conclusions can be summarized:

Hypothesis 1: The introduced species *Cuscuta campestris* has a higher invasive and parasitic potential than the native members of the genus.

1.   The introduced and invasive species *Cuscuta campestris* has a wider distribution, in more diverse climates and a wider range of hosts than the native species. At the same time, it is also of greater importance as a vector and reservoir of plant viruses.

2.   The genetic diversity of *Cuscuta campestris* is relatively high but does not correlate with the distribution or host range of the parasite.

Hypothesis 2: Parasite-host interactions are influenced by abiotic and biotic stressors.

3.   Salinization negatively affects all developmental stages of *Cuscuta*, as the response to salt stress depends on the host species and salinization can lead to a change in host 'quality'.

4.   *Cuscuta* metabolism is highly dependent on host species and to a lesser extent on abiotic stressors.

5.   *Cuscuta* parasitism, in turn, interferes with the ability of hosts to adapt to both salinity and herbivorous insects.

6.   *Cuscuta* parasitism negatively affects host photosynthetic activity in both light-dependent and CO_2 assimilation responses even when the parasite does not have optimal development.

7.   Aboveground parasitism of *Cuscuta* has a significant effect on interactions between the root system and rhizosphere microbial communities by altering root metabolism, relative proportions of microbial taxa, and soil enzyme activity, thereby impairing the metabolism of organic matter and mineral compounds.

Hypothesis 3: Parasites of the genus *Cuscuta* are subject to the influence of biotic factors that significantly alter their metabolism.

8.   Certain factors can induce an active light phase of photosynthesis in the otherwise non-photosynthetic parasite *Cuscuta campestris*. A similar biotic factor is the galls of the *Smicronyx* beetles, which form two functionally and metabolically distinct layers (cortices).

9.   No active antioxidant response is observed in *Smicronyx* galls and there is no evidence that they negatively affect *Cuscuta* growth and development.

10.   Parasitic plants of the genus *Cuscuta* can be active vectors of viruses (Cucumber Mosaic Virus) that transfer to the host without themselves having visible symptoms of viral infection.

In conclusion, it can be considered that all three hypotheses are confirmed.

7. Contributions

Fundamental contributions:

1. For the first time, the problem of the impact of abiotic stress on parasitic plants is defined and the literature available to date is summarized with clear research directions set.
2. Significant host species effects on the adaptation of *Cuscuta* spp. to abiotic stress have been identified.
3. Enzymes and glycoproteins involved in the process of haustorium formation have been confirmed and identified.
4. Data on the changes that occur as a result of the epiparasitism of gall-forming insects of the genus *Smicronyx* in the photosynthetic apparatus of *Cuscuta campestris* have been confirmed and enriched.

Applied contributions:

5. The information on the distribution of the genus *Cuscuta* in Bulgaria, the spectrum of hosts and the collection of herbarium materials in the Herbarium of the University of Sofia has been significantly enriched.
6. The negative impact of the aboveground parasite *Cuscuta* spp. on rhizosphere interactions and soil health has been confirmed.
7. Confirmed the role of *Cuscuta* spp. as a reservoir and vector of plant viruses.

Methodical contributions:

8. Optimized parasite/sensitive host and parasite/ tolerant host model systems for comparative analyses of multiple stressors on *Cuscuta* spp.

Publications related to the thesis

1. 章由陈露茜、李钧敏、柳本·扎戈尔 切夫（**Lyuben Zagorchev**）和杨蓓芬撰写 (Lucy Chen, Junmin Li, **Lyuben Zagorchev** and Beifen Yan) 第9 章 环境因子对菟丝子属植物寄生效果的影响 (Chapter 9 The Effect of Environmental Factors on the Parasitism of Dodder Plants) (2022) In: 植物寄生生态学研究：以菟丝子属研究为例 (Research on the Ecology of Plant Parasitism: Taking Cuscuta as an Example) eds. Ma Jun, Hao Chenyang, Beijing: Science Press. ISBN 978-7-03-073382-5 – collective monograph

2. **Zagorchev L.**, Du Z., Shi Y., Teofanova D., Li J. (2022) *Cuscuta australis* Parasitism-Induced Changes in the Proteome and Photosynthetic Parameters of *Arabidopsis thaliana*. Plants. 11, 2904. (IF = 4.658; SJR = 0.77; Q1) https://doi.org/10.3390/plants11212904

3. Sarić-Krsmanović M., **Zagorchev L.**, Gajić Umiljendić J., Rajković M., Radivojević L., Teofanova D.R., Božić D., Vrbničanin S. (2022) Variability in early seed development of 26 populations of *Cuscuta campestris* Yunck.: The significance of host, seed age, morphological trait, light, temperature and genetic variance. Agronomy. 12 (3) 559. (IF = 3.949; SJR = 0.65; Q1) https://doi.org/10.3390/agronomy12030559

4. **Zagorchev L.**, Albanova I., Lozanova Y., Odjakova M., Teofanova D. (2022) Chitinase profile of Arabidopsis, subjected to the combined stress of soil salinity, Cuscuta campestris parasitism and herbivores. Comptes rendus de l'Academie Bulgare des Sciences. 75 (6), 835-844, (IF = 0.326; SJR = 0.19; Q3) DOI:10.7546/CRABS.2022.06.07

5. Teofanova D., Lozanova Y., Lambovska K., Pachedjieva K., Tosheva A., Odjakova M., **Zagorchev L.** (2022) Cuscuta spp. populations as potential reservoirs and vectors of four plant viruses. Phytoparasitica. 50 (3), 555-566 (IF = 1.809; SJR = 0.41; Q2) https://doi.org/10.1007/s12600-022-00981-9

6. **Zagorchev L.**, Petrova V., Albanova I., Georgieva K., Sarić-Krsmanović M., Muscolo A., Teofanova D. (2022) Salinity modulates crop plants suitability as hosts for *Cuscuta campestris* parasitism. Journal of the Saudi Society of Agricultural Sciences. 21 (5), 324-330, https://doi.org/10.1016/j.jssas.2021.10.008 (SJR = 1.01; Q1)

7. Zhou L., Lu Q., Yang B., **Zagorchev L.,** Li J. (2021) Integrated small RNA, mRNA, and degradome sequencing reveals the important role of miRNAs in the interactions between parasitic plant *Cuscuta australis* and its host *Trifolium repens*. Scientia Horticulturae. 289. 110458 https://doi.org/10.1016/j.scienta.2021.110458 (IF = 4.342; SJR = 0.84; Q1)

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11. Ren Z., **Zagortchev L.,** Ma J., Yan M., Li J. (2020) Predicting the potential distribution of the parasitic *Cuscuta chinensis* under global warming. BMC Ecology. 20:28. DOI: 10.1186/s12898-020-00295-6 (IF = 2.964; SJR = 0.867; Q1)

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12. **Zagorchev L.**, Traianova A., Teofanova D., Li J., Kouzmanova M., Goltsev V. (2020) Influence of *Cuscuta campestris* Yunck. on the photosynthetic activity of *Ipomoea tricolor* Cav. – *in vivo* chlorophyll *a* fluorescence assessment. Photosynthetica. 58 (SI): 237-247. DOI: 10.32615/ps.2020.004 (IF = 3.189; SJR=0.797; Q1)

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14. Denitsa Teofanova, Mariela Odjakova, Nabil Abumhadi, **Lyuben Zagorchev.** Strigolactones. Mediators of abiotic stress response and weakness in parasite attraction. Chapter 8, pp. 115-128. In: Ed. Hasanuzzaman M., Fujita M., Oku H., Islam T. (2019). Plant Tolerance to Environmental Stress: Role of Phytoprotectants. CRC Press, Taylor & Francis Group ISBN 9781138559172

15. **Zagorchev L.**, Albanova I., Tosheva A., Li J., Teofanova D. (2018) Salinity effect on *Cuscuta campestris* Yunck. parasitism on *Arabidopsis thaliana* L. Plant Physiology and Biochemistry. 132: 408-414. DOI: https://doi.org/10.1016/j.plaphy.2018.09.037 (IF = 3.404, SJR = 1.125, Q1 in Plant Science)

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Cited 2 times (in Scopus)

Publication breakdown:

Total number: 20; 4 from the Assoc. Prof. application, 8 used in NACID for Assoc. Prof.;

- With impact factor (WoS): 14 (11 Q1; 2 Q2; 1 Q3)
- With impact rang (Scopus): 3 (1 Q1; 1 Q2; 1 Q3)
- Book chapters: 3 (1 collective monograph in Chinese)
- First author: 13
- Corresponding author: 11
- First or corresponding author: 15
- 4 review papers
- Sum of impact factors: 51.88
- Citations (only in Scopus): 351

Conferences:

Abroad:

1. Denitsa Teofanova, Anita Tosheva, Kalina Pachedjieva, **Lyuben Zagorchev**. Distribution, host range, and genetic variability of the holoparasitic genus Cuscuta in Bulgaria. 4th International Conference on Plant Biology (23rd SPPS Meeting), 6th-8th October, Belgrade, Serbia. Oral presentation. Book of Abstracts p. 137

2. **Lyuben Zagorchev**, Denitsa Teofanova, Margarita Kouzmanova, Vasilij Goltsev. Photosynthetic activity of *Cuscuta campestris*, influenced by gall formation by *Smicronyx* spp., Oral presentation, 16th World Congress on Parasitic Plants, 3rd-8th July, 2022, Nairobi, Kenya. Book of abstracts pp 24-25.

3. **Lyuben Zagorchev**, Denitsa Teofanova, Kristiyana Georgieva, Alexandra Atanassova. Pathogenesis-related proteins of *Arabidopsis thaliana* in response to combination of abiotic (salinity) and biotic (fungus gnats and dodder) stresses. 16th Congress of the Mediterranean Phytopathological Union, 4-8 April 2022, Limassol, Cyprus, Abstract published in Phytopathologia Mediterranea (IF = 2.037), Vol. 62, Issue 1, p. 213.

4. **Lyuben Zagorchev**, Anita Tosheva, Denitsa Teofanova. Response of field dodder (*Cuscuta campestris* Yunck.) to salinity in dependence of the host plants. 11th Weed Science Congress and Symposium on Herbicides and Growth Regulators, Oral presentation, 20-23.09.2021, Palic, Serbia, Book of abstracts p. 24.

5. A. Atanasova, D. Teofanova, I. Albanova, A. Tosheva, **L. Zagorchev**. Parasite-host interaction site–profiling of enzymes and proteoglycans, involved in *Cuscuta campestris* parasitism. 45th FEBS Congress (virtual), 3-8 July, Ljubljana, Slovenia, Abstract published in FEBS Open Bio

(IF = 2.231), Vol. 11, Issue S1, Supplement: 45th FEBS Congress, Molecules of Life: Towards New Horizons, Ljubljana, Slovenia, July 3–8, 2021, p. 387.

6. **Lyuben Zagorchev**, Xue Zhang, Junmin Li. Haustoria formation and changes in photosynthetic structures in parasitic dodders (*Cuscuta* spp.) are induced by blue light alone. 8th International Conference of Ecologists of Montenegro, 2-6 October 2019, Budva, Montenegro, Book of abstracts p. 152.

7. **Lyuben Zagorchev**, Denitsa Teofanova, Junmin Li. Effect of salinity on *Cuscuta campestris* parasitism. 15th World Congress on Parasitic Plants, 30th June–5th July 2019, Amsterdam, the Netherlands. Book of abstracts p. 123.

8. Denitsa Teofanova, Antonii Kaukov, **Lyuben Zagorchev.** Salinity effect on germination of parasitic (*Cuscuta campestris*) and nonparasitic (*Ipomoea purpurea*) members of Convolvulaceae family. 3rd International Conference on Plant Biology. 9-12 June, 2018, Belgrade, Serbia. Book of abstracts pp.60-61.

9. Denitsa Teofanova, Junmin Li, Ivanela Albanova, Anita Tosheva, Mariela Odjakova, **Lyuben Zagorchev**. Antioxidant response of *Arabidopsis thaliana*, subjected to simultaneous salinity stress and *Cuscuta* infection. 3rd International Symposium for Agriculture and Food – ISAF 2017, 18-20 October, 2017, Ohrid, Republic of Macedonia. Book of abstracts p. 354.

National

1. DENITSA TEOFANOVA, IVANELA ALBANOVA, GEORGI GEORGIEV, **LYUBEN ZAGORCHEV**. Salinity stress response of Cuscuta campestris is affected by the host. SCIENTIFIC CONFERENCE "KLIMENT'S DAYS" 5th November 2020 Sofia, Faculty of Biology, Book of Abstracts p. 110

2. IVANELA ALBANOVA, VIKTORIA PETROVA, MARIA STOYANOVA, DENITSA TEOFANOVA, **LYUBEN ZAGORCHEV**. SUSCEPTIBILITY OF MEMBERS OF SOLANACEAE TO CUSCUTA CAMPESTRIS INFECTION. SCIENTIFIC CONFERENCE "KLIMENT'S DAYS" 5th November 2020 Sofia, Faculty of Biology, Book of Abstracts p. 109

3. Alexandra Atanasova, Anelia Traianova, Kristiyana Georgieva, Denitsa Teofanova, Margarita Kouzmanova, Vasilij Goltsev, **Lyuben Zagorchev**. Induction of photosynthetic activity in the non-photosynthetic Cuscuta campestris by gall-forming insects Smycronyx. YOUTH SCIENTIFIC CONFERENCE "KLIMENT'S DAYS" 8th November, 2019 Faculty of Biology, Sofia University, Sofia, Book of Abstracts p. 54

4. **Lyuben Zagorchev**, Georgi K. Georgiev, Kristiyana Georgieva, Denitsa Teofanova. The host quality is decisive for the early developmental stages and success of the parasitic flowering plant *Cuscuta campestris*. International Scientific Conference "Kliment's Days", 8th-9th November, 2018, Sofia, Bulgaria. Book of Abstracts p. 89

5. Anelia Traianova, **Lyuben Zagorchev**, Denitsa Teofanova, Vasilij Goltsev. Changes in the photosynthetic activity of Arabidopsis leaves under different salinity levels as measured in vivo by chlorophyll fluorescence. International Scientific Conference "Kliment's Days", 8th-9th November, 2018, Sofia, Bulgaria. Book of Abstracts p. 118

6. Ivanela Albanova, **Lyuben Zagorchev**, Denitsa Teofanova. Top-down effect of stem parasitic plant *Cuscuta campestris* Yunck. on the roots of its host, *Arabidopsis thaliana* L. under different salinity levels. XXVIII International Scientific Conference "Bulgarian Presidency of the Council of the European Union – innovation and prosperity". 31 May – 1 June. 2018. Starozagorski mineralni bani. Book of abstracts p. 60.

7. Kristiyana Georgieva, **Lyuben Zagorchev**, Denitsa Teofanova. Photosynthetic pigments and carbohydrate metabolism dynamics at early growth stage in the non-photosynthetic parasitic plant *Cuscuta campestris* Yunck. XXVIII International Scientific Conference "Bulgarian Presidency of the Council of the European Union – innovation and prosperity". 31 May – 1 June. 2018. Starozagorski mineralni bani. Book of abstracts p. 61

8. Antoniy Kaukov, Denitsa Teofanova, Ivanela Albanova, Anita Tosheva, Mariela Odjakova, **Lyuben Zagorchev**. *Smicronyx* galls on *Cuscuta campestris* show differential isoenzymes profile. Youth Scientific Conference "Kliment's Days" 16-17 November 2017 Sofia Faculty of Biology Book of Abstracts p. 54

9. Beifen Yang, Xue Zhang, **Lyuben Zagorchev**, Junmin Li, Frank Hagedorn, Mai-He Li. Rhizhosphere soil microbial communities of invasive *Alternanthera philoxeroides* changed by parasitism of *Cuscuta australis* benefit the growth of neighbouring plant. Youth Scientific Conference "Kliment's Days" 16-17 November 2017 Sofia Faculty of Biology Book of Abstracts p. 70

10. Denitsa Teofanova, Ivanela Albanova, Kristiyana Marinova, Anita Tosheva, Junmin Li, Mariela Odjakova, **Lyuben Zagorchev** Arabidopsis – Eutrema pair as model host system to study salt stress response in parasitic plants. XXVII International Scientific Conference 65 Years Maritsa-Iztok. Starozagorski mineralni bani, 1st -2nd June, 2017, Book of abstracts p. 6

Bachelor's and Master's degree theses for which the dissertant is a supervisor (in bold) or project leader

1. Alexandra Yuliyanova Atanasova (2022) Population Diversity and Influence on the Host of the Introduced Invasive Species *Cuscuta campestris* in Bulgaria, M.Sc.
2. **Victoria Petrova Petrova (2021) Comparative assessment of parasitic potential of *Cuscuta campestris* and *Cuscuta europaea* on economically important cultivated plants, B.Sc.**
3. **Yana Ivaylova Lozanova (2021) Interaction between parasitic plant *Cuscuta campestris* and invasive herbivorous insect *Metcalfa pruinosa*, simultaneously parasitizing *Arabidopsis thaliana* under salt stress conditions, B.Sc.**
4. **Georgi Kirchev Georgiev (2020) Response to salt stress of *Cuscuta campestris* depending on the host, M.Sc.**
5. Anelia Trayanova (2019) Influence of *Cuscuta campestris* parasite infection on the photosynthetic apparatus in *Ipomoea tricolor* leaves studied in vivo with chlorophyll fluorescence, MSc
6. Alexandra Yuliyanova Atanasova (2019) Functional characterization of photosynthetic potential of *Smicronyx* sp. galls on *Cuscuta campestris*, B.Sc.
7. **Ivanela Andreeva Albanova (2019) Interrelationships between the parasitic plant *Cuscuta campestris* Yunck. and different host species under salt stress conditions, MSc**
8. **Stefan Emilov Georgiev (2019) Pathogenesis-related proteins in *Arabidopsis thaliana* L. subjected to a combination of abiotic and biotic stress factors, B.Sc.**
9. **Georgi Kirchev Georgiev (2018) Metabolic and functional differences of *Smicronyx* sp. galls on the parasitic plant *Cuscuta campestris*, B.Sc.**
10. Antony Velichkov Kaukov (2018) Effect of salt stress on germination in parasitic (*Cuscuta campestris*) and non-parasitic (*Ipomoea purpurea*) representatives of the family Convolvulaceae, B.Sc. B.Sc.
11. **Ivanela Andreeva Albanova (2017) Influence of the parasitic plant *Cuscuta campestris* Yunck. on *Arabidopsis thaliana* L. under salt stress conditions, B.Sc.**

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