

ISOENZYMATIC PROFILES DURING EARLY STAGES OF HAUSTORIOGENESIS WITHIN THE GENUS *CUSCUTA* ARE TAXONOMICALLY DEPENDENT

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Abstract

Haustorium formation is the key developmental event enabling parasitism in the genus *Cuscuta*, yet comparative data on the enzymatic mechanisms underlying haustoriogenesis across taxonomically distinct species remain scarce. In this study, we investigated whether isoenzymatic profiles associated with early haustorium formation are taxonomically dependent within *Cuscuta*. Nine species representing three major subgenera – *Grammica*, *Cuscuta*, and *Monogynella* – were analyzed. Haustoria were induced using artificial hosts, and soluble protein extracts from the haustorial region were subjected to zymographic analysis of peroxidases, proteases, and cellulases. Peroxidases exhibited the highest diversity, with 16 distinct isoforms detected and clear interspecific and intersubgeneric differences. Protease activity was represented by a limited number of isoforms with species-specific electrophoretic mobility, whereas cellulase activity was detected only in two species. Cluster analysis based on combined peroxidase and protease isoenzyme profiles produced a dendrogram that largely reflected established taxonomic relationships, clearly separating

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the three subgenera. These findings demonstrate that isoenzymatic patterns during haustoriogenesis are not random but are evolutionarily linked to major lineages within *Cuscuta*. The results support the hypothesis that taxonomically related dodder species employ similar enzymatic strategies during host invasion, providing functional biochemical support for existing phylogenetic frameworks.

Key words: dodders, haustoria, parasitic plants, peroxidases, proteases, zymography

Abbreviations: BCA – bicinchoninic acid; DAB – diaminobenzidine; DMBQ – 2,6-dimethoxy-p-benzoquinone; HIF – haustoria inducing factor; RAPD – random amplified polymorphic DNA; ROS – reactive oxygen species; SDS PAGE – sodium dodecylsulphate polyacrylamide gel electrophoresis; UPGMA – Unweighted Pair Group Method with Arithmetic Mean

Introduction. Genus *Cuscuta* (dodders) is a group of obligate parasitic flowering plants in the morning-glory family Convolvulaceae. They are leafless nonphotosynthetic vines that lack functional roots after germination and depend on hosts for water, minerals, and photosynthates. After a seedling contacts a suitable host, it coils around the stem and forms a specialized invasive organ – the haustorium – that penetrates host tissues and connects to the host vascular system, enabling nutrient uptake and extensive physiological “cross-talk” between parasite and host [1]. Within the genus, the most widely used major taxonomic division is into four subgenera. Subgenus *Grammica* is the largest lineage (≈130 species), primarily from the New World, with USA and Mexico as a major diversity centre [2]. Many globally invasive dodders with agricultural impact, such as *C. campestris* Yunck. belong here. Subgenus *Cuscuta* consists of largely temperate species, centred in Eurasia and the Mediterranean–West Asian region, extending into North America by introductions and native ranges depending on the species [2]. Subgenus *Monogynella* expresses more hemiparasitic tendencies in some members [3] and is distributed primarily across Eurasia, with Central Asia highlighted as a centre of diversity in recent phylogenetic work [2]. Finally, the subgenus *Pachystigma* is restricted to Southern Africa and contains about five species [2].

Dodders occur across temperate and tropical regions worldwide, occupying diverse habitats from riparian zones to disturbed agricultural land. Out of over 200 species, relatively few are considered agricultural pests, most notably *Cuscuta campestris* Yunck., but also *C. epithymum*, *C. monogyna*, *C. gronovii*, etc. In particular, *C. campestris*, native to North America, is currently widely distributed worldwide, commonly moved via contaminated crop seed and other pathways [4], and damages various crop plants, including alfalfa/legumes and many vegetables. The primary negative impact is imposed through the nutrient sink, leading to resource exhaustion and reduced fecundity of the hosts, but secondary mechanisms such as reduction in hosts’ photosynthesis [5] are also exerted.

Although widely considered generalists, e.g., infecting a wide variety of host plant species, not all hosts are equally beneficial and environmental variables

may impose host preferences [6]. The central event behind *Cuscuta* parasitism is the formation of functional haustoria, required for successful nutrient extraction. Haustoriogenesis in *Cuscuta* is both a developmental program (induced by host contact cues such as tactile signals and host-derived chemicals) and a cell-wall invasion process [7]. A strong involvement of cell-wall remodelling enzymes was suggested and extensively studied. These include, but are not restricted to pectin-modifying enzymes like pectin methylesterases [8], cellulases/ β -1,4-glucanases [9], xyloglucan endotransglucosylase/hydrolases [10], and others. Recently, it was also reported that dodders are able to adjust the enzymatic cocktail for haustoriogenesis in response to different hosts [11]. However, the possibility that taxonomically distinct *Cuscuta* species express differing isoenzymatic profiles in the site of haustoriogenesis, evolved in the course of host-parasite arm race under different host availability and environmental conditions has not been explored so far.

Material and methods. Plant material. In the present study, we explored a total of nine *Cuscuta* species, distributed equally between the major three subgenera: subg. *Grammica* (*C. campestris*, *C. chinensis*, and *C. platyloba*), subg. *Cuscuta* (*C. europaea*, *C. approximata*, and *C. epithymum*), and subg. *Monogynella* (*C. japonica*, *C. monogyna*, and *C. lupuliformis*). Seeds of all but one, *C. lupuliformis*, kindly provided by Monika Tothova from the Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, belong to the laboratory collection in the Department of Biochemistry, Faculty of Biology, Sofia University, where they are continuously maintained as in-house lines. To induce germination, the common method of chemical scarification with concentrated sulphuric acid for 30 min was employed, after which the thoroughly washed seeds were placed into a peat substrate:perlite mixture at a 4:1 ratio and left to germinate at 25 °C in the dark. All three species, belonging to the subgenus *Cuscuta* were also subjected to cold stratification at 4 °C for 14 days, which was found to be a critical condition for germination, probably affected by the predominantly temperate origin. After the seeds were germinated, the seedlings were transferred under blue LED light and paper straws were offered as artificial hosts to induce coiling and initial haustorial development. Then the haustorial sections for each species were collected, frozen in liquid nitrogen.

Protein isolation and zymographic analyses. Total soluble proteins were isolated by grinding the tissue and extracting with 20 mM Tris.HCl, pH 7 (0.1 mL per 0.1 g tissue), centrifugation at 10 000 $\times$ g and collecting the supernatant. Protein concentration was measured using BCA Protein estimation kit (Pierce™) according to producer's manual and equalized quantities of 15 μ g protein were mixed with sample buffer, and separated on 12.5% T SDS PAGE [12] on an omniPAGE Mini Vertical Protein Electrophoresis System (Cleaver Scientific). Zymograms were acquired after renaturation in 10% (v/v) Triton X-100 essentially according to protocols available in Handbook of Detection of Enzymes on Electrophoretic Gels [13]. Peroxidases (EC 1.11.1.x) were visualized with Sig-

maFast DAB tablets with metal enhancer. Proteases (EC 3.4.21.x) were separated on polyacrylamide gel, containing 0.2% (w/v) gelatin, incubated overnight to allow in-gel gelatin hydrolysis, and visualized as achromatic bands after Coomassie staining. Cellulases (EC 3.2.1.x) were analyzed in a similar way, with 0.2% (w/v) carboxymethylcellulose in the gel and Congo Red staining. All gels were photographed, transformed to greyscale (protease and cellulase gels were also subjected to colour inversion) and analyzed with GelAnalyzer 23.1.1. The isoenzymatic profiles were used to construct a binary matrix (1 for presence, 0 for absence of a particular isoform), which was used to construct a UPGMA dendrogram by the neighbour-joining cluster method (Jaccard coefficient), using PAST software version 5.1.

Results and discussion. The peroxidase profile shows a large number of isoforms that differ significantly between different *Cuscuta* species (Fig. 1A). The most intense bands are observed in *Cuscuta japonica* (subgenus *Monogynella*). A total of 16 isoforms were identified, with the highest number (seven) observed in *C. japonica*, followed by *C. monogyna* and *C. approximata* (six each). Some similarity in the peroxidase profile was observed among representatives of the same subgenus, but there were also significant differences. Unlike peroxidases, the zymogram analysis of proteases is significantly poorer (Fig. 1B). In all *Cuscuta* species, only one achromatic band is observed, which, however, differs in electrophoretic mobility, respectively, in molecular weight, between the individual representatives. Among the nine species of *Cuscuta*, a total of three isoforms with differing molecular weights were detected. The picture for cellulases is radically different, with only two isoforms (Fig. 1C) found in only two of the studied parasitic plant species – *Cuscuta monogyna* and *Cuscuta platyloba*.

Unlike proteases and cellulases, the role of peroxidases in haustorial formation is not straightforward. Plant peroxidases play a variety of functions, including cell wall metabolism and lignification, ROS detoxification, thus being implicated in

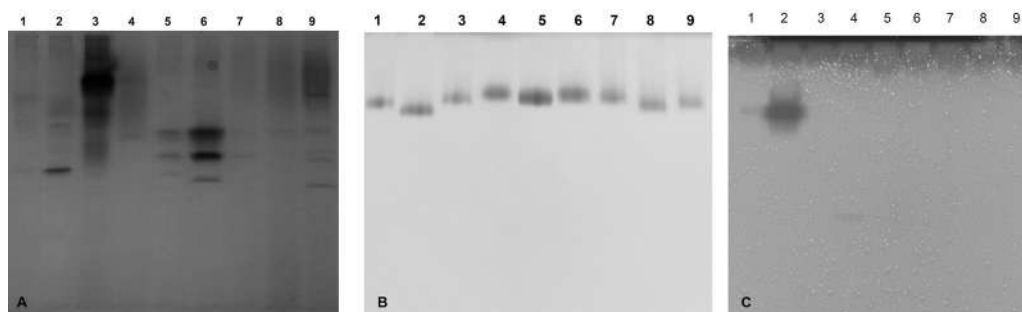


Fig. 1. Peroxidase (A), protease (B) and cellulase (C) profiles in the area of haustorium formation. 1. *Cuscuta lupuliformis*; 2. *C. monogyna*; 3. *C. japonica*; 4. *C. platyloba*; 5. *C. chinensis*; 6. *C. campestris*; 7. *C. europaea*; 8. *C. epithymum*; 9. *C. approximata*. 10% T SDS PAGE, renaturation in Triton X-100

abiotic and biotic stress response and tolerance [14]. In this respect, it is not surprising that they are widely expressed in the host plants during haustoriogenesis, and it was previously reported that peroxidases are widely expressed in host tissue during parasitic invasion [15], reinforcing the cell walls as a defense mechanism. LÓPEZ-CURTO et al. [16] proposed an important role for peroxidases during host penetration by *Cuscuta jalapensis*. According to this research, dodder's peroxidases are involved in the generation of the signals needed for the development of the haustorial cushion and the haustorium itself, in the reorganisation of the tissue of the parasite and in creating invasion structures and the generation of an entrance pathway, with the penetration and exploration of the haustoria in the tissue of the host. For example, a HIF (haustoria inducing factor), 2,6-dimethoxy-p-benzoquinone (DMBQ), during haustoria formation in *Striga hermonthica* is produced by oxidation of its precursor, syringic acid, and that reactive oxygen species (ROS) and peroxidases are involved in the process [17].

Although there are several studies on the enzymes, involved in haustoria formation in *Cuscuta* [8,10], to our knowledge this is the first comparative study of several taxonomically different dodder species. The lack of variety in the isoenzymatic profile of proteases and cellulases may be explained by the use of artificial host, eliminating the need for host-related adjustment of the enzymatic cocktail. However, the variety of peroxidases and proteases was meaningful enough to produce a phylogenetic tree (Fig. 2), with a clear clustering of species within subgenera.

It is clearly shown that representatives of the subgenus *Monogynella* and the subgenus *Grammica* are grouped together and are distant from each other and from the subgenus *Cuscuta*. In the subgenus *Cuscuta*, *C. europaea* and *C. epithymum* were grouped together, but *C. approximata* is relatively distant from them

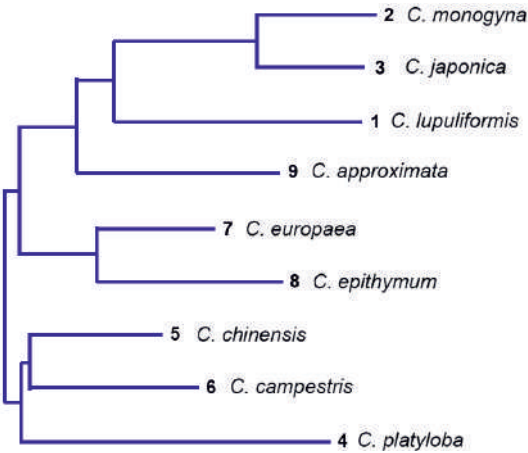


Fig. 2. Phylogenetic tree of nine *Cuscuta* species based on the isoenzyme profile of proteases and peroxidases in the haustorium formation region

and closer to the subgenus *Monogynella*. This particular clustering of the members of the subgenus *Cuscuta* is in agreement with our previous studies, involving RAPD molecular markers [18], as well as plastome sequences [19]. It also confirms, more or less, previous works on the taxonomy of this parasitic genus [20]. However, the purpose of this study and the expectations behind it were not to challenge the existing phylogeny of dodders, based on nuclear and chloroplast genomes. The main purpose was to check the hypothesis that enzymes, involved in haustoriogenesis are evolutionarily linked to the main lineages in the genus, and taxonomically close dodder species employ similar mechanisms of host invasion. However, current studies on host overlapping do not support the idea that phylogenetically related dodder species share similar host range [6], suggesting that host preference might be dependent and guided by other mechanisms.

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