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Germinating Seeds of the Root Parasite *Orobanche aegyptiaca* Pers. Excrete Enzymes with Carbohydrase Activity

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Abstract

Germinating seeds of the root parasite *Orobanche aegyptiaca* Pers. were analyzed for the ability to excrete cell wall degrading enzymes, in a sterile environment, and without the host roots. Enzymatic activity was detected by exposing the radicles and haustoria to different substrates representing cell wall components. The extracellular activities of polygalacturonase, carboxymethyl-cellulase and β-glucosidase were detected. Optimum activity was observed on the second or third day from germination and it coincided with the time required by the parasite to penetrate the root cortex.

Keywords: *Orobanche aegyptiaca* Pers., haustorium, excretion, carboxymethyl-cellulase, β-glucosidase, poly-galacturonase

1. Introduction

The Orobanchaceae are root-parasitic angiosperms that lack chlorophyll and photosynthetic function. They acquire their carbon, water and minerals entirely from a photosynthetic host (Musselman, 1980; Press et al., 1990). The genus *Orobanche*, with an estimated 150 species, is widespread in both temperate and semitropical regions. Many of the hosts are economic plants that suffer great loss of yield. In the last decades, these parasitic weeds have become a serious agricultural pest (Sauerborn, 1991).

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The seeds of *Orobanche* ($\sim$ 0.3 mm) germinate in response to stimuli in the rhizosphere of the host plant. The parasite radicle develops a haustorium that is responsible for attachment to the host root, penetration and establishment of apoplastic continuity between host and parasite. This continuity enables acquisition of water, minerals and solutes (Kuijt, 1977; Musselman, 1980; Stewart and Press, 1990).

Haustorial progress has been credited to both mechanical and enzymatic action. Growth is responsible for the mechanical pushing in, but there is no convincing evidence of extracellular enzymatic activity. Increase in the activity of cellulase has been demonstrated in different parasite-infested hosts (Kuijt, 1977; Gordon-Ish-Shalom, 1990). Activity of acid phosphatase has been demonstrated in the contact zone between the host and the haustoria of two mistletoes (Kuijt, 1977). In all cases the reaction product may have originated from the dying host cells. Furthermore, plant cells are known to express specific defense mechanisms against external biotic or abiotic elicitors (Ryan, 1987). Possible defense responses are the induction of β-glycanohydrolases (Mauch et al., 1988) and a change in membrane properties that leads to cell death (Young and Kauss, 1983). Haustorial penetration could induce the plant defense mechanism and thus trigger synthesis of glycanohydrolases.

A set of experiments was performed, without the host, to clarify the mechanism of haustorium penetration. Germinating seeds of *Orobanche aegyptiaca* Pers. were exposed to different substrates representing components of cell walls, and the depolymerization ability was measured.

2. Materials and Methods

Seeds of *Orobanche aegyptiaca* Pers. were surface disinfested in 2.5% sodium hypochlorite plus 0.01% Tween 20, for 5 min and then thoroughly rinsed with autoclaved distilled water. The seeds were spread over sterilized, water moistened glass or paper filters, in petri dishes for a 10 day preconditioning in darkness at 20° C. Then, an analogue of strigol, termed GR-24 (5 ppm), a sesquiterpene that stimulates germination, was added (Johnson et al., 1976). Sixty-five hours later, after approximately 80% germination, the seeds were transferred to agitated sterilized 0.02 Hoagland solution for 2–3 hr. The activity of different extracellular cell-wall-degrading enzymes was analyzed by exposing the germinating seeds to different filter sterilized substrates representing cell wall components, and the accumulation of the products was analyzed at intervals from the time of germination. The following controls were applied: (1) different substrates in their buffers, without germinating seeds; (2) germinating seeds in different buffers, with no substrates; (3) non-germinating seeds

that were preconditioned for 5 days only, without the stimulant treatment, and then exposed to the different substrates. In each treatment, the amount of non-specific degradation product was substracted from the results. The seeds and the stimulant GR-24 were a generous gift from Dr. R. Jacobsohn, Volcani Center, Israel.

Experiments were repeated at least three times with three or four biological replicates in each set. Enzyme activity was linear in respect to the amount of seeds.

3. Carbohydrase Activity

Polygalacturonase (EC3.2.1.15)

The enzyme attacks 1,4 linked D-galacturonide chains of pectic substances and cleaves them by hydrolysis. The pH optimum is at 6.0 or lower. Enzyme activity was demonstrated by the release of reducing sugars from the substrate polygalacturonic acid (Sodium salt, Sigma). The reducing groups released were measured spectrophotometrically at 530 nm after reaction at 100° Cwith 3,5-dinitrosalicylic acid and centrifugation according to Ng and Zeikus (1988).

Different amounts of germinating seeds were exposed to an agitated solution containing 0.05–0.3% of polygalacturonic acid in 10 mM NaCl, pH 5.1. Aliquots were sampled for reducing groups. D-galacturonic acid was used as the standard.

Cellulase (EC3.2.1.4)

Cellulase activities were measured according to Wood and Bhat (1988), with some modification. Different amounts of germinating seeds were exposed to an agitated medium containing 1 mM citrate buffer at pH 5.1, with the following substrates specific to the enzyme activity analyzed.

Exocellulase

The substrate was 0.4% Avicell (Sigmacell 100).

Endocellulase

The substrate was 0.01–0.3% Carboxymethyl-cellulose sodium salt (CMC, BDH). Aliquots were sampled for released reducing sugars for both cellulases. D-glucose was used as the standard.

β-glucosidase (EC3.2.1.21)

The enzyme cleaves glucose residues from cellobiose and higher cellodextrines. The extracellular activity of 1,4-β-glucosidase was measured according to Deshpande and Eriksson (1988). The substrate was 0.2–2 mM 4-nitrophenyl-β-D-glucopyranosid (Merck, Darmstadt) in the same 1 mM citrate buffer, pH 5.1. Aliquots were sampled and measured at 405 nm for p-nitrophenol.

Hemicellulase-xylanase (EC3.2.1.8)

The substrate representing hemicellulose, was 0.3% xylan (BDH), in the same citrate buffer. Aliquots were sampled for released reducing groups. Reducing groups were reacted with 3,5-dinitrosalicylic acid and measured spectrophotometrically at 530 nm.

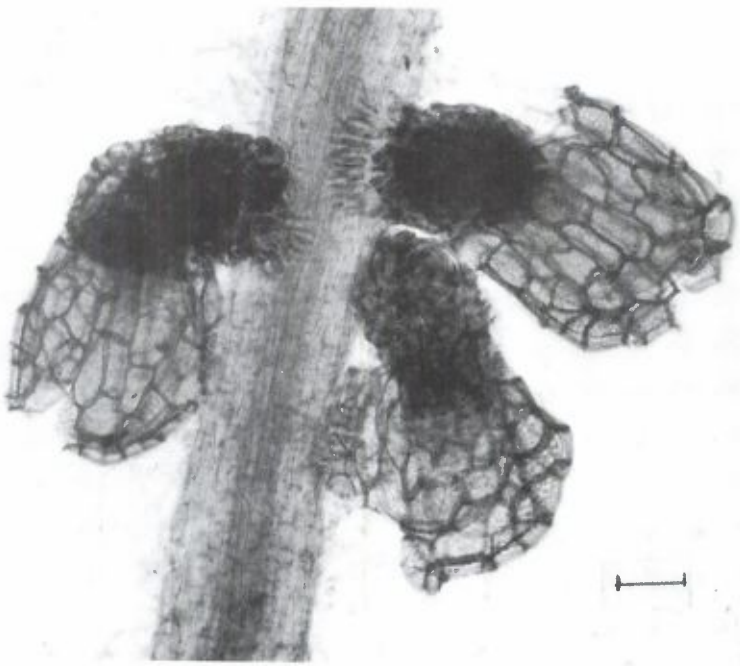

Figure 1. A cleared tomato root infested (2^{nd}d) with three parasite broomrapes (*Orobanche aegyptiaca*). Scale bar = 140 μm.

Anatomy of infested tomato roots

Seeds of *O. aegyptiaca* on the first day of germination were spread at the root zone of three-week-old tomato seedlings. The tomato plants were grown in a glass sandwich (Gordon-Ish-Shalom, 1989), on 0.5 Hoagland's solution, in a controlled growth chamber (25° C, 12 hL, 250 $\mu Em^{-2}min^{-1}$). Infested tomato roots were sampled every day during the first 2 weeks. The infested roots were cleared in lactic acid and then stained with Safranin and Fast green (Jensen, 1962).

4. Results

Germination

Approximately 80% germination was observed in the seeds preconditioned for 10 days in darkness and then treated with the stimulant. Three to four percent germination was observed in seeds preconditioned for 12 days, without chemical stimulation, and thus they could not serve as controls. Seeds preconditioned for only 5 days did not germinate and could serve as a control for at least the next five days. The degree of radicle elongation and the time (5–24 hr) and size of haustorial development differed, depending on the substrate and its concentration.

Infested host roots

Analyzing the anatomy of the cleared infested host roots enabled us to follow the sequence of events. A day after germination and infestation, seeds could be found attached to the host root (Fig. 1). Afterwards the intrusive haustorial cells were seen at different stages of penetration (Figs. 2b,c). On the 5th day, small tubercles could be found.

Extracellular enzymatic activity

Germinating seeds performed extracellular pectinolytic and other carbohydrase activity while the ungerminated seeds did not. The cellulolytic activities are usually achieved by a complex of enzymes attacking different bond types on the cellulose polymer. Exposing the germinating seeds to different specific substrates (Wood and Baht, 1988) enabled detection of the activity of an endocellulase (CM-cellulase) but not that of an exocellulase (Fig. 3). The activity of other carbohydrases was analyzed. No activities of the hemicellulase-xylanase could be detected (Table 1).

The activity of β-glucosidase which cleaves cellobiose, an inhibitor of cellulase, was detected immediately after radicle appearance, while that of the

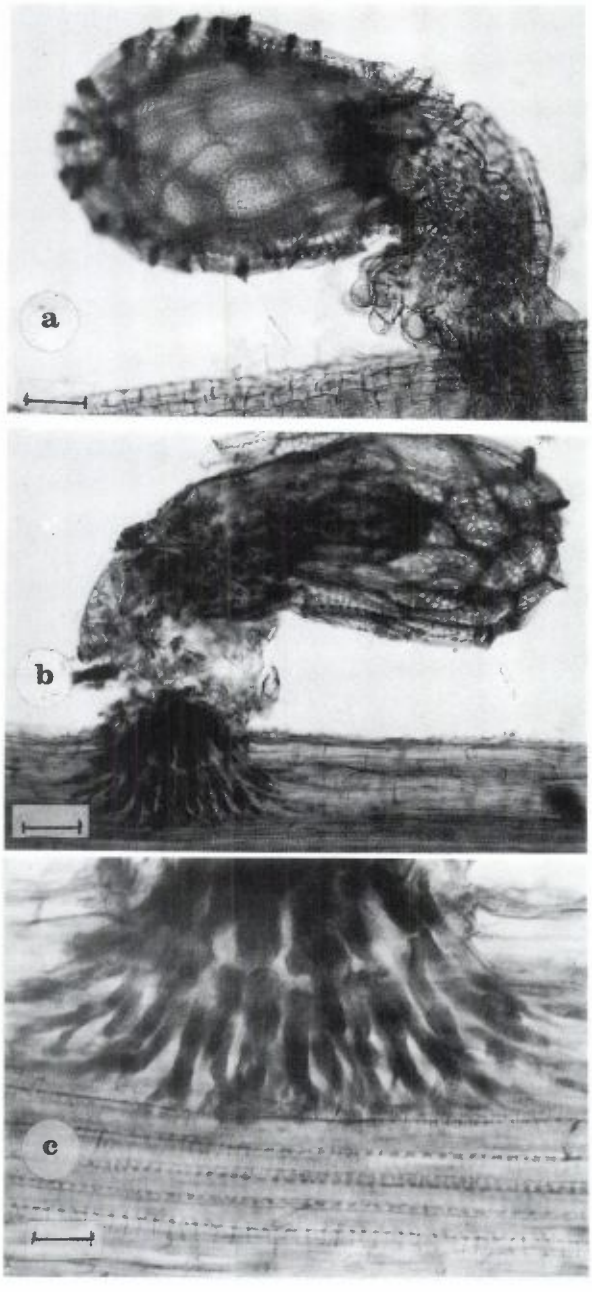

Figure 2. Cleared tomato roots infested with *Orobanche aegyptiaca* showing the intrusive haustorial cells on (a) the second day. (b;c) the 3rd day from infestation. Scale bar (a) = 40 μm; (b) = 60 μm; (c) = 20 μm.

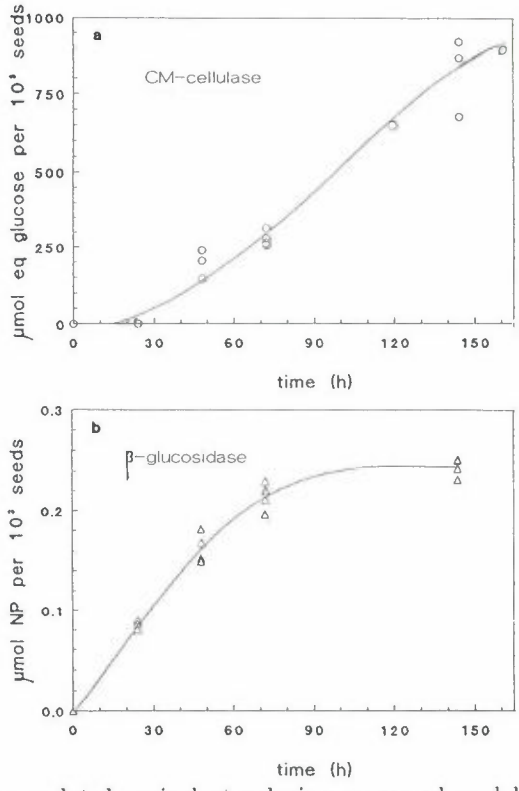

Figure 3. (a) The accumulated equivalent reducing sugars released by endocellulase from 0.2% CM-cellulose, with time from germination.
(b) Accumulation with time of p-nitrophenol released by β-glucosidase from 1 mM p-nitrophenyl-β-D-glucopyranosid. Each point is the mean of 3–4 biological replicates in an experiment

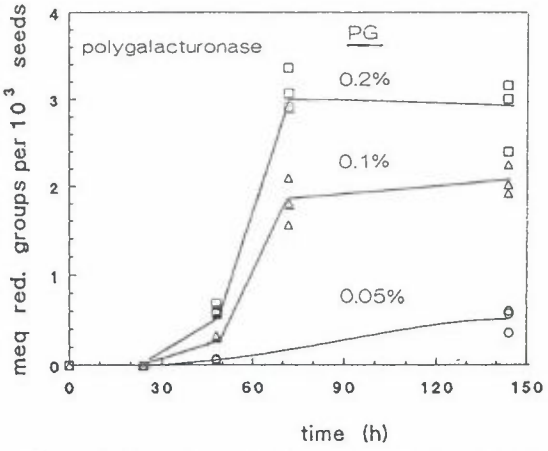

Figure 4. Demonstration of the extracellular pectinolytic activity of the *Orobanche aegyptiaca* haustoria.

Table 1. Specific activity of different carbohydrases (μmol eq released groups per 10^3 seed per day) excreted by the haustoria of *Orobanche aegyptiaca* on the second or the third day after germination

Enzyme	Substrate	Specific Activity
Polygalacturonase	Polygalacturonic acid (0.2%)	2400
Exocellulase	Avicell (0.2–0.4%)	no activity
Endocellulase	CM-Cellulose (0.2%)	170
β-glucosidase	p-Nitrophenyl-β-D-glucopyranosid (1 mM)	0.07
Xylanase	Xylan (hermicullolose) (0.2–0.4%)	no activity

endocellulase (CM-cellulase) was detected only 48 hr after germination. Both activities were relatively low, but significant (Fig. 3).

The hydrolytic activity of polygalacturonase (pH 5.1) was detected 24 hr after germination and increased as time elapsed (Fig. 4). It seems to be the major contributor (Table 1) to the cell wall hydrolysis at the point of haustorium penetration. A very low and insignificant activity of pectate lyase was detected at pH 8.0 and it was not cation dependent.

Optimum activity of the excreted carbohydrases was observed on the second or third day from germination. It coincides with the time required for the haustorial cells to penetrate the root cortex (Figs. 1,2). A similar extracellular activity of polygalacturonase was observed with germinating seeds of *O. crenata* Forsk.

5. Discussion

Orobanche haustorial intrusive cells seem to grow only intercellularly (Kujt, 1977). They have to push their way through branched and cross-connected microfibers of lignin, pectin, hemicellulose, all of which might be cross-linked with ferulic acid esters (Meyer et al., 1991). What are the possible mechanisms enabling the *Orobanche* intrusive body to penetrate the host root?

1. The intrusive cells' progress might proceed via a mechanical pushing only.
2. The resistance to such a mechanical mechanism could be decreased by the aid of host cellulases, excreted in response to toxic compounds (Kuijt, 1977) or, as a response of the plant defense mechanisms (Olivier et al., 1991).

3. The parasite itself excretes cell-wall degrading enzymes to reduce host resistance and enable a smooth mechanical penetration. This was suggested by Ikonkwo and Nwoke (1978) and Maiti et al. (1984) without conclusive evidence.

The data demonstrated in this paper favor the third hypothesis. They do not exclude a combination that at first the parasite excretes cell-wall degrading enzymes and later,host glycanohydrolases are synthesized.

In conclusion, we may say that the *Orobanche* haustorium excretes enzymes that depolymerize pectin (polygalacturonic acid) and cellulose (CM-cellulose). These extracellular enzymes with their carbohydrase activity have the ability to loosen the cell wall micro-fibers and thus enable a smoother pushing through the cortex towards the conducting elements. They seem to be a key factor of the parasite/host biology.

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